THE UTILIZATION OF ECTO 5'-NUCLEOTIDASE AS A MONITOR OF CELL SURFACE ALTERATIONS

By

ROBERT COURTLAND DOSS

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1975

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1979

THE UTILIZATION OF ECTO 5'-NUCLEOTIDASE AS A MONITOR OF CELL SURFACE ALTERATIONS

Thesis Approved:

Kit Conaway
Thesis Adviser

Ulrich Melcher

Ta-hsin Lin

Richard C. Essenberg

E. A. Funkhouser

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation for the guidance, patience and inspiration received from his major advisor, Dr. Kermit L. Carraway, during the course of these investigations and the preparation of this thesis. It has been a truly rewarding experience to work under Dr. Carraway's direction. The author also acknowledges and thanks Dr. Ulrich K. Melcher, Dr. Ta-hsiu Liao, Dr. Richard C. Essenberg and Dr. Edward A. Grula for their valuable time spent as members of the advisory committee and for their contributions towards the preparation of this thesis.

The excellent technical assistance provided by Frank Corrado throughout these investigations is deeply appreciated. Frank's enthusiasm and dedication will always be remembered and his friendship treasured. The author also wishes to express his gratitude to Charlene Bymaster, Grant Gamel, Edwin Li, Sandy McGuire, Bill Smith and Elizabeth Wright for their technical assistance during portions of this study. Thanks are also extended to Dr. Anne Sherblom for her assistance with the electrophoresis in the MFGM studies. The stimulating discussions with Dr. Sherblom, Dr. John Huggins, Dr. Don Yeltman and Dr. Coralie A. C. Carraway were also of great value to the author. Financial assistance and facilities provided by the Oklahoma State University Biochemistry Department are gratefully acknowledged.

Special appreciation is expressed to my wife, Connie, for her

constant love, faith, support and patience during the course of this study. Her excellent typing skills and helpful comments in the preparation of this thesis were invaluable and will not be forgotten. It is to Connie that I dedicate this thesis.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

NOMENCLATURE

ABP - actin binding protein

ADP - adenosine 5'-diphosphate

AMP - adenosine 5'-monophosphate

ATP - adenosine 5'-triphosphate

ATPase - adenosine 5'-triphosphatase

BHK-21 - baby hamster kidney fibroblasts

cm - centimeter

Con A - Concanavalin A

cyclic AMP - adenosine 3',5'-monophosphate

Dg - digitonin

DNase I - deoxyribonuclease I

DOC - deoxycholate

DPH - 1,6-diphenylhexatriene

dpm - disintegrations per minute

EDTA - ethylenediaminetetraacetic acid

EGTA - ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid

F_1 - Fraction 1 (0.9-32% interface)

F_2 - Fraction 2 (32-36% interface)

F_1DF_1 - Digitonin Fraction 1 (0.9-32% interface) from Dg treatment of F_1

F_1DF_3	- Digitonin Fraction 3 (36-40% interface) from Dg treatment of F_1
F_2DF_3	- Digitonin Fraction 3 (36-40% interface) from Dg treatment of F_2
g	- gravity
GEM	- 5 mM glycine-1 mM EDTA-5 mM β-mercaptoethanol (pH 9.5)
Hepes	- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
K	- one thousand
kcal	- kilocalories
K_m	- Michaelis-Menten constant
M	- molar
MAT-A, -C1	- 13762 mammary ascites adenocarcinoma sublines
MFGM	- milk fat globule membranes
mg	- milligram
ml	- milliliter
αMM	- α-methylmannoside
mM	- millimolar
NC	- noncompetitive
nm	- nanometer
PBS	- phosphate buffered saline
Pi	- inorganic phosphate
rpm	- revolutions per minute
SDS	- sodium dodecyl sulfate
TCA	- trichloroacetic acid
Tris	- tris-(hydroxymethyl)-aminomethane
TX-100	- Triton X-100
UV	- ultraviolet

v	- velocity
v_o	- maximum or uninhibited velocity
μ	- micro or 10^{-6}
μCi	- microcurie
μg	- microgram
% (w/v)	- percent (weight to volume)

CHAPTER I

INTRODUCTION

The Cell Surface

The cell surface plays a critical role in many of the essential processes of animal cells. A substantial amount of data has implicated the cell surface as the primary site for control of cell growth, division, development, communication, differentiation, and death (1,2). Cells must react to their environments and to extracellular stimuli through their surfaces, particularly their plasma membrane, so this cellular structure has evoked considerable interest (1-8).

An understanding of cell surface dynamics is dependent on a thorough knowledge of the organization of the plasma membrane and its associated structures. The interpretation of most cell surface phenomena has been based on models, which for the present serve as useful conceptions to explain an enormous variety of physical, chemical, and biological data on membrane structure (3-8).

Biological membranes are composed mainly of proteins, lipids, and carbohydrates. Since the sugar residues are attached to either the protein or lipid components, or both, one can actually consider membranes to be made of proteins and glycoproteins and of lipids and glycolipids (5). It has been observed that the protein to lipid ratio increases with increasing membrane function and specialization (5). For example,

myelin, whose main function is to act as an insulator, contains much more lipid than protein while the inner mitochondrial membrane which contains a number of functional activities possesses a very large amount of protein (9,10). A substantial fraction of membrane protein probably plays an important role in determining membrane structure. Therefore, the structural properties of these proteins are of great importance.

The early plasma membrane models of Danielli and Davson endeavored to explain the physical and chemical aspects of membrane organization and were based, in part, on the characteristics of lipid films (11,12). The idea that membrane lipids are formed into a bimolecular leaflet with the long chain hydrocarbon moieties of the fatty acids interacting hydrophobically and with the ionized, hydrophilic head-groups oriented outward was later advanced by Robertson as the "unit membrane model" (13). The protein components of the membrane are depicted in this model in the form of extended sheets, bound to the phospholipid bilayer essentially by ionic interactions with the hydrophilic head-groups of the phospholipids. However, these early membrane models were unable to explain a large number of experiments which indicated that the hydrophilic portions of membrane phospholipids were susceptible to enzymatic attack and were not complexed to any great degree to membrane proteins (14,15). In fact, most membrane proteins are globular and are not β-structures or "random coils" as originally proposed (4,14). These globular proteins were found to be stabilized in low polarity solvents or in solutions containing chaotropic agents or detergents (16). Some proteins span the membrane and are accessible to chemical and enzymatic modification at either surface (17,18).

Membrane associated proteins are generally divided into two broad

categories, peripheral and integral (6), which differ in their position with respect to the lipid bilayer and therefore in their mode of association with the lipid. Peripheral membrane proteins do not penetrate the membrane bilayer, but are held at the surface of the membrane by predominantly electrostatic or hydrophilic interactions (6). Two examples of peripheral proteins include cytochrome c of the mitochondrial inner membrane (19) and spectrin of the red cell membrane (20), both of which can be removed from their respective membranes by reagents which disrupt electrostatic interactions. These and all peripheral proteins are characteristically soluble in aqueous solution, once liberated from the membrane.

Integral membrane proteins penetrate into and sometimes completely through the interior bilayer and are bound to the membrane through hydrophobic interactions with the lipids (6). The observation that the major glycoprotein of the human red cell membrane can be chemically labeled from both sides of the membrane supports the concept that this protein spans the bilayer (21). This protein, and indeed all intrinsic membrane proteins, can only be liberated from their respective membranes by reagents which disrupt hydrophobic interactions. They are insoluble in aqueous solutions in the absence of detergents or other solubilizing agents, although in some cases polymerization occurs which yields a water soluble aggregate. Further, intrinsic membrane proteins have the unique ability to recombine with lipid to form membranes.

Several kinds of experiments indicate that protein-lipid interactions play a direct role in a variety of membrane functions. Many membrane-bound enzymes require lipids, often specific phospholipids, for the expression of their activities (22). Furthermore, the nature of the

fatty acids incorporated into phospholipids affect the functions of certain membrane-bound proteins in bacterial membranes (23). However, it has been shown that several properties of the bulk of the phospholipids are not affected by proteins inserted into the membranes (4), suggesting that the majority of phospholipids do not interact with membrane proteins.

From the collection of data involving the structural and thermodynamic considerations of the interactions between lipids and proteins, the "fluid mosaic model" of membrane structure was postulated (6,24). The fluid mosaic membrane model proposes that membranes are composed of a fluid lipid bilayer matrix which allows lateral movements of membrane components through a somewhat viscous environment. The viscosity of the membrane lipid matrix is determined by the lipid composition, temperature, and other factors (6). The globular integral protein molecules are postulated to be amphipathic, as are the phospholipids, with the ionic amino acid residues of the polar region in contact with the aqueous phase and the nonpolar residues embedded in the hydrophic interior of the lipid matrix. The amphipathic structure adopted by a particular integral protein molecule, and therefore the extent to which it is embedded in the membrane, are under thermodynamic control so that the free energy of the system as a whole is at a minimum (6).

The mosaic structure can be readily diversified in several ways. Protein-protein interactions may be important in determining the properties of the membrane. Such interactions may result either in the specific binding of a peripheral protein to the exterior exposed surface of a specific integral protein or in the association of two or more integral protein subunits to form a specific aggregate within the membrane

(6). Likewise, the cooperative nature of the cell membrane (25) can be explained by this model. Cooperativity in membranes is described as an effect which is initiated at one site of the membrane and transmitted to another remote site by some structural coupling between the two sites. Thus, localized environmental changes or signals may influence the rest of the cell surface and thereby affect other cell properties.

It has therefore become increasingly evident that past conceptions of the plasma membrane, regarding it as a static structure and presuming all biological membranes to possess sameness and uniformity of structure and function are totally invalid. In contrast, the current concept of the plasma membrane is one of a dynamic three-dimensional mosaic, which reflects the genetic and phenotypic diversities of cells. This is clearly illustrated by the fact that nature has endowed the cell surface with a variety of specificity markers, receptors, transport mechanisms, junctional specializations for intercellular communication, and enzymes (1,26), giving it the capabilities for manifesting responsiveness to a multitude of environmental changes and signals.

Transmembrane Control of Cell Surface Receptor Mobility

Although it is well established that cell surface components such as proteins and glycoproteins are mobile in the membrane plane, the mechanisms responsible for the organization of the cell surface and for the mobility of molecules within it are still not well understood. After the development of the "fluid mosaic model" it was soon evident that the model was much too simple to explain membrane dynamics. Cellular control of the free lateral movements of at least some surface molecules must

occur since certain types of cell-mediated surface movements, such as the capping of surface receptors via lectins, cannot be explained simply by the "fluid" nature of the membrane. Nicolson (27) postulated several mechanisms for the restriction of lateral movements of membrane components such as proteins and glycoproteins: (a) association of membrane components by noncovalent forces in the membrane plane leading to the formation of aggregates or ordered lipid arrays; (b) sequestration or exclusion of membrane components to or from specific lipid domains; (c) restraint by peripheral components of the inner or outer membrane surfaces; or (d) restraint by membrane-associated cytoskeletal elements at the inner membrane surface. All of these restraints may be temporary and would have the effect of reducing the diffusion of membrane components that would be determined purely by the lipid viscosity of the membrane.

The interaction of membrane macromolecules with cytoplasmic structures may not only restrict movement of surface molecules but may enhance their displacement by "nondiffusional" mechanisms, as in the well-known processes of patching and capping (28). The membrane-associated cytoskeletal restraining components differ operationally from restraints by peripheral membrane components. In peripheral systems the restraining forces are probably due to the formation of peripheral lattices across large portions of the membrane inner or outer surfaces. These lattices or networks may crosslink specific components or an array of different components and impede their lateral mobilities. The membrane-associated cytoskeletal system differs from peripheral restraints due to (a) its ability to reorganize rapidly under cellular influence, (b) its dependency on cell energy systems for maintenance, (c) its sensitivity to drugs which disrupt cytoskeletal components, and (d) its structural link-

age to other cell organelles as detected by electron microscopic studies (27). Therefore, the cytoskeletal system has been given considerable attention toward explaining the mechanism for control of receptor mobility.

Nicolson describes three basic types of cytoskeletal components that are at times associated with cell membranes (27): thin filaments or microfilaments, intermediate filaments, and small tubules or microtubules. Microfilaments are cytoplasmic protein polymers arranged in double helical filamentous structures, some 4-7 nm in diameter and of variable length up to several μm. Microfilaments are frequently found in dense bundles or networks closely associated with the plasma membrane. Concentrations of microfilaments are also found at cleavage furrows during cell division, and in pseudopods, which are the cytoplasmic extensions by which ameboid-type locomotion is achieved (29). Movement of the cleavage furrow and pseudopod is believed to be due in part to changes in microfilaments. The association between microfilaments and cellular movements is reinforced by the observation that nonmuscle actin has been identified in microfilament regions by the heavy meromyosin binding (30), and fluorescent antibody techniques (31). It has also been observed that microfilaments closely resemble the thin actin filament of muscle, one of the two main types of filaments in muscle. Actin monomers are single polypeptide proteins of about 43,000 molecular weight which contain Ca^{++} or Mg^{++} and ATP. Upon polymerization the bound ATP is rapidly hydrolyzed to ADP and free phosphate (32). Nonmuscle actin filaments are involved in a variety of cellular processes such as cell motility and morphogenic movements, cytokinesis, secretion, cytoplasmic streaming, and endocytosis (33).

Nonmuscle myosin *in vivo* has been shown to be associated with microfilaments and has also been localized outside microfilament regions in a less structured organization which could be intermediate filaments (34). Nonmuscle myosins are complex proteins that bind reversibly to actin filaments forming actomyosin complexes and possessing an actin-activated ATPase (27). Intermediate filaments have been observed in a wide variety of cells. Intermediate filaments possessing a diameter of 10 nm are often found deep in the cell cytoplasm with a few extending to the plasma membrane (35).

Microtubules are polymeric complexes of the protein tubulin arranged into long tubular structures possessing an outer diameter of 25 nm and an inner diameter of 15 nm (36). Both microfilaments and microtubules are polymers of smaller protein subunits, however microtubules are more complex structures as they appear to be hollow cylinders whose walls contain several strands of polymerized tubulin. Tubulin dimers of about 110,000 molecular weight probably assemble to form the tubular structures. The assembly and disassembly of microtubules are sensitive to temperature, Ca^{++} concentration, pH, pressure and other blocking agents (27). Microtubular structures are involved in nuclear events during mitosis, but they are also present in the cytoplasm of most non-dividing cells (37). Microtubules have in some cases been found in association with microfilaments and cell membranes (27) and appear to be involved in the control of certain cell surface components. In addition to these functions microtubules are the basic building blocks of centrioles, basal bodies, cilia, and flagella (29).

The concept of the cell having transmembrane control over its cell surface through its cytoskeletal components is relatively new, but there

exists a large amount of evidence which suggests that this cytoskeletal control system must be dealt with in any proposal for cell surface organization. Recently this membrane-associated cytoskeletal system has also been implicated in the mobility of cell surface receptors for immunoglobulins and lectins (38). This concept is based on the observations that drugs which alter either microfilaments, such as a group of mold metabolites called cytochalasins (39), or microtubules, such as the alkaloid drugs colchicine, colcimid, vinblastine, and vincristine (36), interfere with the ligand-induced movements of these surfaces receptors. Thus, the cytoskeletal system has come under intense investigation, since microfilaments and microtubules are proposed to play important interconnected roles in maintaining the distribution and mobility of cell surface receptors.

Concanavalin A as a Probe of Cell Surface Dynamics

Concanavalin A is one of a group of plant proteins, termed lectins, that has been a particularly useful probe in studies of cell surface dynamics and properties of a wide variety of cell types. The diverse biological effects of Con A are complex and include the agglutination of some cell lines (40), the induction of mitosis of lymphocytes (41), and the inhibition of growth of some cells (42). The various biological activities of Con A all appear to involve initial binding of the lectin to saccharide-containing receptors on the cell surface since this binding can be inhibited by the addition of specific simple sugars. Con A binds α-D-mannopyranose, α-D-glucopyranose, D-fructofuranose, their glycosides and sterically related structures, and it complexes with many other

substances that contain these saccharides (40). It appears to recognize terminal as well as internal saccharide residues. In addition to sugars, Con A binds Mn^{++} and Ca^{++}. Transition metal ions are required for the binding of Ca^{++}, and both are necessary for the binding of saccharides (40).

Unlike most purified lectins, Con A is devoid of covalently bound carbohydrate and thus is not a glycoprotein. Previous studies have elucidated the subunit structure, amino acid sequence, and three-dimensional structure of Con A (43-45). At physiological pH, this lectin is a tetramer composed of identical globular subunits of 25,000 molecular weight. Four saccharides can bind to the tetrameric molecule, each subunit containing one saccharide-binding site. Studies of the physical structure of Con A are complicated by the reversible pH- and temperature-dependent association of the subunits (40). At pH values below 5.6 and also at temperatures below 20°C Con A exists in solution as a dimer with a molecular weight of 55,000. Above pH 5.6 the protein forms tetramers of about 112,000 molecular weight. Con A is present in dimer and tetramer forms between 4° and 36°C, the tetramer form becoming more predominant with increasing temperature.

Techniques utilizing Con A as a probe of cell surface structural organization and dynamics include cell agglutination, quantitative labeling, ultrastructural localization, and perturbation of receptor and enzyme function. Many types of bacteria and cells agglutinate with Con A (46). Unfortunately, there do not seem to be simple reasons for susceptibility to agglutination by Con A. Agglutination appears to be controlled by a variety of cell and environmental factors including the amount and distribution of the Con A receptors, the transmembrane re-

straints on the receptors, temperature, pH and ionic strength (46). Radioisotope-labeled Con A has been employed by a number of investigators as a more quantitative assay for lectin binding, monitoring lectin receptor changes in the cell cycle (47), at various developmental stages (48), or after transformation (49). Since agglutination and quantitative labeling data by themselves cannot determine the dynamics of cell surface receptors, fluorescence and electron microscopy have provided further insight into surface component redistribution (50). The distribution of Con A receptors on cell surfaces can be evaluated by fluorescence microscopy, using fluorescein-labeled Con A and by electron microscopy using ferritin-labeled Con A, peroxidase-labeled lectin and localization of bound Con A by its reaction with hemocyanin.

Using fluorescent lectin and lectin-anti-lectin indirect techniques, it was reported that Con A binding sites appeared to be uniformly distributed on the cell surfaces of fibroblasts and lymphocytes at low temperatures, but quickly migrated into multiple complexes or "patches" upon warming the cells to 37°C (51,52). These observations supported the fluid mosaic model since the receptors were shown to be more mobile at the higher temperatures. Inbar et al. (53) showed that upon binding fluorescein Con A to normal lymphocytes, the Con A receptors of a proportion of the lymphocytes accumulated into a single compact mass at one pole of the cell. This process is known as capping and is evidence for Con A receptor mobility on several different types of cells (54). The physiological significance of the capping phenomenon remains unknown, but the process continues to attract much attention.

There are a variety of environmental factors and drugs which block capping in lymphoid cells, but seem to have little effect at all on the

formation of patches. Specific metabolic inhibitors, such as azide, cyanide, and dinitrophenol, will inhibit capping but will not affect the patching process or short-range receptor movements. This indicates that capping requires a more metabolically active cell, probably due to the incredible distances the receptors must travel (55). Capping is inhibited to varying degrees in different cells by the cytoskeletal-disrupting drug cytochalasin B, which is known to act on microfilaments (39). de Petris (56) has found that preformed Con A caps can be abolished by cytochalasin B either in the presence or in the absence of vinblastine sulfate, a microtubule disrupting drug. Drugs that disrupt microtubules have been shown to have little effect on capping or may even enhance the capping process somewhat. When cytochalasin B plus colchicine or vinblastine are administered simultaneously to lymphocytes or polymorphonuclear leukocytes, capping is strikingly hindered (38). These results demonstrate that the membrane-associated cytoskeletal system is, at least in part, involved in the control of lymphoid cell surface receptor mobility.

Although a considerable amount of successful experimentation has been done utilizing labeled Con A to detect receptor movements on the cell surface, this approach suffers from two major difficulties. First of all, since Con A binds to any surface component containing α-D-mannose or α-D-glucose residues, the binding reactions are often complex, involving multiple receptors with possibly different properties (28). In many cases such as capping, the observed effect is the end result of multiple, sequential processes, making interpretation difficult. Secondly, quantitation of the lectin reactions is often very difficult. It is critical to the interpretation of these and other experiments con-

cerned with the redistribution of lectin-receptor complexes that lectin accumulations associated with the cell surface be distinguished from those which have been formed intracellularly as a result of direct endocytosis of the bound lectin (57). Considering the difficulties in interpreting the data involving multiple Con A receptors, it would be advantageous to focus on a single Con A receptor.

Since a large number of enzymes, particularly those of plasma membranes, contain associated carbohydrate, it seems likely that lectins such as Con A might be able to alter cell surface enzyme activities. Con A might interact directly with the enzymes or it may act to alter enzymatic activity by interacting with other membrane components. Con A-induced alterations have been demonstrated for a galactosyltransferase (58) and Mg^{++}-ATPase (59). It has also been reported that Con A is able to inhibit 5'-nucleotidase in plasma membrane-enriched fractions of rat mammary gland, demonstrating that 5'-nucleotidase is also a Con A receptor (60). This inhibition was prevented by the presence of α-methylmannoside. The mechanisms of the Con A effects on these enzymes are of interest not only because of the diverse biological activites of Con A, but also because the interactions of Con A with these enzymes may be used as a probe for cell surface alterations and receptor mobility.

Ecto 5'-Nucleotidase as a Cell Surface Reporter Group

The utilization of cell surface enzymes, or ecto-enzymes, as reporter groups for monitoring membrane alterations offers several advantages. First of all, the cell surface enzymes can be assayed in intact cells without cellular disruption or membrane isolation; these methods

tend to alter the systems under investigation. Secondly, several enzymatic properties can be monitored, including enzyme activity, kinetic parameters, allosteric or cooperative effects, and inhibition or activation by external agents. Finally, in order to make comparisons to naturally occurring phenomena, the cells or their membranes can be subjected to defined experimental perturbations. The success of these procedures depends on both the enzymatic sensitivity to environmental changes and the understanding of the nature of the enzymatic responses to cellular perturbing agents (61).

Considering the difficulties in interpreting the redistribution of a heterogeneous population of Con A receptors which possess different properties, it is of great advantage to focus on a single kind of Con A receptor. In light of this advantage and the advantages of employing cell surface enzymes in monitoring membrane changes, the ecto-enzyme 5'-nucleotidase appears to be a likely Con A receptor to study.

5'-nucleotidase, or 5'-ribonucleotide phosphohydrolase (EC 3.1.3.5), was first detected in heart and skeletal muscle in the early 1930's by Reis (62) in France. While studying the deamination of nucleotides in these tissues, Reis discovered that adenosine was produced by the hydrolysis of adenosine 5'-monophosphate and deduced that the reaction was catalyzed by a phosphatase specific for the 5'-monophosphate on the ribose moiety of purine nucleotides. Since the time of its discovery, 5'-nucleotidase has been found to be widely distributed in nature and has been studied in a crude form from a number of sources (63).

The 5'-nucleotidase of rat and mouse liver plasma membranes has been isolated and shown to be a glycoprotein, whose molecular weight has been determined to be 140,000-150,000 by gel filtration and sucrose density

gradient centrifugation (64,65). Recently, 5'-nucleotidase has been purified from the plasma membranes of lymphocytes by utilizing affinity chromatographic techniques, involving Lens culinaris lectin-Sepharose 4B and 5'-AMP-Sepharose 4B columns (66). This enzyme has also been shown to be a glycoprotein with a molecular weight of 130,000. All purification procedures have required the employment of detergents as initial steps in order to solubilize the enzyme which is strongly associated with the membrane.

Although there has been much speculation concerning the function of ecto-5'-nucleotidase, outside of its extracellular hydrolytic action, the results substantiating these speculations are rather sparse. It is generally agreed that nucleotidase is located at the cell surface (67). The localization of nucleotidase at the cell surface has implicated it in adenosine translocation across the plasma membrane. Since cells are generally impermeable to 5'-nucleotides, the translocation process involves the dephosphorylation of nucleotides to the readily transported nucleosides (68). The nucleotidase would therefore increase nucleotides available for uptake into cells. Regulation of this uptake would be very important in some cells, since adenosine produced by the enzyme is vasodilatory and has also been shown to affect cellular cyclic AMP levels (68). It is obvious that the proposed adenosine translocase activity of nucleotidase is not the only available uptake mechanism for nucleosides into cells, since cells which lack significant nucleotidase survive and take up nucleosides (69).

There has also been considerable interest in the utilization of nucleotidase as a clinical marker for various neoplastic and disease states (70). Kim et al. (71) found that the activity of the nucleotidase

decreased with increasing metastatic potential and suggested that the loss in enzymatic activity resulted from a shedding or sloughing of cell surface material in the metastatic cells. This finding was supported recently by Ip and Dao (72) who found increased levels of 5'-nucleotidase in the serum of breast cancer patients with metastasis. The increase in this enzyme in the circulation would presumably indicate accelerated breakdown and release of cell surface material. Sequential studies in cancer patients with liver metastases (70) have also indicated that serum 5'-nucleotidase determinations are useful in following the progress of the disease. Lower serum enzyme levels are found in association with tumor regression after successful courses of radio- or chemotherapy. Levels which fall after therapy and rise once more suggest the need for further therapy. Although it appears that nucleotidase may be of clinical significance as a diagnostic tool in detecting tumor progression and metastases, the loss in nucleotidase has been shown not to be a universal marker for metastasis (73).

Since 5'-nucleotidase is known to be a cell surface constituent and has been shown to be rapidly inhibited by Con A via a direct interaction with a carbohydrate moiety on the nucleotidase (60), the Con A inhibition of the enzyme might be a useful adjunct to the utilization of the nucleotidase as a cell surface reporter group. Con A has also been shown to noncompetitively inhibit 5'-nucleotidase of plasma membrane from rat liver (74), of lymphocytes from pig mesenteric lymph nodes (75), of bovine milk fat globule membrane (76), and of a 13762 rat mammary ascites adenocarcinoma cell line and its isolated membrane envelopes (61). Since the inactivation of the enzyme by Con A occurs at the cell surface, the inhibition process can be studied without prior disruption of the

cell (61,75).

Carraway et al. (61,77) have previously shown that if the data involving the Con A inhibition of the nucleotidase are analyzed by a Hill plot (78) for possible cooperative effects, the calculated Hill coefficient indicates cooperativity which is dependent on conditions that alter the "state" of the isolated membranes. For example, cell surface envelopes prepared from 13762 mammary ascites cells using the Zn^{++}-stabilization technique do not show cooperativity in the inhibition process, exhibiting a Hill coefficient of about 1. These Zn^{++}-stabilized envelopes, which are representative of unfragmented plasma membrane, show much greater quantities of membrane-associated high molecular weight cytoskeletal proteins than do plasma membrane vesicles prepared from the same type of cells (79). Alternatively, membrane vesicles, prepared by EDTA extraction of membrane envelopes, and solubilized nucleotidase, prepared by incubating membrane envelopes with deoxycholate, show differences in the behavior of the nucleotidase toward Con A even though the K_m of the enzyme and the noncompetitive kinetics of the inhibition are essentially unchanged. The Hill coefficient for the inactivation process in solubilized membranes and in membrane vesicles increases, indicating significant cooperativity in the Con A-enzyme interaction (61). The Hill coefficients from previous cell and membrane preparations displaying the cooperative nature of the Con A-nucleotidase interaction in the various membranes can be seen in Table I. It can also be seen in Table I that the kinetic parameters involving the inhibition process remain unchanged among the different membrane perturbations.

Two explanations have been suggested for the apparent cooperative effect (77). The first is the classical explanation involving a change

TABLE I

PROPERTIES OF 5'-NUCLEOTIDASE OF VARIOUS MEMBRANE SOURCES

Membrane Source Perturbation	K_m	Inhibition Characteristics	
		Kinetics	Hill Coefficient
	μM		
13762 Cells[a]	25	NC	1.0
13762 Envelopes[a]	25	NC	1.1
DOC	25	NC	1.9
GEM extraction	25	NC	1.8
MFGM[b]	25	NC	1.0
DOC	25	NC	1.9

NC, noncompetitive; DOC, deoxycholate; GEM, glycine-EDTA-mercaptoethanol.

[a]Carraway et al. (61).

[b]Snow (76).

in the subunit interactions of a multisubunit nucleotidase in the membrane resulting from interactions with the lectin (80). The second explanation involves a Con A-induced association or "clustering" of membrane enzyme molecules due to the multivalence of the Con A molecules—a situation analogous to the patching of Con A receptor sites on cell surfaces and similar to the polymerization model for allosteric effects in enzymes proposed by Nichol et al. (81). This second explanation is consistent with the increased cooperativity observed with the deoxycholate solubilized 13762 rat mammary ascites membrane envelopes, since cooperativity would be favored by increased enzyme mobility. Therefore, induction of cooperativity by Con A-promoted association of the enzyme molecules would be dependent not only on the enzyme structures and interaction with the lectin, but also on the multivalency of the lectin and the control of nucleotidase mobility in the membrane. It is of considerable interest that the EDTA extraction of 13762 cell surface envelopes induces cooperativity, since this treatment has been shown to remove substantial quantities of cytoskeletal proteins from the envelopes, including actin, α-actinin, and actin binding protein (82). These results are of significance in relationship to previous hypotheses concerning transmembrane control of cell surface receptors (27), suggesting that the cytoskeletal proteins may be involved in restrictions on the mobility of 5'-nucleotidase. In this context the change in cooperativity of the lectin-nucleotidase interaction could be explained by the absence of mobility of the nucleotidase in the intact membrane vesicles and cells. This restraint on the mobility of nucleotidase would result from the interactions of cytoskeletal elements with the membrane (61).

5'-Nucleotidase-Membrane Interactions

The studies involving the cooperativity of the Con A-nucleotidase inhibition reaction also suggest an intimate association of the nucleotidase with the membrane. Although the molecular details of the nucleotidase-membrane association are unclear, the properties of several other integral membrane enzymes have been shown to be influenced by their interactions with the membrane. It has been reported that the $(Na^+ + K^+)$-stimulated ATPase activity of the human erythrocyte membrane depends on its association with phosphatidylserine (22). Membrane bound galactosyltransferase, along with many other membrane bound enzymes, loses its catalytic activity when removed from the membrane (83). When the enzyme is replaced in an appropriate lipid environment provided by lipopolysaccharide-phosphatidylethanolamine, not only does it undergo a conformational change in moving from an aqueous to a lipid environment but activity is restored as well. This suggests that induced conformational changes may explain the observed requirement for phospholipids in membrane enzyme systems. Therefore, modification of the lipid composition could play a role in modulating the conformation and thereby regulating the function of membrane enzymes (83).

Purified rat liver 5'-nucleotidase has been shown to contain only sphingomyelin and is inactivated irreversibly upon removal of this lipid. This suggests that sphingomyelin is required for activity (64). The ability to solubilize 5'-nucleotidase with a phosphatidylinositol-specific phospholipase has suggested a role for this phospholipid in the anchoring of the enzyme in the membrane (84). Other studies have found no such specificity for a particular phospholipid (65).

Arrhenius plots have also been used to study enzyme-membrane interactions by looking at the effects of temperature on enzymatic activity. The activity of nucleotidase is modulated by the physical properties of the bilayer in that Arrhenius plots of its activity show a well defined break at 28°C, characteristic of a lipid phase transition occurring in the outer half of the bilayer (85). Addition of the local anesthetic, benzyl alcohol, increases the fluidity of the bilayer and depresses the temperature of the lipid phase separation by about 6°C. This causes an activation of 5'-nucleotidase and a downward shift in the temperature of the break in its Arrhenius plot to about 22°C (85). In this way Arrhenius plots can be utilized to study the intimate associations of enzymes with membranes.

The primary interest in 5'-nucleotidase has been in its use as a "marker" enzyme for isolation of plasma membranes (86). The enzyme is usually treated as a single species in assessing the purity of the membrane isolation. However, there is considerable variability in the properties of the enzyme from different membrane sources (63). For example, multiple forms of 5'-nucleotidase have been shown in milk fat globule membrane (87). Such results also indicate a need to understand the association of nucleotidase with the membrane and the influence of that association on the properties of the enzyme.

Research Objectives

The objective of this study was to develop a greater understanding of the complex processes involved in the control of cell behavior by the cell surface. Utilizing the cooperativity in the Con A inhibition of 5'-nucleotidase, the changes that occur at the cell surface due to exper-

imentally defined perturbations can be monitored. The inhibition of the nucleotidase represents one of the few examples in which cell surface changes can be followed by a single, defined process. This particular study has focused on the cytoskeletal system and the role it plays in the transmembrane control of the cell surface, further testing the validity of the postulations set forth by Carraway et al. (61). If these cytoskeletal proteins do restrict the mobility of 5'-nucleotidase, it should then be possible to induce cooperativity in the enzyme inhibition by treatment of intact cells with specific agents which perturb cytoskeletal structures in the cells.

In order to understand the membrane-nucleotidase association and the effects of this association on the properties of the enzyme, plasma membranes isolated from lactating rat mammary gland have been studied. Previously unpublished observations by C.A.C. Carraway on the isolation and characterization of plasma membranes from rat mammary gland have shown variations in the kinetics of the nucleotidase depending on the membrane fraction and assay techniques used. Therefore, further analyses of the rat mammary membrane fractions have been performed in order to assess the nature of the variations in the kinetics of the nucleotidase. Knowledge of the cytoskeletal-membrane and membrane-nucleotidase interactions may therefore yield a better understanding of membrane forces that are involved in cellular control mechanisms.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

The 13762 mammary ascites adenocarcinomas (Mason Research Institute Tumor Bank, Worcester, Massachusetts), were maintained in Fischer 344 strain female rats (40-45 days old) by intraperitoneal injection of 0.2 to 0.3 ml of ascites fluid from rats 6-10 days post-injection (61). The cells were removed from the peritoneal cavity by aspiration and washed three times in cold Hepes-buffered saline with centrifugation at 210 g·min before use. Lymphocytes were isolated from the spleens of adult rats. BHK-21 was obtained from the American Type Culture Collection, Rockville, Maryland, and maintained in McCoy's 5a modified medium containing ten percent calf serum in the presence of penicillin and streptomycin and grown in a humidified 37°C chamber containing a 95% O_2 and 5% CO_2 atmosphere. Raw cream for the MFGM studies was obtained from the Oklahoma State Dairy. Partially purified rat mammary plasma membranes (F_1 and F_2) were obtained as previously described (88), and subjected to further fractionation by the digitonin shift procedure to yield a highly purified plasma membrane fraction and a Golgi fraction (88).

Cytochalasins, colchicine, dibucaine, theophylline, dibutyryl cyclic AMP, Con A, enzyme substrates, digitonin, detergents, and buffers were

from Sigma Chemical Company. Ionophore A23187 was a gift of Dr. Robert Hosley of Lilly Research Laboratories. Adenosine 5'-mono [^{32}P] phosphate was obtained from Amersham. L-[4,5-^{3}H] Leucine was purchased from ICN. Chemicals for electrophoresis were obtained from Bio-Rad. All other chemicals were reagent grade or highest purity available.

Lumicolchicine was prepared by UV irradiation of a dilute ethanolic solution of colchicine and characterized by its spectrum as reported previously (89).

Methods

5'-Nucleotidase Spectrophotometric Assay

Enzyme activity for ascites cells, MFGM, and lymphocytes was determined by a modification of the coupled assay of Ipata (90), measuring the production of inosine from adenosine at 265 nm in a 3 ml volume containing 50 mM Tris (pH 8.0), 1 mM $MgCl_2$, 125 mM sodium chloride and 4-5 units adenosine deaminase. Samples were preincubated at 37°C before assaying at 37°C with 0.1 mM AMP (61).

5'-Nucleotidase [^{32}P]-AMP Assay

A more sensitive, radioactive assay was required of the rat mammary gland membrane and solubilized fractions in order to observe a wider range of AMP concentrations for kinetic analyses. For the assay of 5'-nucleotidase mammary membranes or solubilized fractions at an appropriate concentration were suspended in assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.0) and incubated for 10 min at 37°C. Subsequently 5 mM β-glycerophosphate was added prior to initiation of the reaction. The reaction

was started by adding AMP (containing 1-10 μCi/ml of [^{32}P]-AMP) to make a total assay volume of 0.4 ml. The reaction was run at the appropriate temperature and terminated by adding 1 ml of ice cold 10% (w/v) trichloroacetic acid solution containing 10% (w/v) acid-washed charcoal. The assay tubes were then centrifuged at 5,000 x g for 15 min to sediment the charcoal, and a 500 μl aliquot of the supernatant in Aquasol-2 was counted in a Packard Tri-Carb Model 3320 Liquid Scientillation Counter. Blanks were made in which no membranes or enzymes were added.

5'-Nucleotidase [^{32}P]-AMP Assay of BHK-21 Fibroblasts Attached to Culture Plates

In the experiments involving BHK-21 fibroblasts the sensitivity of the radioactive assay was still required but the assay had to be modified for cells attached to culture plates. Each assay was performed in a separate 35 mm well of a Costar cluster plate in which the fibroblasts were grown. Fibroblasts in each of the assay wells were washed twice with incubation buffer (50 mM Tris-HCl, 1 mM Mg Cl_2, 125 mM NaCl, pH 7.4) at 37°C. The final wash was aspirated, and the reaction was initiated by adding 1 mM β-glycerophosphate and 0.1 mM AMP (containing 1-10 μCi/ml of [^{32}P]-AMP) to the incubation buffer to make a total assay volume of 1 ml. The reaction was allowed to progress at 37°C and was terminated by the addition of 0.5 ml of 20% TCA. A 1 ml aliquot was transferred to a plastic tube containing 50 mg of acid-washed charcoal and vortexed. The tubes were then centrifuged at 5,000 x g for 15 min to sediment the charcoal, and a 0.5 ml aliquot of the supernatant in Aquasol-2 was counted in a Beckman Model LS-3150 T Liquid Scintillation Counter. Blanks were made in which no cells were present. As described elsewhere in this chapter

the cells were allowed to incorporate [^{3}H]-leucine during their growth in order to quantitate the number of cells present, since counting fibroblasts was difficult. Enzymatic activity was therefore expressed as a function of dpm of ^{3}H present. After taking the 1 ml aliquot from the reaction well for counting ^{32}P, the TCA precipitated material was washed twice with incubation buffer and then solubilized with hot 1% SDS. A 0.5 ml aliquot of this solubilized material was added to a scintillation vial containing 1 ml of NCS tissue solubilizer (Amersham) and incubated for 1 hr at 60°C before adding Aquasol-2. The vials were then placed in the dark overnight before counting in a Beckman Model LS-3150 T Liquid Scintillation Counter.

Maintenance and Preparation of BHK-21 Fibroblasts

BHK-21 fibroblasts were grown to subconfluency in milk dilution bottles on McCoy's 5a modified medium plus ten percent calf serum. The old medium in the bottles was aseptically removed and the cells treated with 3 ml of 0.25% trypsin for 3-5 min in order to help round up and disaggregate cells. This process was monitored with an inverted microscope. Then 10 ml of fresh growth medium was added to each bottle. The cells were harvested by scraping with a rubber policeman. To insure single cells, rather than cell aggregates, the cell-medium suspension was pipetted gently about ten times. Aliquots of the cell suspension were transferred to individual 35 mm wells of Costar cluster plates and were incubated with 5 μCi L-[4,5-^{3}H]-leucine per well in a CO_2-0_2 incubator at 37°C for two and one-half days before assaying.

Preparation of Milk Fat Globule Membranes

Raw cream was washed by three suspensions in 0.25 M sucrose, 10 mM imidazole, 2 mM $MgCl_2$ (pH 7.0) at room temperature (91). The washed cream was suspended to a concentration of 33 percent cream in the same buffer and subjected to one cycle of freezing at -20°C for 20 hr and thawing at 37°C. Membranes were isolated from the thawed 33 percent cream suspension by centrifugation at 40,000 x g for 1 hr at 5°C and washed twice by centrifugation. Membranes were dialyzed overnight against water at 4°C before protein assays were performed.

Isolation of Lymphocytes

Rats were sacrificed and their spleens perfused with Hepes-buffered saline to remove the red blood cells. The spleens were then excised and teased apart with a scalpel. Pieces of solid tissue were pressed through a sterile metal screen bathed in Hepes-buffered saline. This yielded a free cell suspension in Hepes which was then centrifuged at 1,100 x g for 10 min to pellet the cells. The pelleted cells were suspended in 4-5 ml of 10% fetal calf serum in PBS in a glass centrifuge tube and 4-5 ml of Ficoll-Hypaque solution (Sigma) was carefully pipetted to the bottom of the tube. This rendered a discontinuous gradient with the top layer containing the cell suspension and the bottom layer the Ficoll-Hypaque solution. The gradient was centrifuged in a clinical centrifuge at 3650 rpm for 20 min. The centrifuge was accelerated and deaccelerated slowly so as not to disturb the gradient. The upper layer of the gradient was carefully removed, leaving the pure lymphocyte layer undisturbed at the interface. The lymphocytes were then removed with a bent needle syringe

and washed twice in Hepes-buffered saline before counting and assaying for nucleotidase activity. All steps were performed at room temperature.

Isolation and Purification of Rat Mammary Plasma Membrane Fractions

Rat mammary gland tissue was excised from normal lactating rats which were about two weeks postpartum (92). All subsequent steps including the digitonin shift procedure which yielded a highly purified plasma membrane fraction and a Golgi fraction were performed as previously described (88).

Whole Cell and Membrane Perturbations

The various cytoskeletal perturbing drugs, metabolic inhibitors, or detergents were incubated with the particular cells or membranes under investigation for 30 min to 1 hr at 37°C. Subsequently, Con A was added and the mixture incubated an additional 10 min at 37°C prior to assaying for 5'-nucleotidase activity at the same temperature.

The MFGM extractions with 1.5 M $MgCl_2$, 1 M KCl/10 mM Tris (pH 7.4), and glycine-EDTA-mercaptoethanol (pH 9.5) were performed at 4°C for 16 hr. Aliquots from the extraction tubes were incubated for 15 min at 37°C. Con A was then added and incubated another 10 min at 37°C before assaying at the same temperature.

Electrophoresis of MFGM Extracted Samples

MFGM samples were incubated in the various extraction buffers for 16 hr at 4°C and centrifuged at 100,000 x g for 1 hr. The pellets were resuspended in 0.25 M sucrose, 10 mM imidazole, 2 mM $MgCl_2$ (pH 7.0). The

supernates from the extractions were dialyzed and concentrated in a Collodion Bag Apparatus (Schleicher and Schuell, Keene, NH) and the samples solubilized by addition of an equal volume of solution containing 0.125 M Tris, pH 6.8, 3% SDS and 5% β-mercaptoethanol and immersion in a boiling water bath.

Slab gels (13.5 cm x 16.0 cm x 0.75 mm) were prepared with a linear 5-15% acrylamide gradient using the gel buffer system of King and Laemmli (93). A 2 cm 3.5% acrylamide stacking gel was used. Following electrophoresis, samples were soaked in 25% isopropanol-10% acetic acid for 30 min, stained in solution of 25% isopropanol, 10% acetic acid, and 0.025% Coomassie blue R250 for 30 min, and destained in 10% acetic acid (usually complete within 30-45 min). The gel was then soaked in 2-3 changes of 10% methanol and once in 10% methanol, 1% glycerol.

Fluorescence Polarization Measurements of Mammary Membrane Fractions

Mammary membrane fractions were diluted to about 0.5 mg protein/ml. Membranes were suspended in 40 mM Tris, pH 7.4. The fluorophore 1,6-diphenylhexatriene was prepared as a stock solution in 1 mM tetrahydrofuran. Then 10 µl DPH stock solution per mg membrane protein was injected into tubes containing membrane fractions to make a total volume of 1 ml and incubated for 10 min at the appropriate temperature. All fluorescence measurements were made in quartz cuvettes with the samples continuously stirred under constant temperature. Control samples without DPH present were used throughout the experiment to correct for scattered light. Fluorescence measurements were obtained with an instrument built by the OSU Physics Department. Excitation of samples was with a 150 watt

Xenon lamp with 20 nm bandpass monochrometers and appropriate Corning glass color filters. Fluorescent light was detected with a similar monochrometer-filter arrangement and a 1P28 phototube. All excitations were at 360 nm and depolarization measurements were made by passing the entire fluorescence emission band. Fluorescence lifetimes were measured as previously described (94).

Protein Determination

Protein was determined by the method of Lowry et al. (95).

CHAPTER III

RESULTS

Cooperativity of the Concanavalin A Inhibition of 13762 Rat Mammary Adenocarcinoma 5'-Nucleotidase

Effects of Cytoskeletal Perturbants on Cooperativity

Previous studies on the Con A inhibition of 5'-nucleotidase have shown no cooperativity (Hill Coefficient, 1) in intact 13762 rat mammary ascites adenocarcinoma cells and their Zn^{++}-stabilized cell surface envelopes, which retain attached cytoskeletal elements (61). If the enzyme from these envelopes was solubilized in deoxycholate, cooperativity was observed (Hill Coefficient, 2). More significantly, pronounced cooperativity was also observed when envelopes were extracted with glycine-EDTA-mercaptoethanol. This treatment removes substantial quantities of cytoskeletal proteins (82).

Since these results suggest an involvement of the cellular cytoskeleton in the behavior of 5'-nucleotidase, the effects of specific cytoskeletal perturbants on the cooperativity of the Con A inhibition of the enzyme have been investigated. If either MAT-A or MAT-C1 rat mammary ascites cells are treated with an appropriate concentration of colchicine, a microtubule disrupter (36), cooperativity is induced (Figure 1

and Table II). Lumicolchicine, a structural analog of colchicine which does not alter microtubule polymerization (89,96), is not effective in inducing cooperativity (Figure 2 and Table II). Lumicolchicine was employed as a control against any nonspecific membrane effects colchicine might have on the cells. Since lumicolchicine did not induce cooperativity the induction of cooperativity by colchicine was probably due to the drug's disruption of microtubule structures. The concentration dependence of the colchicine effect on the cooperativity parameter, or Hill coefficient, is shown in Figure 3. As can be seen in Figure 3, colchicine exhibits a half maximal effect at a concentration of 0.4 μM. The low concentration necessary for the effect is additional evidence for microtubule disruption rather than some nonspecific perturbation as the cause of the change in cooperativity.

Cytochalasins B and D are effective perturbants of cell morphology as a result of their ability to alter microfilament structures (39). Cytochalasin D apparently has greater specificity for the disruption of microfilament structures, since it is not as effective in inhibiting hexose transport as is cytochalasin B (97). Both cytochalasins B and D were tested on MAT-A and MAT-C1 cells, and cooperativity was induced with both drugs (Table II). Dimethylsulfoxide, in which the cytochalasins were dissolved for addition to the suspended cells, has no effect at the concentrations used for cell incubations. The concentration dependence of the cytochalasin D effect on the cooperativity is shown in Figure 4. As was the case with colchicine, the low half-maximal concentration of 0.3 μM Cytochalasin D, required for the induction of the cooperative effect, suggests a specific microfilament disruption rather than a nonspecific membrane perturbation as the cause of the cooperativity change.

Figure 1. Effect of Colchicine on the Con A Inhibition of the 5'-Nucleotidase of 13762 MAT-C1 Cells. Cells, prepared as described in Experimental Procedures, were suspended in assay buffer (50 mM Tris, 125 mM NaCl, 1 mM $MgCl_2$, pH 8.0) at 2 x 10^5 cells/assay and incubated for 30 min in a shaking water bath at 37°C with 0.01 mM colchicine. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at 37°C. The inset shows the Hill plot (Hill Coefficient, 2.1).

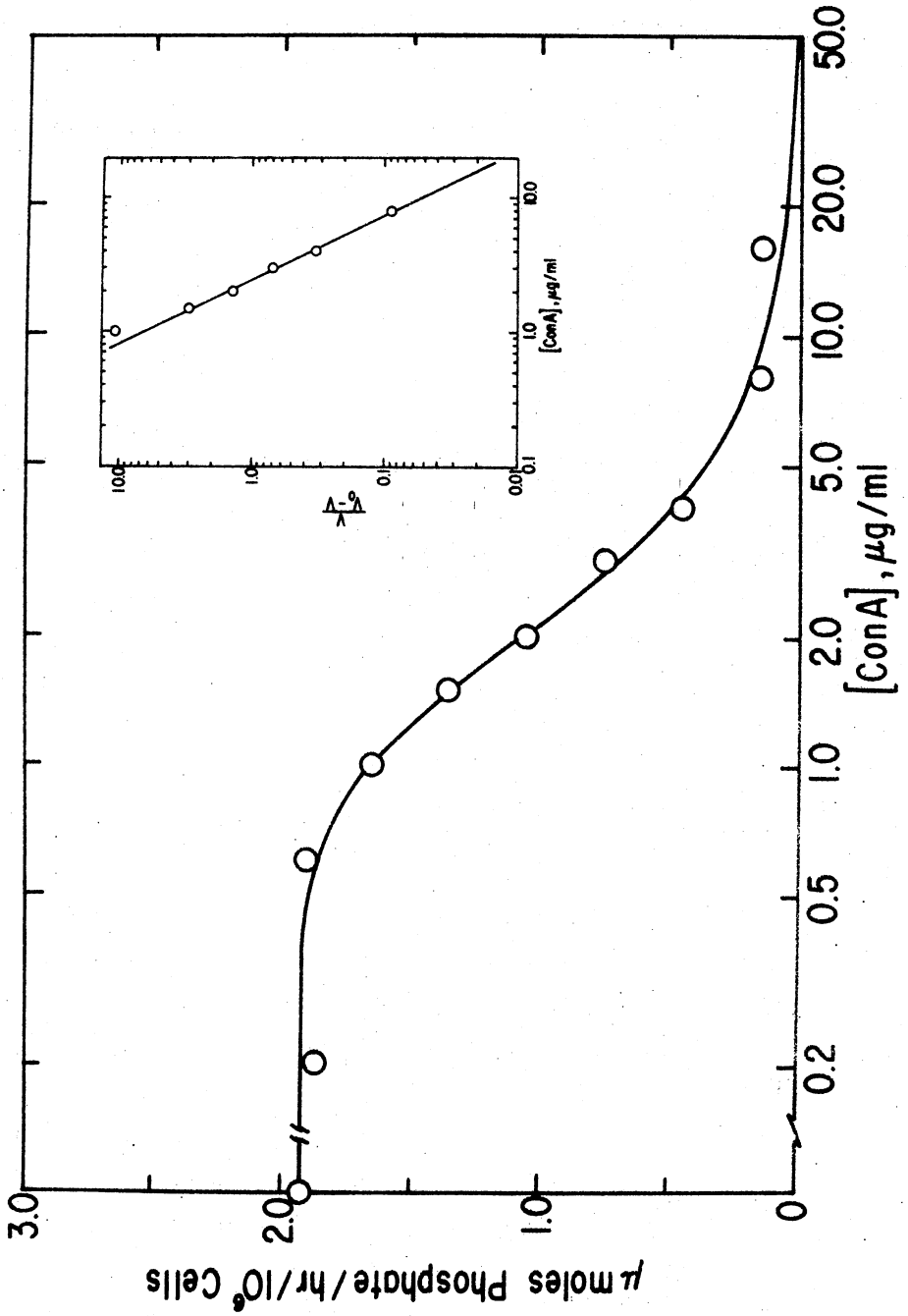

μ moles Phosphate / hr / 10⁶ Cells
3.0
2.0
1.0
0
0.2
0.5
1.0
2.0
5.0
10.0
20.0
50.0
[ConA], μg/ml
ν/ν₀-ν
[ConA], μg/ml

TABLE II

EFFECTS OF CYTOSKELETAL PERTURBANTS ON HILL COEFFICIENTS OF CON A INHIBITION OF 5'-NUCLEOTIDASE OF MAT-A AND MAT-C1 CELLS

Perturbant	Concentration mM	Cell Type	Hill Coefficient
None	----	A	0.87±0.06 (3)
None	----	C1	0.85±0.08 (4)
Cytochalasin B	0.02	A	2.1 ±0.1 (2)
Cytochalasin B	0.02	C1	1.9 ±0.4 (4)
Cytochalasin D	0.02	A	2.1
Cytochalasin D	0.02	C1	2.0
Colchicine	0.1	A	1.9 ±0.05 (3)
Colchicine	0.1	C1	2.05±0.08 (2)
Colchicine	0.01	A	2.05
Colchicine	0.01	C1	2.1
Dibucaine	0.2	C1	2.05±0.14 (2)
Dimethylsulfoxide	28 (0.2%)	C1	0.98
Lumicolchicine	0.1	C1	0.93

*Numbers in parentheses denote number of determinations.

Figure 2. Effect of Lumicolchicine on Con A Inhibition of the 5'-Nucleotidase of 13762 MAT-C1 Cells. Cells were prepared as described in Figure 1 and incubated with 0.1 mM lumicolchicine for 30 min at 37°C. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at 37°C. The inset shows the Hill plot (Hill Coefficient, 0.93).

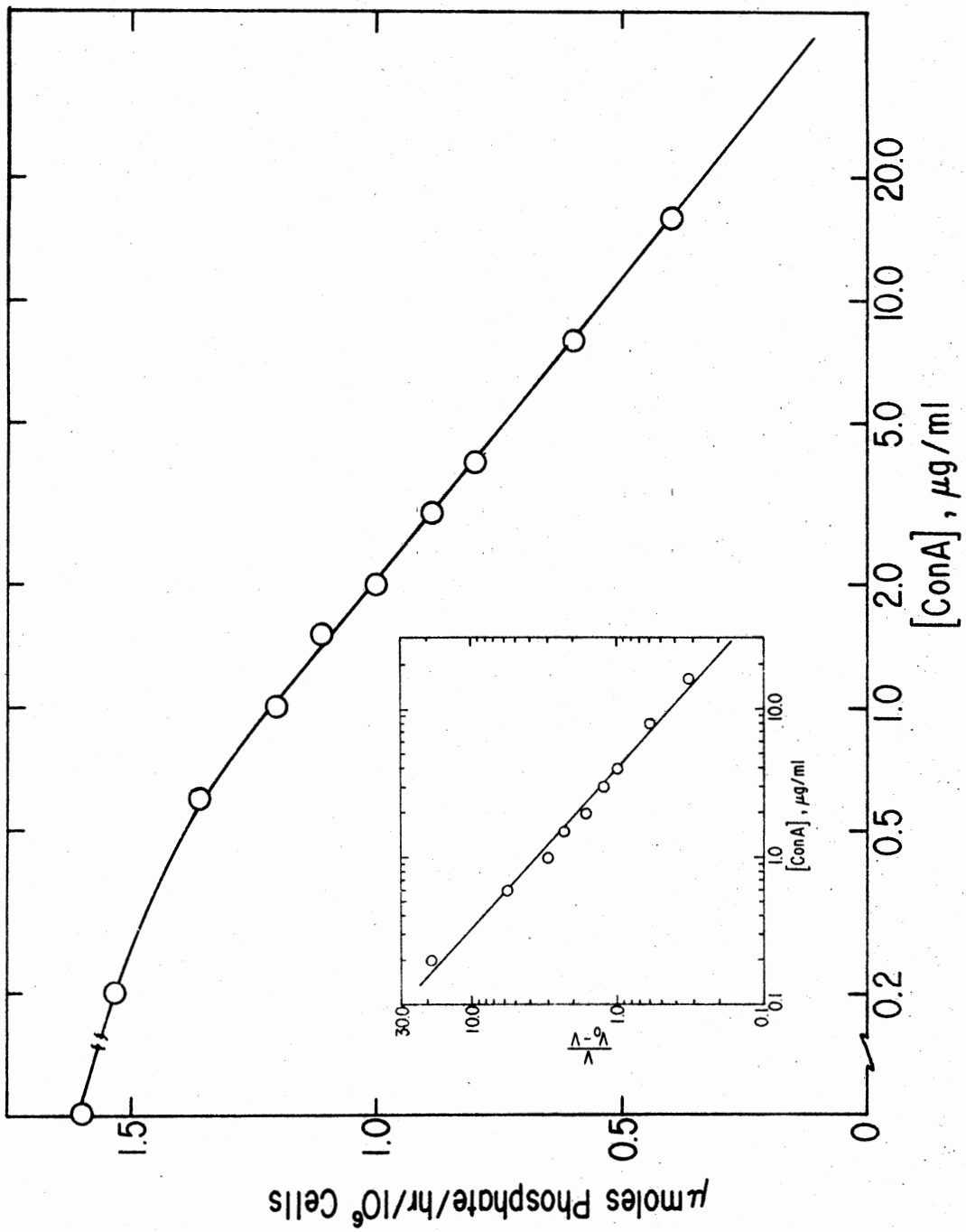

μmoles Phosphate/hr/10⁶ Cells
1.5
1.0
0.5
0
0.2
0.5
1.0
2.0
5.0
10.0
20.0
[ConA], μg/ml
V/V₀-V
30.0
10.0
1.0
0.1
0.1
1.0
10.0
[ConA], μg/ml

Figure 3. Concentration Dependence of the Effect of Colchicine on the Cooperativity of the Con A Inhibition of 5'-Nucleotidase for MAT-C1 Cells.

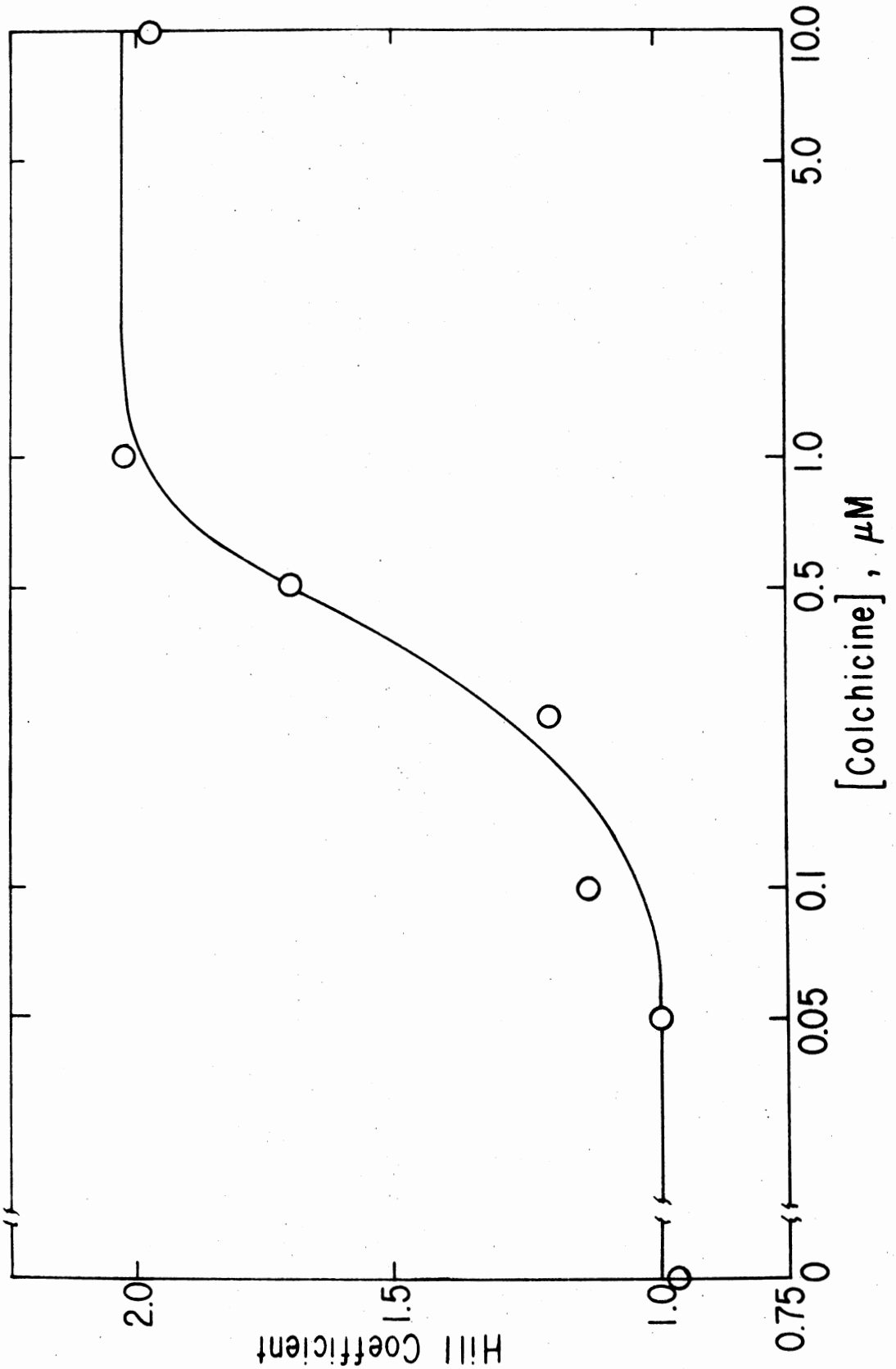
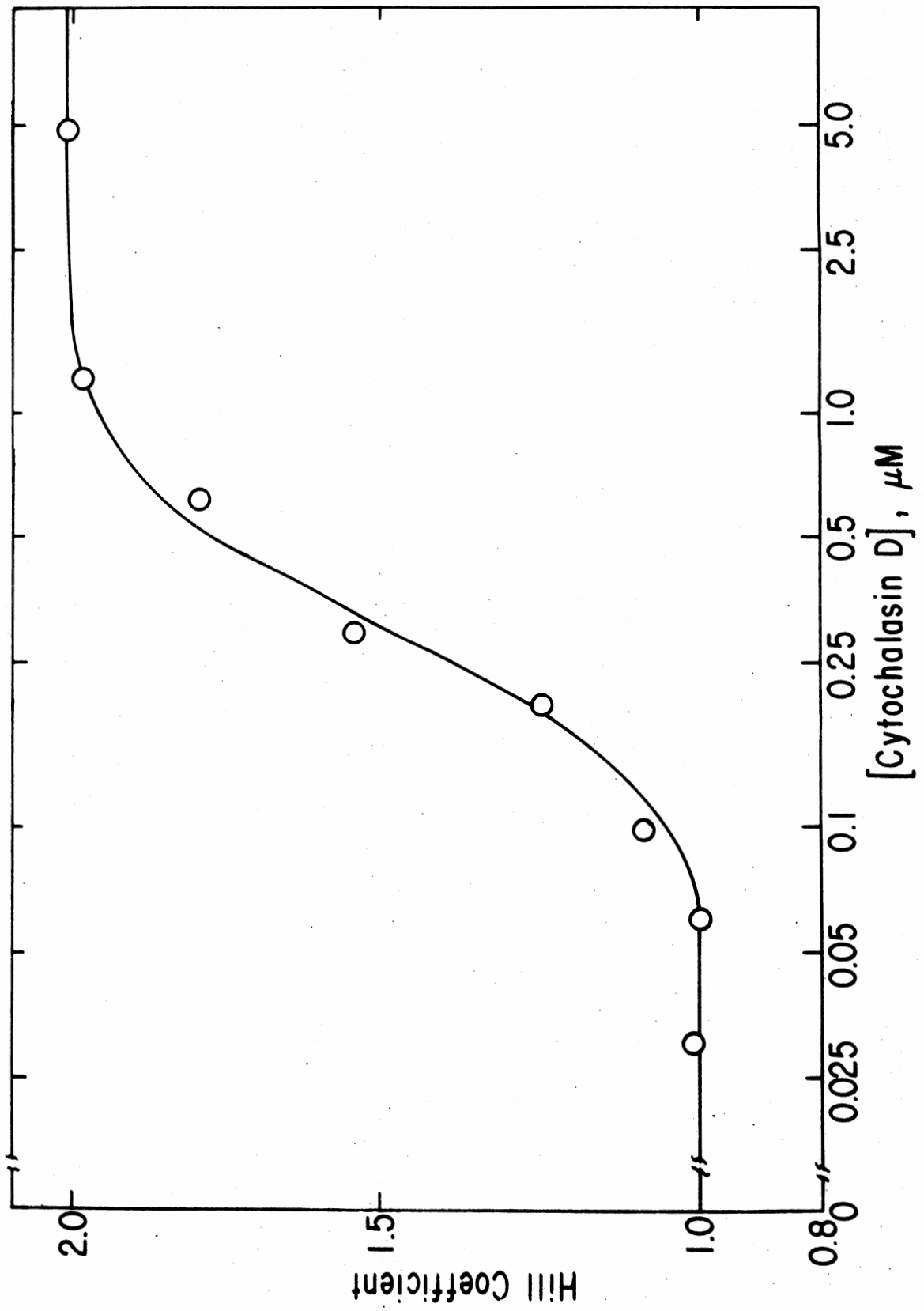

Hill Coefficient
2.0
1.5
1.0
0.75
0
0.05
0.1
0.5
1.0
5.0
10.0
[Colchicine], μM

Figure 4. Concentration Dependence of the Effect of Cytochalasin D on the Cooperativity of the Con A Inhibition of 5'-Nucleotidase for MAT-C1 Cells.


Hill Coefficient
2.0
1.5
1.0
0.8
0
0.025
0.05
0.1
0.25
0.5
1.0
2.5
5.0
[Cytochalasin D], μM

A further indication of the specificity was obtained by comparing the effects of cytochalasins B, D and E at the concentration observed for the half-maximal effect for cytochalasin D (Table III). The order of effectiveness for inducing cooperativity is cytochalasin E>D>B. This order is exactly that found for binding to the spectrin-actin cytoskeletal complex of the erythrocyte membrane (98) and for promoting morphological changes of 3T3 fibroblasts (99).

The local anesthetic dibucaine which is known to disrupt cellular cytoskeletal elements also induces cooperativity as shown in Table II.

Effect of Hypotonic Swelling on Cooperativity of Con A Inhibition of 5'-Nucleotidase

It has recently been shown by Huggins et al. (100) that hypotonic swelling of MAT-C1 cells allowed redistribution and shedding of cell microvilli. Therefore, the effect of this swelling on the cooperativity of the Con A inhibition of 5'-nucleotidase was studied. When the inhibition reaction was examined in hypotonic solutions, it was found that the swollen cells exhibited cooperativity (Table IV). This suggests that swelling perturbs the membrane or its associated cytoskeletal components in such a way as to alter the nature of the Con A inhibition of the enzyme.

When ascites cells were pre-swollen then incubated with 1 mM $ZnCl_2$ for 15 min and subsequently assayed in hypotonic media, cooperativity was not observed (Table IV). The pronounced cooperativity observed with swollen cells is therefore prevented by including Zn^{++} in the incubation mixture. Zn^{++} does not alter the nucleotidase activity significantly in these cells. The mechanism of Zn^{++}-stabilization of the membrane and its

TABLE III

EFFECTS OF THE VARIOUS CYTOCHALASINS ON THE HILL COEFFICIENT FOR INHIBITION OF MAT-C1 5'-NUCLEOTIDASE BY CON A[1]

0.3 μM Cytochalasin	Hill Coefficient
B	0.84
D	1.58
E	1.90

TABLE IV

EFFECT OF HYPOTONIC SWELLING ON THE COOPERATIVITY OF THE INHIBITION OF 5'-NUCLEOTIDASE BY CON A

Treatment	Cell Type	Hill Coefficient
Hypotonic Assay Medium	MAT-C1	2.40±0.02 (2)
Hypotonic Assay Medium	MAT-A	1.92±0.05 (2)
Zn^{2+}-Treated Cells in Hypotonic Assay Medium	MAT-C1	1.10±0.07 (2)
Zn^{2+}-Treated Cells in Hypotonic Assay Medium	MAT-A	1.29±0.01 (2)

*Numbers in parentheses denote number of determinations.

The assay medium contained the same components used with drug treatments except that NaCl and the drug were omitted. The incubations were the same as for Figure 1. The cells which were stabilized in 1 mM $ZnCl_2$ for 15 min (79) were washed and then incubated in the same hypotonic buffer as in the swelling experiment.

associated structures is not well understood, but the data showing that Zn^{++} prevents the induction of cooperativity are consistent with results indicating that Zn^{++}-stabilized ascites cell surface envelopes retain their membrane-associated cytoskeletal proteins (79).

Effects of Increased Intracellular Calcium on Cooperativity

Studies from several groups have suggested that there is a relationship between calcium levels in cells and the organization of the cell surface (101). Cell surface properties which are known to be sensitive to intracellular calcium, such as receptor redistributions, can be altered by treatment of cells with calcium in the presence of ionophores. Calcium has also been shown to prevent the assembly of tubulin into microtubules *in vitro* (36). Therefore, rapid assembly and disassembly of cytoplasmic microtubules is regulated by the intracellular calcium levels which may also affect the contractibility and polymerization of microfilaments (102).

Treatment of MAT-A or MAT-C1 cells with 1.5 μM ionophore A23187 and 2 mM calcium causes pronounced cooperativity in the inhibition of 5'-nucleotidase by Con A as seen in Figure 5 and Table V. If the cells are treated with ionophore alone, 2 mM Ca^{++} alone, or ionophore plus 1 mM EGTA no cooperativity is observed (Table V). These results indicate that the increased intracellular calcium concentration, resulting from the uptake of externally added calcium using ionophore A23187, causes a perturbation of the cell surface which is reflected in the behavior of the Con A-nucleotidase inhibition reaction. The ionophore alone has been shown to release Ca^{++} from intracellular storage compartments (103). However,

Figure 5. Con A Inhibition of 5'-Nucleotidase of 13762 MAT-C1 Cells with or without Ca^{++}. A) MAT-C1 cells were incubated in assay medium containing 1.5 μM ionophore A23187, with no added Ca^{++} for 30 min at 37°C in a shaking water bath. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at the same temperature. The inset shows the Hill plot (Hill Coefficient, 0.92). B) MAT-C1 cells were incubated in assay medium containing 1.5 μM ionophore A23187 and 2 mM $CaCl_2$ for 30 min at 37°C. Con A was added as an additional incubation of 10 min at 37°C was performed prior to assaying at the same temperature. The inset shows the Hill plot (Hill Coefficient, 2.4).

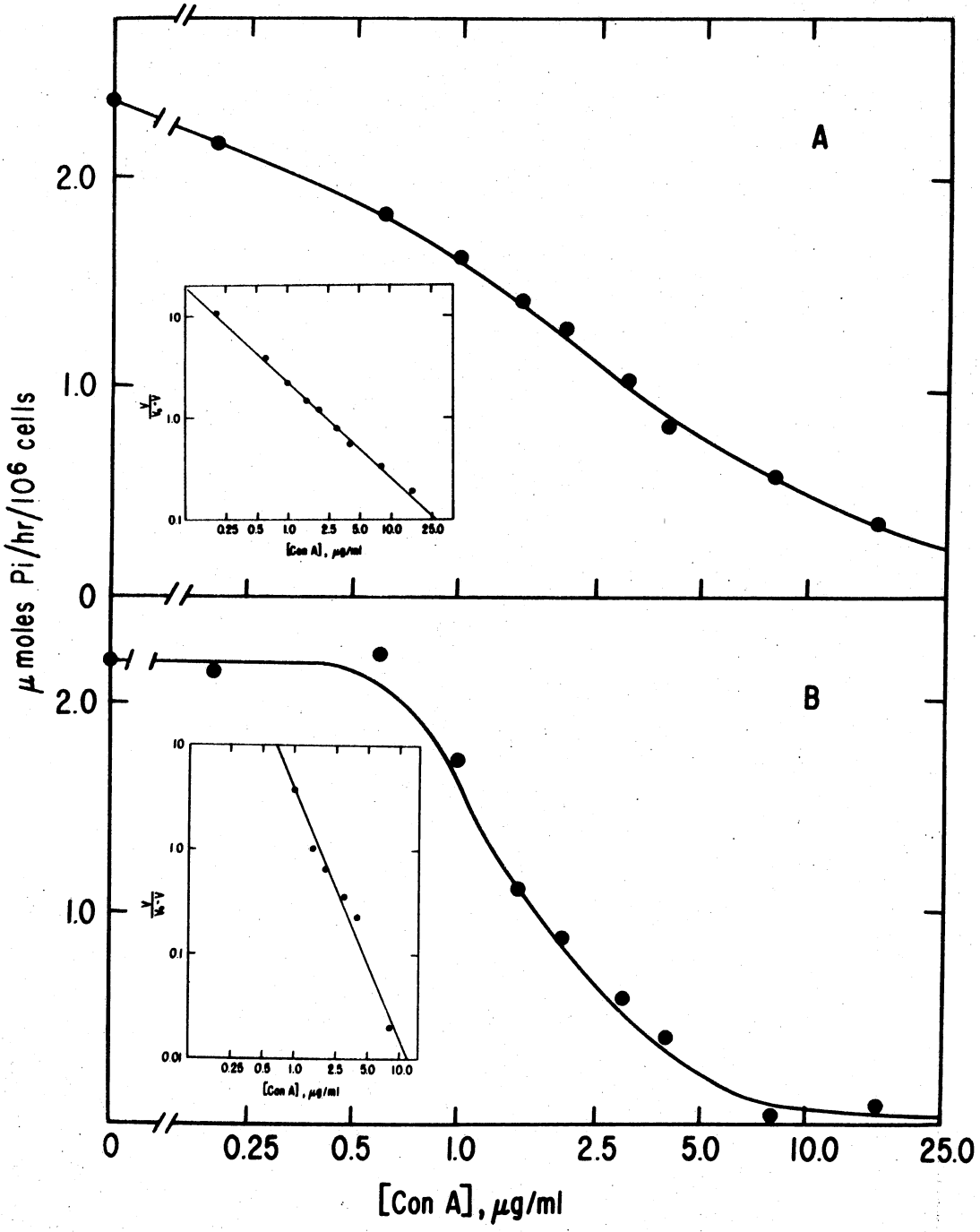

A
B
2.0
1.0
0
μ moles Pi/hr/10⁶ cells
0
0.25
0.5
1.0
2.5
5.0
10.0
25.0
[Con A], μg/ml

TABLE V

EFFECT OF IONOPHORE A23187 AND Ca^{++} ON HILL COEFFICIENTS OF CON A INHIBITION OF MAT-A AND MAT-C1 5'-NUCLEOTIDASE

Treatment	Cell Line	Hill Coefficient
A23187	MAT-A	1.1
2 mM $CaCl_2$	MAT-A	1.1
A23187 + 2 mM $CaCl_2$	MAT-A	2.4
A23187 + 1 mM EGTA	MAT-A	1.1
A23187	MAT-C1	0.92
2 mM $CaCl_2$	MAT-C1	0.75
A23187 + 2 mM $CaCl_2$	MAT-C1	2.4
A23187 + 1 mM EGTA	MAT-C1	1.1

it appears that this release is not sufficient to induce cooperativity.

The concentration dependence of the Ca^{++} effect is shown in Figure 6. Induction of cooperativity occurs between 10 and 50 μM. Since the number of cells present in the assay mixture is small, their contribution to the total Ca^{++} concentration and volume is not significant. Provided there is equilibration across the cellular membranes, the intracellular Ca^{++} concentration should be very close to that added externally.

Effects of Energy Poisons on Cooperativity

As previously discussed, metabolic inhibitors have been shown to inhibit cell surface receptor redistributions, such as the capping of Con A receptors (55). Therefore, ascites cells were incubated with sodium azide, potassium cyanide, and 2-deoxyglucose in order to see if these energy poisons would have an effect on the Con A inhibition of 5'-nucleotidase. Incubation of the cells with either of these compounds resulted in induction of cooperativity, although azide and cyanide showed a more pronounced change than did 2-deoxyglucose (Table VI). Since these poisons affect the cell by inhibiting ATP production, it appears that a metabolically active cell is required for the noncooperative expression of the Con A inhibition of the nucleotidase as seen in unperturbed cells.

Effects of Theophylline and Dibutyryl cAMP on the Cooperativity Change Induced by the Cytoskeletal Perturbants

It has previously been demonstrated that transformed fibroblasts can be changed back to a morphology resembling a normal fibroblast by

Figure 6. Concentration Dependence of the Effect of Ca^{++} on the Cooperativity of Con A Inhibition of 13762 MAT-C1 5'-Nucleotidase. Cells were prepared, incubated and assayed as described in Figure 5, with increasing amounts of $CaCl_2$, added to the incubation medium, containing 1.5 μM ionophore A23187.

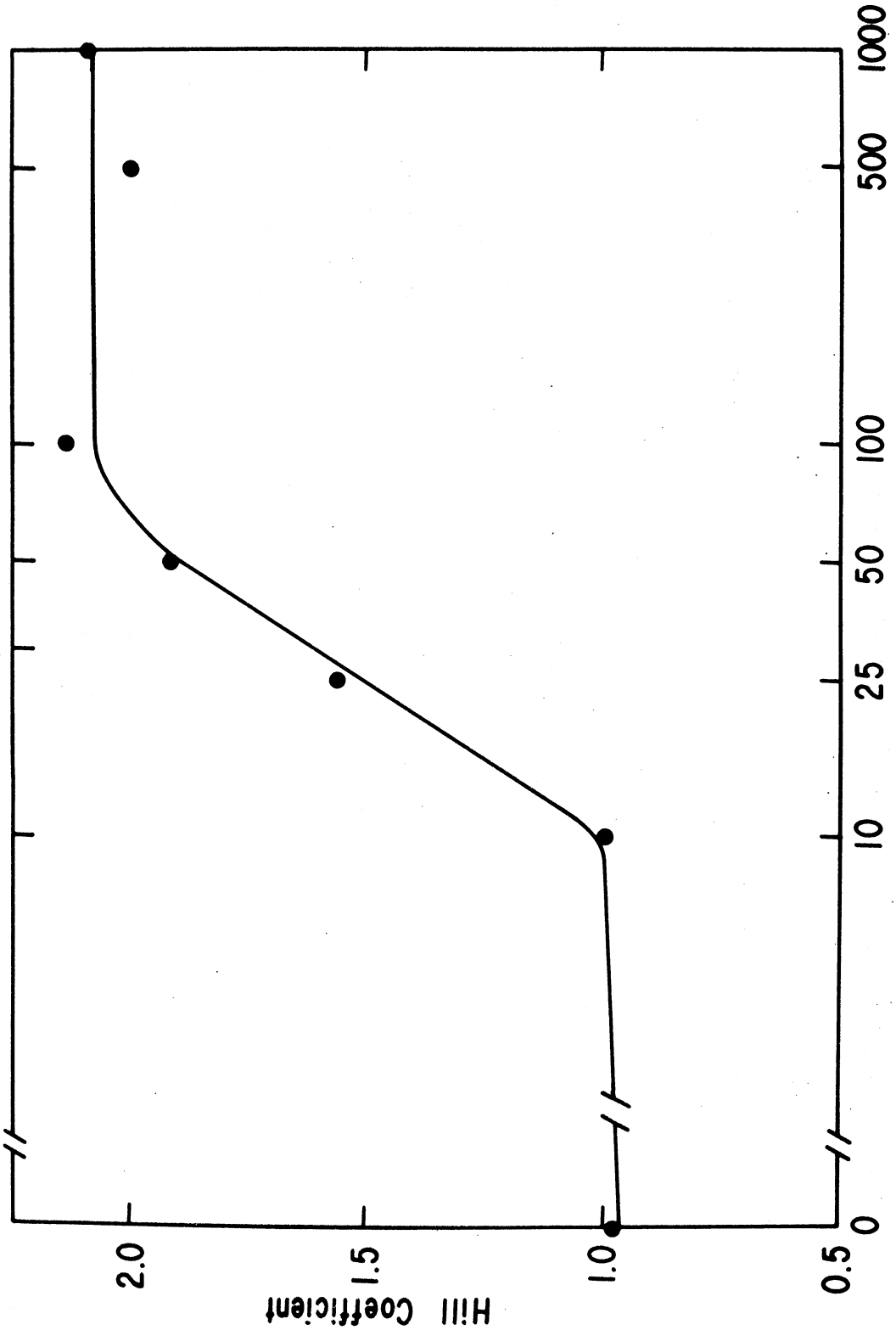

Hill Coefficient
2.0
1.5
1.0
0.5
0
10
25
50
100
500
1000
[Ca2+], μM

TABLE VI

EFFECT OF ENERGY POISONS ON COOPERATIVITY OF CON A INHIBITION OF 5'-NUCLEOTIDASE

Treatment	Hill Coefficient
10 mM NaN_3	2.03
1 mM KCN	2.04
1 mM 2-deoxyglucose	1.53

increasing the cellular cyclic AMP concentration (104). This process of "reverse transformation" involves organization of the network of microtubules and microfilaments throughout the cell cytoplasm. It was proposed that cyclic AMP plays a necessary role in the formation and operation of the cytoskeletal system, and that disruption of the cellular cyclic AMP levels disorganizes the overall cytoskeletal structures (104).

Since alterations in cytoskeletal elements have been shown to be sensitive to cyclic AMP, the ability of drugs that increase the intracellular cyclic AMP levels to alter the induction of cooperativity of the Con A inhibition of 5'-nucleotidase have been studied. Theophylline, which inhibits cyclic AMP phosphodiesterase (105), and dibutyryl cyclic AMP have been utilized in this study. Both drugs are known to increase cell cyclic AMP levels (104,105). Theophylline or dibutyryl cyclic AMP alone has little effect on the cooperativity parameter (Table VII). If cells are preincubated with 0.1 mM theophylline or dibutyryl cyclic AMP before addition of cytochalasin or colchicine, the ability of the cytoskeletal perturbants to induce cooperativity is prevented (Table VII). These results suggest that an increase in cellular cyclic AMP is able to overcome the effects of the cytoskeletal perturbants on the cooperativity of the nucleotidase inhibition. This provides further support for previous studies that have implied cytoskeletal responsiveness to changes in cellular cyclic AMP.

TABLE VII

EFFECT OF THEOPHYLLINE AND DIBUTYRYL CYCLIC AMP ON HILL COEFFICIENT OF MAT-C1 CELLS INCUBATED WITH COLCHICINE OR CYTOCHALASIN D

Treatment	Hill Coefficient
0.02 mM Cytochalasin D	2.0
0.01 mM Colchicine	2.1
0.1 mM Theophylline	1.18
0.1 mM Theophylline + 0.02 mM Cytochalasin D	1.03
0.1 mM Theophylline + 0.01 mM Colchicine	1.00
0.1 mM Dibutyryl cAMP	1.26
0.1 mM Dibutyryl cAMP + 0.01 mM Colchicine	1.10
0.1 mM Dibutyryl cAMP + 0.02 mM Cytochalasin D	0.92

*MAT-C1 cells were incubated in assay buffer containing 0.1 mM theophylline or dibutyryl cyclic AMP for 15 min at 37°C followed by an additional 30 min at 37°C with or without cytochalasin D or colchicine. Subsequently, Con A was added to the tubes which were incubated another 10 min at 37°C before assaying at the same temperature.

Effects of Cytoskeletal Perturbants on the Cooperativity of the Concanavalin A Inhibition of BHK-21 Fibroblast 5'-Nucleotidase

BHK-21 fibroblasts were studied in order to determine whether 5'-nucleotidase could be utilized as a reporter of cell surface alterations in other cell types. A radioactive assay utilizing 5'-[^{32}P]-AMP was employed for greater sensitivity in these studies. The 5'-nucleotidase reaction was linear with time under all conditions used. Con A treatment of BHK-21 attached to culture plates resulted in essentially complete inhibition of 5'-nucleotidase that displayed no cooperativity (Table VIII). The inhibition was reversed with α-methyl mannoside, indicating a specific interaction of Con A with carbohydrate on the enzyme. Treatment of BHK-21 with 10 μM colchicine caused a substantial induction in cooperativity (Table VIII), although the drug did not have as drastic an effect on the cooperativity as it did with the ascites cells (Table II). Incubation of BHK-21 with 20 μM cytochalasin D produced only a slight increase in cooperativity (Table VIII) which was markedly reduced from that observed with the ascites cells (Table II). Therefore, it seems as though the cooperativity of the Con A inhibition of 5'-nucleotidase may also be used as a monitor of cell surface perturbations in these cells. Although the BHK-21 fibroblasts are not as responsive to cytoskeletal perturbants, as are the 13762 ascites cells, the results further support the idea that the alterations in nucleotidase cooperativity may be a fundamental parameter responsive to changes in cellular regulatory mechanisms which affect transmembrane control of the cell surface.

TABLE VIII

EFFECTS OF CYTOSKELETAL PERTURBANTS ON HILL COEFFICIENTS OF CON A INHIBITION OF 5'-NUCLEOTIDASE IN BHK-21 FIBROBLASTS

Perturbant	Concentration mM	Hill Coefficient
None	----	0.98
Colchicine	0.01	1.71
Cytochalasin D	0.02	1.48

BHK-21 fibroblasts were incubated with the drug for 30 min at 37°C. Subsequently Con A was added and the cells incubated an additional 10 min before initiating the reaction with 0.1 mM [^{32}P]-AMP. The reaction was allowed to proceed for 20 min before terminating with 20% TCA.

Effects of Cytoskeletal Perturbants and Extraction of Putative Cytoplasmic Surface Coat Proteins on the Cooperativity of the Con A Inhibition of Bovine MFGM 5'-Nucleotidase

The bovine MFGM is useful for studies on certain membrane components and functions because it can be obtained in large quantities. The fat globules of bovine milk are surrounded by a membrane (MFGM), derived primarily from plasma membranes during milk fat secretion from secretory mammary cells (106). This process has been described previously (76). Although MFGM does not contain detectable actin and probably has no tubulin, it does show fibrillar material attached to the previous cytoplasmic membrane surface (106). The nature and function of this material is unknown. Keenan (107) has been able to extract fibrillar material from MFGM and show similarities to actin filaments, although no actin polypeptide was detected by electrophoresis. It has previously been noted that Con A inhibition of MFGM 5'-nucleotidase does not exhibit cooperativity (76). Thus, it seems likely that the membrane-associated fibrillar proteins must exert restraints upon the membrane components in this system also. Since the components responsible for this restriction are not known, extraction and cytoskeletal perturbation studies have been performed to determine if cooperativity can be induced and related to any particular polypeptides.

Neither colchicine nor cytochalasins induced cooperativity of the Con A inhibition of MFGM 5'-nucleotidase (Table IX). However, extraction of MFGM with glycine-EDTA-mercaptoethanol does induce cooperativity

TABLE IX

EFFECTS OF CYTOSKELETAL PERTURBANTS AND EXTRACTION ON THE COOPERATIVITY OF THE INHIBITION OF BOVINE MFGM 5'-NUCLEOTIDASE BY CON A

	Hill Coefficient
10 μM Colchicine	1.11
100 μM Colchicine	1.24
10 μM Cytochalasin D	1.08
20 μM Cytochalasin D	1.14
5 mM Glycine-1 mM EDTA mM β-Mercaptoethanol, pH 9.5	1.85 (3)
1.5 M $MgCl_2$	1.10 (2)
1 M KCl-10 mM Tris, pH 7.4	1.20 (2)

*Numbers in parentheses denote number of determinations.

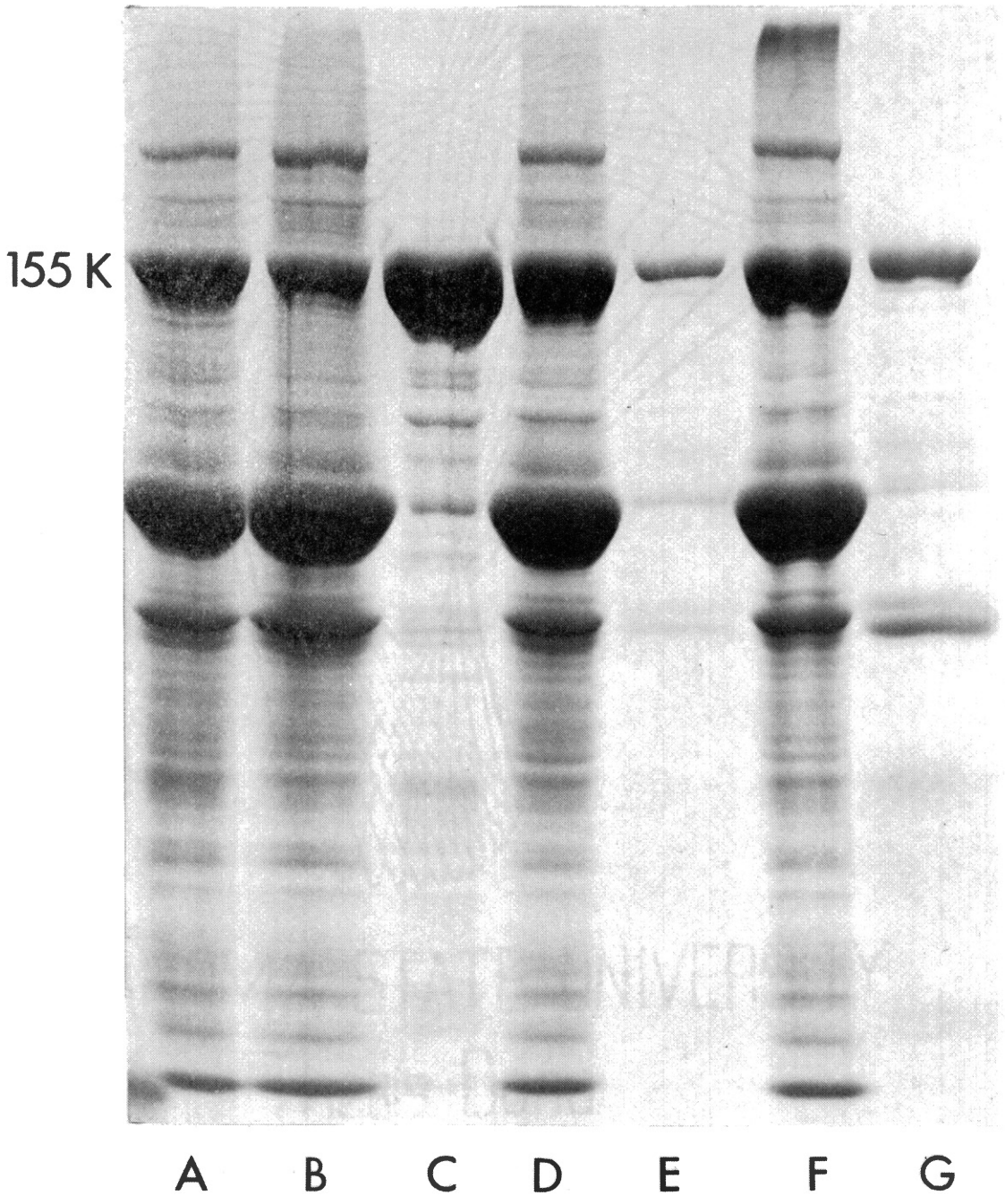
(Table IX), as it does with 13762 ascites cell surface envelopes. Since the MFGM does not contain significant quantities of the cytoskeletal proteins which are removed by extraction from ascites membranes, it was of interest to determine the components that are extracted from MFGM. Electrophoresis on polyacrylamide gels in dodecyl sulfate showed that the predominant component eluted from MFGM was one with a molecular weight of 155,000 (Figure 7).

The induction of cooperativity is not due to nonspecific membrane perturbation by extraction. Neither 1.5 M $MgCl_2$ nor 1 M KCl, which have also been used to extract MFGM components (91,108), were effective in inducing cooperativity (Table IX). These treatments also did not extract significant quantities of the 155,000 dalton polypeptide (Figure 7).

Cooperativity of the Concanavalin A Inhibition of Lymphocyte 5'-Nucleotidase

It was previously reported that the Con A inhibition of 5'-nucleotidase in intact lymphocytes and plasma membranes displayed high positive cooperativity (75). The inhibition of the purified 5'-nucleotidase from lymphocytes by Con A was shown to be a non-competitive process which exhibits no cooperativity. These results are very interesting since they are directly opposite to the noncooperative behavior seen with the 13762 ascites cells, the MFGM and the BHK-21 fibroblasts. The Con A inhibition of 5'-nucleotidase in intact ascites cells, MFGM, and BHK-21 did not exhibit cooperativity, while the solubilized enzyme displayed cooperativity. Therefore, it was of interest to see under what conditions the highly cooperative Con A inhibition of 5'-nucleotidase in intact lymphocytes could be reversed and made noncooperative.

Figure 7. Electrophoresis of MFGM Extracted with Glycine-EDTA-Mercaptoethanol, $MgCl_2$, or KCl. A) MFGM; B) glycine-EDTA-mercaptoethanol pellet; C) glycine-EDTA-mercaptoethanol supernatant; D) $MgCl_2$ pellet; E) $MgCl_2$ supernatant; F) KCl pellet; G) KCl supernatant.


155 K
A
B
C
D
E
F
G

Incubation of the lymphocytes from rat spleen with 20 μM cytochalasin D was unable to prevent the cooperative effect seen with the intact cells (Table X).

Treatment of the lymphocytes with 0.1 mM theophylline also produced no decrease in the cooperativity. However, the Con A inhibition of the enzyme in cells treated with 1.0 mM theophylline or 1.0 mM dibutyryl cyclic AMP showed no cooperativity (Table X). Therefore, the highly cooperative nature of the inhibition process in intact lymphocytes can be prevented by drugs that increase intracellular cyclic AMP levels. This further supports the role cyclic AMP may play in regulating cytoskeletal integrity.

The Influence of the Membrane on the Properties of 5'-Nucleotidase

The results illustrating the cooperative nature of the Con A inhibition of 5'-nucleotidase indicate a need to understand the association of nucleotidase with the membrane and the influence of that association on the properties of the enzyme. Toward this end a different system, plasma membranes isolated from normal lactating rat mammary gland, have been studied.

Multiple Enzyme Forms in Partially Purified Mammary Membrane Fractions

Plasma membrane-enriched material from the lactating rat mammary gland is obtained in a light fraction (F_1) and a heavy fraction (F_2) after flotation of a microsomal preparation on a discontinuous sucrose gradient (92). In order to increase the range of substrate concentra-

TABLE X

EFFECTS OF VARIOUS DRUG TREATMENTS ON COOPERATIVITY OF CON A INHIBITION OF LYMPHOCYTE 5'-NUCLEOTIDASE

Treatment	Concentration mM	Hill Coefficient
None	----	2.10 (3)
Cytochalasin D	0.02	1.89 (2)
Theophylline	0.1	1.91 (2)
Theophylline	1.0	1.16 (2)
Dibutyryl Cyclic AMP	1.0	1.12

*Numbers in parentheses denote number of determinations.

Lymphocytes were incubated with the drug for 30 min at 37°C. Then aliquots were taken and incubated with Con A in assay media for 10 min at 37°C before assaying at the same temperature with 0.1 mM AMP.

tions and the sensitivity of the nucleotidase assay in these membrane fractions, a radiochemical assay for the cleavage of [^{32}P]-AMP was used. The assay was linear with time under all conditions used. The enzyme exhibited a broad pH optimum (6.5-8) over the entire range of substrate concentrations. When the partially purified rat mammary plasma membrane F_1 fraction (0.9-32% interface) was assayed by the radiochemical procedure, complex kinetic behavior was observed for the 5'-nucleotidase. The Lineweaver-Burk plot of the activity exhibited two distinct lines (Figure 8). This suggested the presence of two enzyme forms with apparent Km's of 25 and 130 μM.

When kinetic analyses were performed on the F_2 fraction (32-36% interface), an essentially hyperbolic response was observed for the substrate concentration dependence of the nucleotidase activity. Analysis by the Lineweaver-Burk plot revealed only one enzyme form with a K_m of 27 μM, essentially identical to the low K_m form of the enzyme in F_1 (Figure 9). It should be noted that the Lineweaver-Burk plot shows a slight "tailing" at the high substrate end. This suggests the presence of a small amount of enzyme with a higher K_m and may be due to some F_1 membrane contamination. In fact, the F_2 fraction, upon recentrifugation, will re-equilibrate to yield some F_1 (109).

5'-Nucleotidase Behavior in Membrane Fractions Purified by Digitonin Shift Fractionation

The studies involving the kinetics of the nucleotidase in the partially purified mammary membrane fractions suggest that there are two forms of the enzyme present in the F_1 fraction. It has previously been shown that the partially purified F_1 fraction contains two different

Figure 8. Lineweaver-Burk Plot of F_1 Membrane Fraction 5'-Nucleotidase Kinetics. Membranes were prepared, incubated and assayed as in Experimental Procedures. Membrane protein concentration was 22 µg/assay.

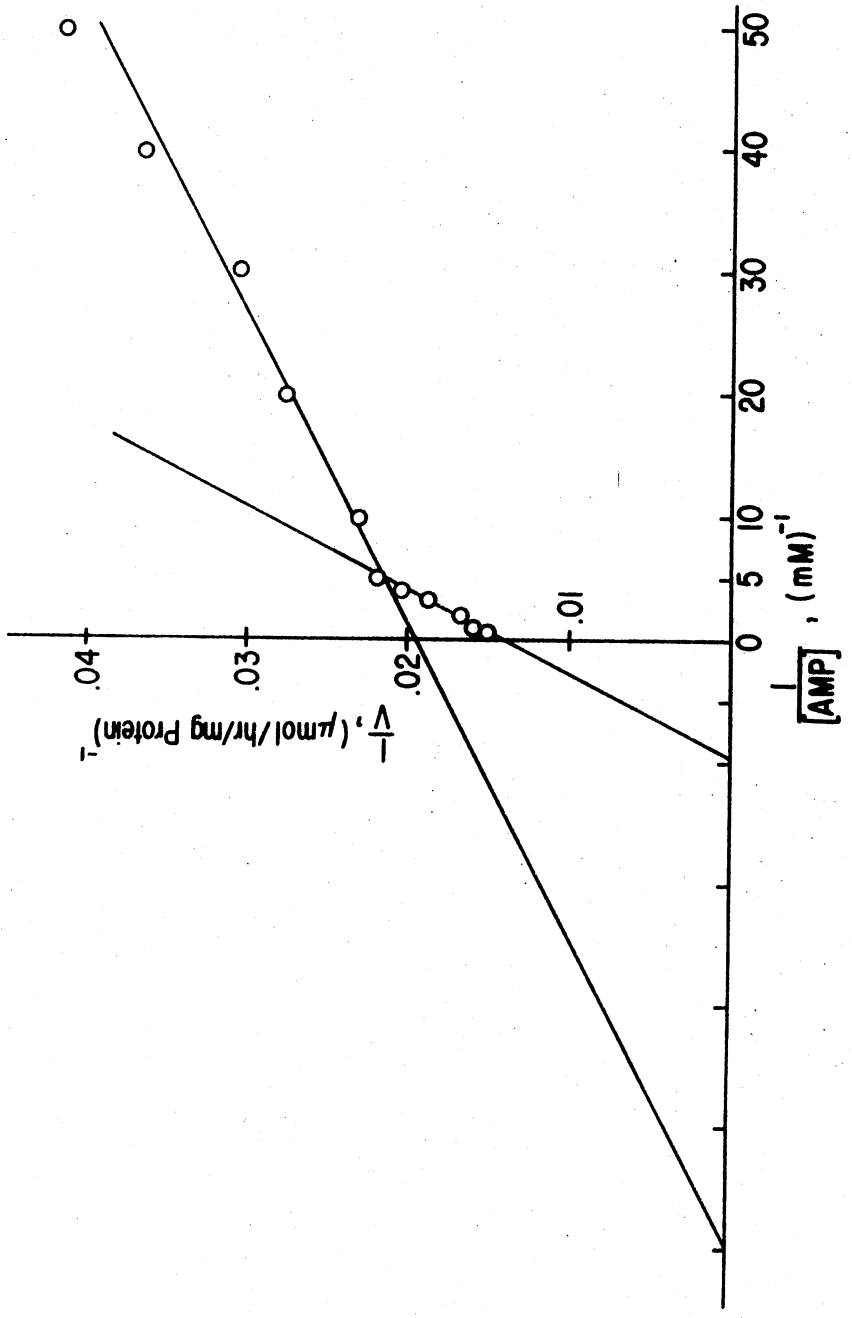
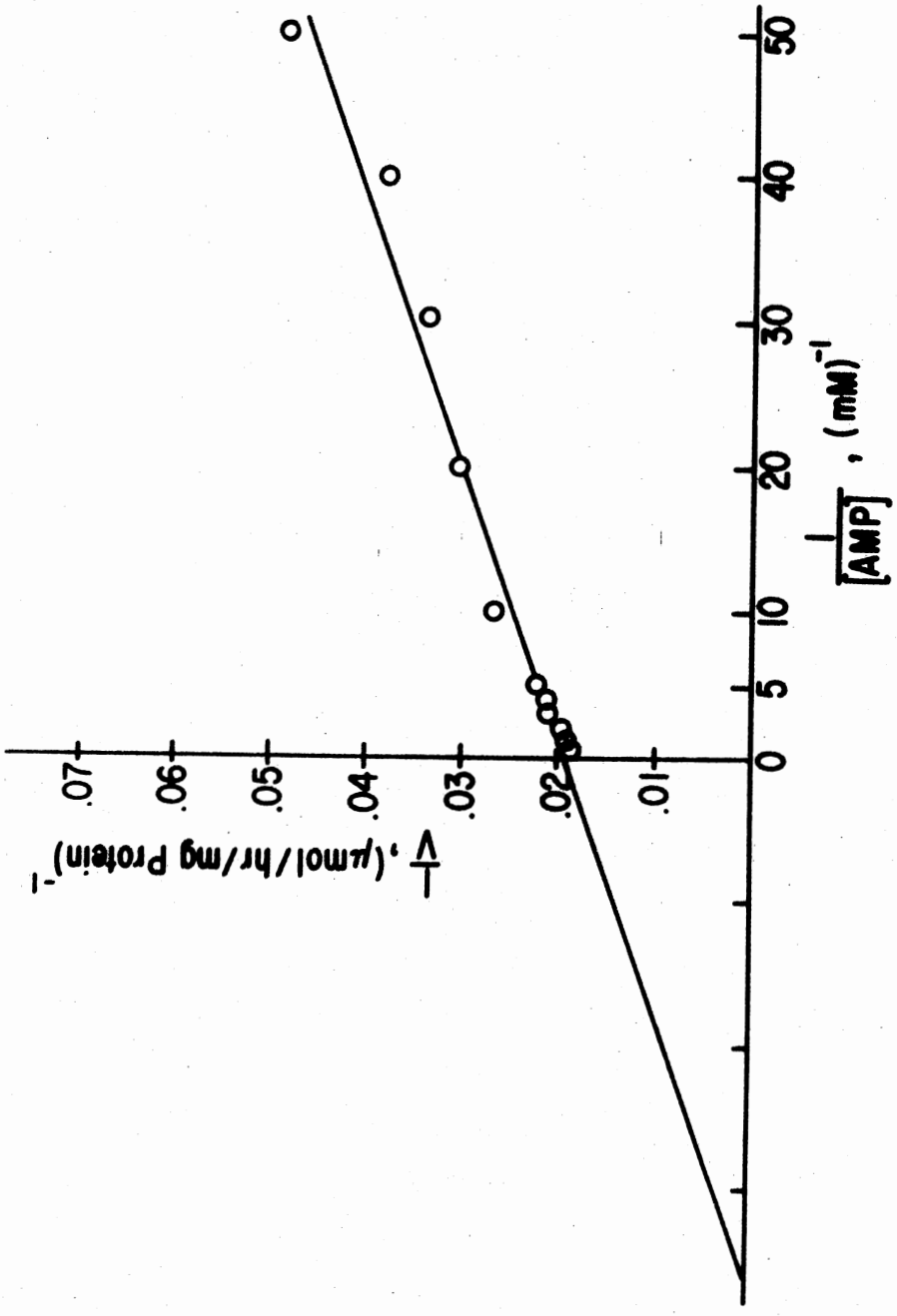

.04
.03
.02
.01
0
5
10
20
30
40
50
1/V, (μmol/hr/mg Protein)^-1
1/[AMP], (mM)^-1

Figure 9. Lineweaver-Burk Plot of F_2 Membrane Fraction 5'-Nucleotidase Kinetics.


$\frac{1}{V}$, (μmol/hr/mg Protein)$^{-1}$
.07
.06
.05
.04
.03
.02
.01
0
5
10
20
30
40
50
$\frac{1}{[AMP]}$, (mM)$^{-1}$

types of membranes, which can be separated by digitonin treatment and reflotation on a density gradient (88). One of the membrane types (F_1DF_3) is isolated from the 36-40% interface of the digitonin-treated density gradient and possesses properties expected of a highly purified plasma membrane. The other membrane type (F_1DF_1) is taken from the 0.9-32% interface and has properties expected of Golgi elements. In order to determine if the two nucleotidase forms are segregated by this digitonin treatment, it was first necessary to determine if the digitonin by itself altered the enzyme behavior.

Figure 10 shows the Lineweaver-Burk plot for digitonin-treated F_1 membranes, demonstrating that digitonin did not alter the biphasic nature of the F_1 kinetics. The two K_m's remained essentially unchanged as seen in Table XI.

Separate kinetic analysis of the purified plasma membrane (F_1DF_3) and Golgi (F_1DF_1) fractions indicates that each form of the nucleotidase is present in a different type of membrane (Table XI). The low K_m form is associated with the putative Golgi fragments, while the high K_m form is associated with putative plasma membrane.

Treatment of the partially purified F_2 fraction with digitonin and reflotation on a sucrose density gradient also yields a plasma membrane fraction with an increased density, designated F_2DF_3 (36-40% interface). The nucleotidase of the F_2DF_3 fraction has a K_m of 26 μM, essentially unchanged from the parent F_2 fraction (Table XI).

Effects of Membrane Solubilization on the Enzyme Forms

There are two likely explanations for the different enzyme forms

Figure 10. Lineweaver-Burk Plot of Digitonin-Treated F_1 Membrane 5'-Nucleotidase Kinetics.

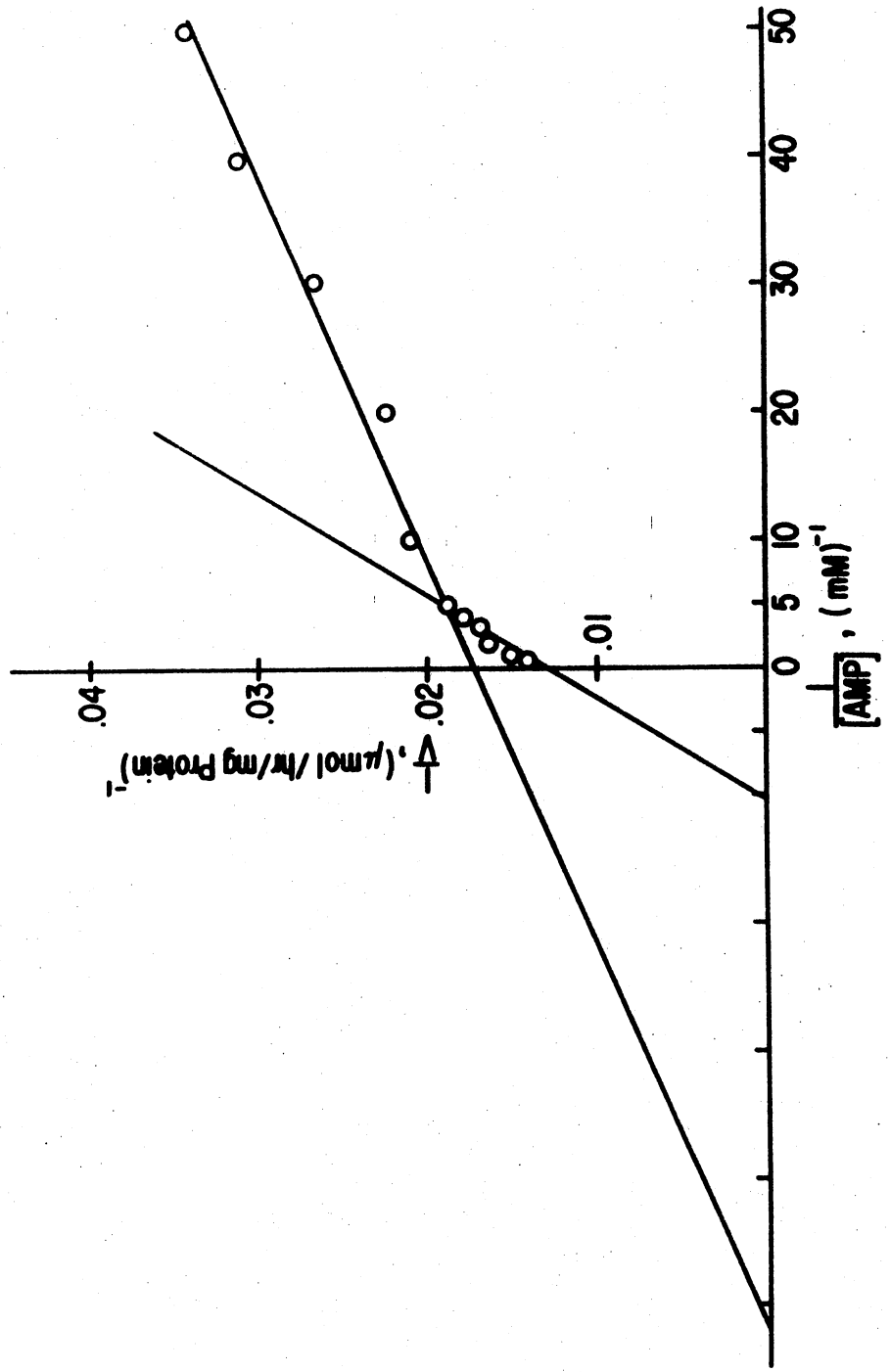

.04
.03
.02
.01
0
5
10
20
30
40
50
[AMP], (mM)$^{-1}$
$\frac{1}{V}$, (μmol /hr/mg Protein)$^{-1}$

TABLE XI

K_m VALUES FOR 5'-NUCLEOTIDASE OF MEMBRANE AND SOLUBILIZED FRACTIONS OF NORMAL RAT MAMMARY GLAND

Membrane Fraction	K_m
	μM
F_1 High K_m	130
F_1 Low K_m	22
F_1 + DOC	28
F_1 + TX-100	23
F_1 + Digitonin:	
High K_m	120
Low K_m	18
F_1DF_1	28
F_1DF_1 + TX-100	21
F_1DF_3	120
F_1DF_3 + DOC	37
F_1DF_3 + TX-100	34
F_2	27
F_2DF_3	26

observed in the F_1DF_3 and F_1DF_1 membrane fractions. 1) Two different 5'-nucleotidase entities exist with different properties, also known as isoenzymes. 2) There is only one type of 5'-nucleotidase, but its properties vary with its mode of association with the membrane and with the properties of the membrane. If the second explanation is correct, it should be possible to eliminate the observed differences between the two enzyme forms by disrupting the membrane structure and thus freeing the enzyme from its membrane-bound environment. This can be accomplished by utilizing detergents for solubilizing the membrane structure. Two different detergents, deoxycholate and Triton X-100, with different solubilizing properties were employed in separate solubilization experiments in order to reduce the chance that the detergent caused its effect by acting directly on the enzyme.

Figure 11 shows the 5'-nucleotidase activity of the F_1 fraction solubilized by deoxycholate. The solubilization procedure released more than 80% of the enzyme activity from the membrane. The high K_m and the low K_m forms were assayed at 1.0 mM and 0.1 mM AMP respectively. Although Figure 11 only shows the results of the solubilization of the low K_m form, the solubilization curve of the high K_m form was identical. It can be seen from the solubilization curve that 1% DOC is required for maximum solubilization of the enzyme. Kinetic studies of the soluble enzyme from the DOC-treated F_1 membranes showed essentially hyperbolic behavior. Lineweaver-Burk analysis of the kinetics (Figure 12) indicated a K_m of 28 μM (Table XI). Thus, the low K_m enzyme predominated, although there was again a slight "tailing" at higher substrate concentrations. This "tailing" indicated the presence of a minor amount of the high K_m enzyme, possibly the result of incomplete solubilization. Incubation of

Figure 11. Solubilization of 5'-Nucleotidase Activity with Deoxycholate. F_1 membranes were suspended in 50 mM Tris (pH 8.0) containing various concentrations of DOC. After a 1 hr incubation at 37°C, the suspensions were centrifuged at 40,000 x g for 2.5 hr. The supernatant was assayed for 5'-nucleotidase activity in the presence of 0.1 mM AMP (containing 1-5 μCi/ml [^{32}P]-AMP. This is referred to as the solubilized activity (Δ-Δ). The total activity (0-0) represents the activity present before the centrifugation.

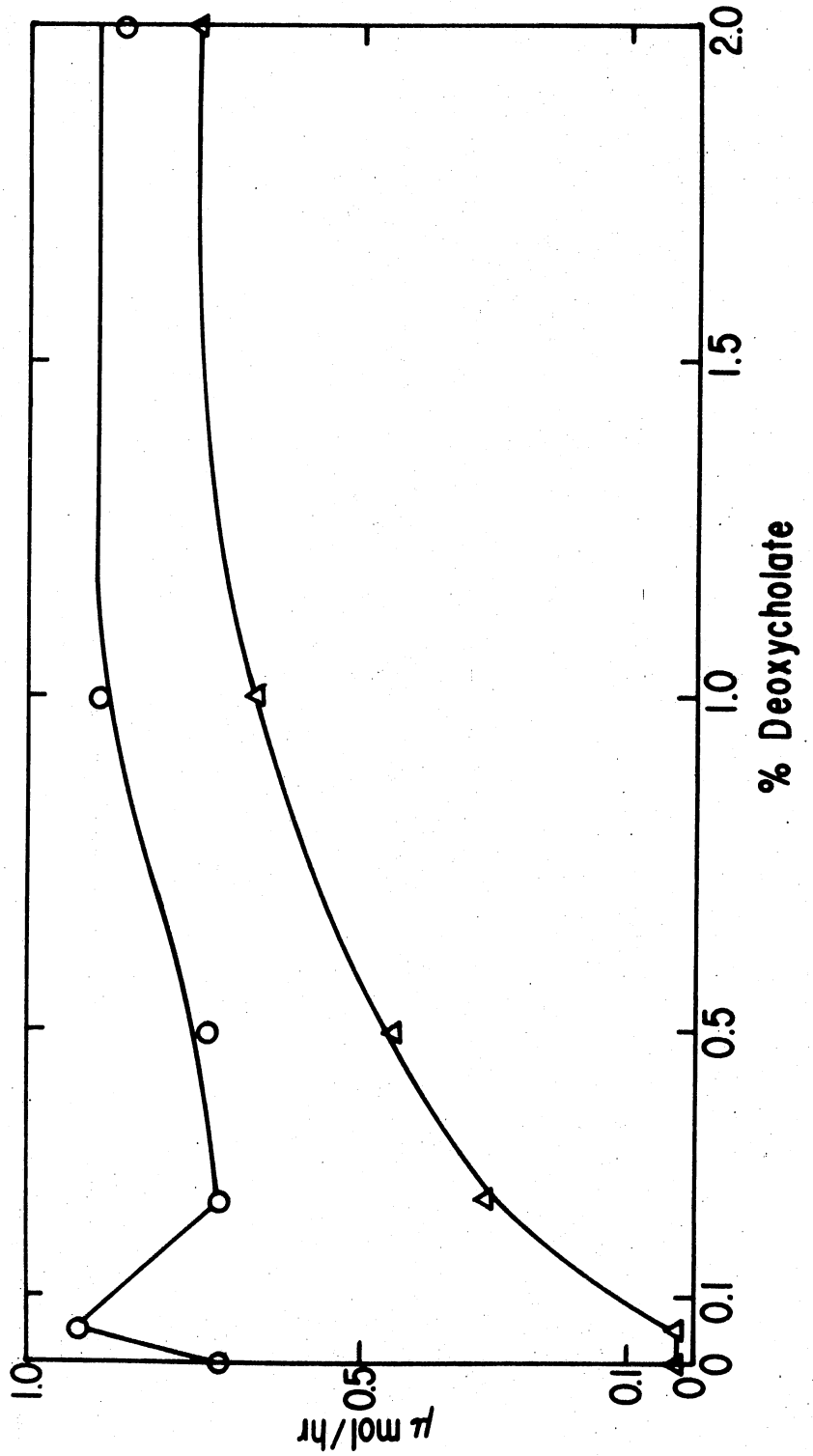
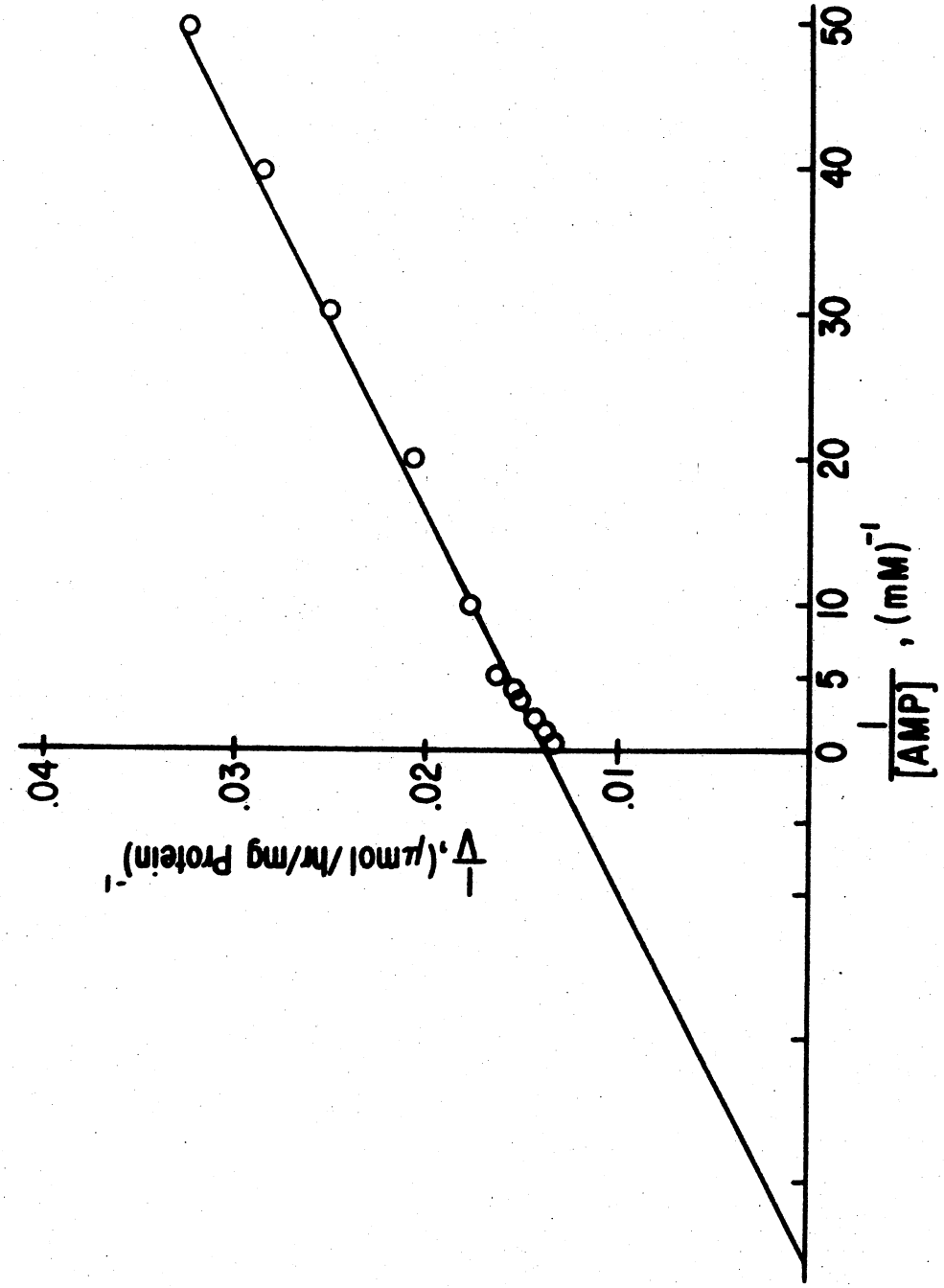

1.0
0.5
0.1
μ mol/hr
0
0.1
0.5
1.0
1.5
2.0
% Deoxycholate

Figure 12. Lineweaver-Burk Plot of Deoxycholate-Solubilized F_1 Membrane 5'-Nucleotidase Kinetics. F_1 membranes were suspended in 50 mM Tris (pH 8.0) containing 1% DOC and incubated for 1 hr at 37°C before assaying.


.04
.03
.02
.01
0
5
10
20
30
40
50
$\frac{1}{V}$, (μmol/hr/mg Protein)$^{-1}$
$\frac{1}{[AMP]}$, (mM)$^{-1}$

the F_1DF_3 fraction with 1% DOC resulted in a dramatic decrease in the K_m of the nucleotidase (Table XI). This suggests that the high K_m form of the enzyme in the F_1DF_3 fraction is a result of its association with the membrane, since the enzyme resembles the low K_m form when the membrane is solubilized.

Similar results were obtained when the nonionic detergent Triton X-100 was used. Triton solubilization studies closely paralleled behavior in deoxycholate and revealed that 1% Triton X-100 was required for maximum solubilization of the nucleotidase in F_1 membranes (Figure 13). When F_1 and F_1DF_3 membranes were solubilized in Triton X-100, essentially one nucleotidase form was observed (Table XI). The Triton-solubilized enzyme possessed a low K_m not significantly different from that found with the DOC-solubilized enzyme.

When membranes containing only the low K_m form of nucleotidase (F_1DF_1, F_2, F_2DF_3) were solubilized in either DOC or Triton X-100, the K_m values for the enzyme were not substantially changed (Table XI).

Temperature Dependence of the Nucleotidase Activity

As a further indication of the influence of the membrane-enzyme association the temperature dependence of the nucleotidase in the various membrane fractions was examined. Arrhenius plots for the F_1 fraction assayed with 1.0 mM AMP (High K_m enzyme) showed breaks at 15.3° and 36.7°C (Figure 14).

Table XII shows the transition temperatures and activation energies for nucleotidase in the F_1 fraction and the other membrane and solubilized fractions. As shown in Table XII, the F_1 fraction assayed at 0.1 mM

Figure 13. Solubilization of 5'-Nucleotidase Activity with Triton X-100 (1.0 mM AMP). F_1 membranes were suspended in 10 mM Tris (pH 8.0) containing various concentrations of Triton X-100. After a 1 hr incubation at 37°C, the suspensions were centrifuged at 100,000 x g for 1.5 hr. The supernatant was assayed for 5'-nucleotidase activity in the presence of 1.0 mM AMP (containing 1-5 μCi/ml [^{32}P]-AMP). This is referred to as solubilized activity (Δ-Δ). The total activity (0-0) represents the activity present before centrifugation.

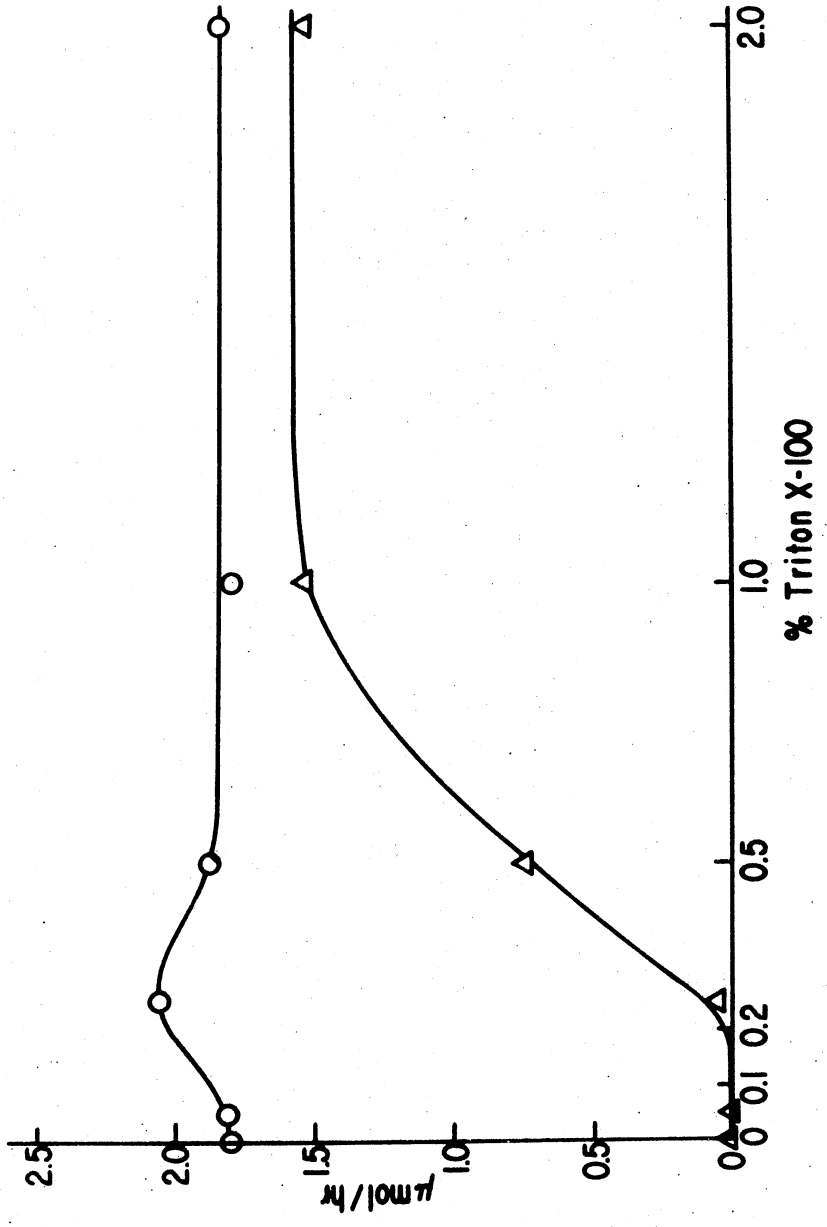

2.5
2.0
1.5
1.0
0.5
0
μmol/hr
0 0.1 0.2 0.5 1.0 2.0
% Triton X-100

Figure 14. Arrhenius Plots for Mammary Membranes and Triton-Solubilized Fractions. A) Membranes were incubated at the appropriate temperature in assay media for 10 min prior to assaying at the same temperature. F_1DF_3 membranes (●) and F_1 membranes (○) were assayed with 1.0 mM AMP, while F_2 membranes (■) were assayed with 0.1 mM AMP. B) Membranes were suspended in 10 mM Tris (pH 8.0) containing 1% Triton X-100 and incubated for 1 hr at 37°C. The solubilized membranes were then incubated at the appropriate temperature in assay media for 10 min prior to assaying at the same temperature. F_1 Triton-solubilized membranes (○) were assayed with 1.0 mM AMP, and F_2 Triton-solubilized membranes (●) were assayed with 0.1 mM AMP.

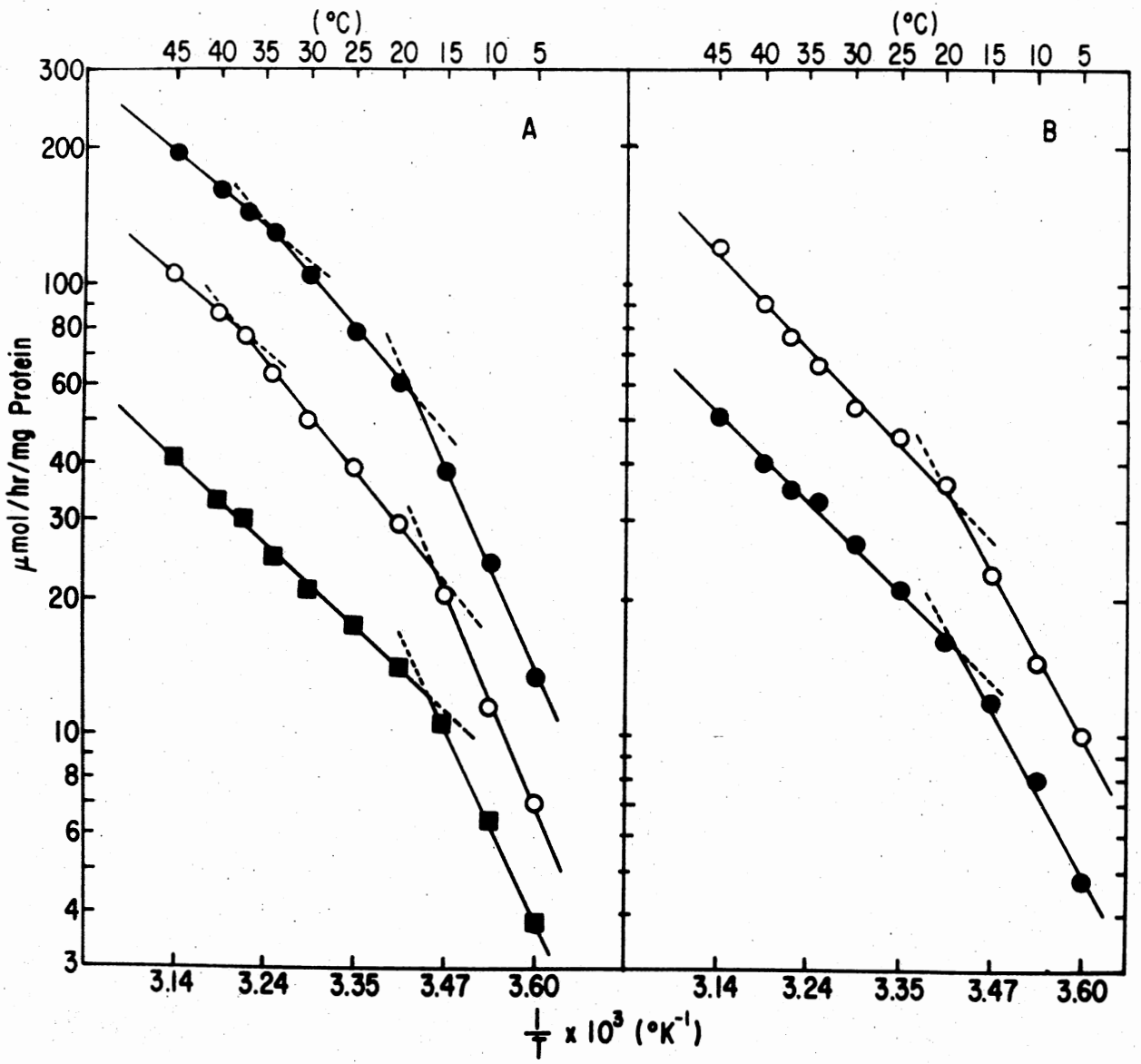

(°C)
45 40 35 30 25 20 15 10 5
A
B
300
200
100
80
60
40
30
20
10
8
6
4
3
μmol/hr/mg Protein
3.14 3.24 3.35 3.47 3.60
1/T x 10³ (°K⁻¹)

TABLE XII

TRANSITION TEMPERATURES AND ACTIVATION ENERGIES FOR NUCLEOTIDASE IN VARIOUS MEMBRANE AND SOLUBILIZED FRACTIONS

Fraction	Treatment	AMP	Activation Energy			Transition Temperature	
			Ea_1	Ea_2	Ea_3	T_1	T_2
		mM	------kcal/mole-----			-----C°-----	
F_1	----	1.0	17.8	10.5	7.4	15.3	36.7
F_1	----	0.1	13.4	7.4	----	18.0	----
F_2	----	0.1	14.3	8.0	----	18.2	----
F_1	Dg	0.1	14.8	7.3	----	18.3	----
F_1	Dg	1.0	17.7	9.2	6.8	17.6	37.7
F_1DF_3	----	1.0	16.9	10.1	7.3	18.2	34.4
F_1	DOC	0.1	14.0	7.7	----	19.2	----
F_1	DOC	1.0	14.4	9.0	----	19.2	----
F_2	DOC	0.1	14.0	8.6	----	19.1	----
F_1	TX-100	1.0	13.6	7.6	----	20.7	----
F_1	TX-100	0.1	13.2	7.1	----	19.2	----
F_2	TX-100	0.1	13.7	8.0	----	18.8	----

All values reported are averages of 2 or 3 separate determinations on different membrane preparations with standard deviations of less than 10% of the reported value. The blanks indicate no treatment or a failure to observe a corresponding value for that sample. Dg, digitonin.

AMP (low K_m enzyme) and the F_2 fraction gave only one break at 18°C. Digitonin treatment of the F_1 fraction did not alter the behavior of the enzyme, except for a slight shift in the lower transition temperature. Purified F_1DF_3 also showed both transitions (Figure 14). Treatment of the F_1 fraction with DOC or Triton X-100 abolishes the high temperature transition observed in the presence of 1.0 mM AMP (Table XII). There is essentially no change in the low temperature transition resulting from detergent treatments. Therefore, the altered temperature dependence of the high K_m enzyme was converted to the characteristics of the low K_m enzyme with detergents. It was further observed that DOC or Triton X-100 solubilization of membranes containing only the low K_m enzyme form of nucleotidase did not alter the temperature dependence of this form (Table XII). Thus, the loss of the high temperature transition on detergent treatment is correlated with the change in K_m previously noted, although there is no obvious relationship between the low temperature transition and K_m. Data for the transition temperatures and activation energies of the various membrane and solubilized fractions, presented in Table XII, show the similarity of the solubilized forms to the forms observed in membranes exhibiting a low K_m enzyme.

Cooperativity of the Con A Inhibition

Previous studies have shown that the cooperativity of the Con A inhibition of 5'-nucleotidase is dependent on the "state" of the membrane or the membrane-enzyme association (61,110). Therefore, the various mammary membrane fractions were examined in order to determine whether there was a relationship between changes in K_m and changes in cooperativity. Con A caused essentially complete inhibition of the high K_m

nucleotidase (Figure 15) and the low K_m nucleotidase (Figure 16) in F_1 membranes. The inhibition of both enzyme forms was reversed with α-methylmannoside, indicating a specific interaction of Con A with carbohydrate on the enzyme. Hill plot analysis of the inhibition reaction showed that the degree of cooperativity was different for the two enzyme forms (Figure 15 and 16 insets). A Hill coefficient of 1.4 was seen with the high K_m enzyme, while a Hill coefficient of 2.4 was observed for the low K_m form. Purified F_1DF_3, which possesses the high K_m nucleotidase, also exhibited decreased cooperativity (Table XIII).

Deoxycholate solubilization of membranes containing the high K_m enzyme (F_1 and F_1DF_3) caused a pronounced increase in cooperativity (Table XIII) along with the decrease in K_m previously observed. The DOC-solubilized F_1 and F_1DF_3 fractions exhibited increased cooperativity when assayed either under high K_m (1.0 mM AMP) or low K_m (0.1 mM) conditions (Table XIII), indicating that the substrate concentration had no effect on the observed cooperativity. In addition the F_2 membrane fraction, which has only the low K_m enzyme, exhibits cooperativity for the Con A inhibition (Table XIII). Thus, there appears to be a relationship between the K_m of the enzyme and the display of cooperativity.

Figure 15. Concentration Dependence of Con A Inhibition of the High K_m F_1 Membrane 5'-Nucleotidase. Membranes, prepared as described in Experimental Procedures, were incubated 10 min with Con A before assaying with 1 mM AMP (containing 1-5 μCi/ml [^{32}P]-AMP). 50 mM α-methyl mannoside (α-MM) was added to a tube containing the highest Con A concentration to show reversibility of the reaction. The α-methyl mannoside was added after incubation with Con A and the membranes incubated an additional 10 min before assaying. The inset shows a Hill plot for Con A inhibition of F_1 membrane high K_m 5'-nucleotidase (Hill Coefficient, 1.4).

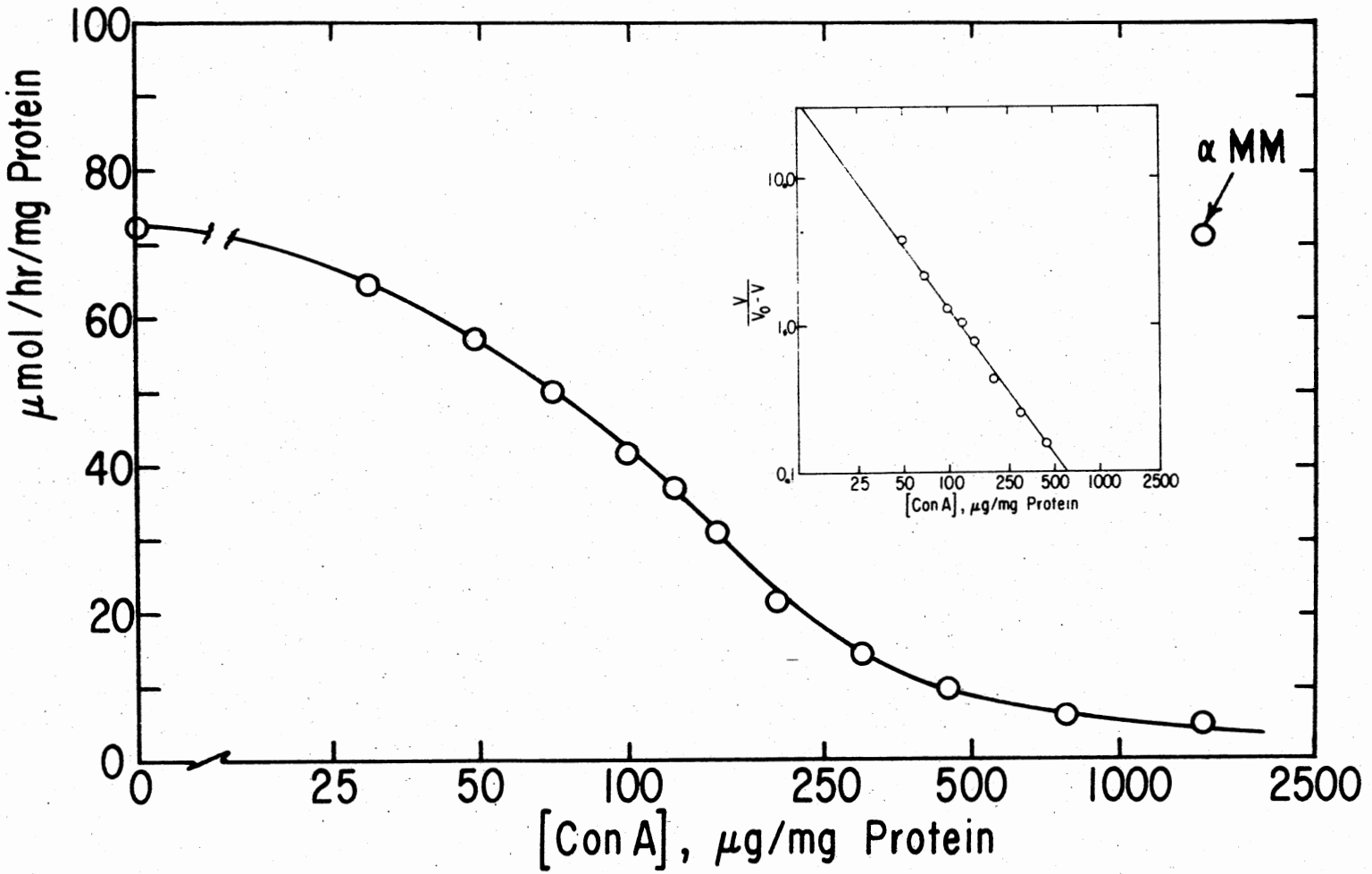

μmol/hr/mg Protein
100
80
60
40
20
0
0
25
50
100
250
500
1000
2500
[Con A], μg/mg Protein
α MM
10.0
1.0
0.1
V/(V0-V)
25
50
100
250
500
1000
2500
[Con A], μg/mg Protein

Figure 16. Concentration Dependence of Con A Inhibition of the Low K_m F_1 membrane 5'-Nucleotidase. Membranes were prepared, incubated and assayed as in Figure 15 except that 0.1 mM AMP (containing 1-5 μCi/ml [^{32}P]-AMP) was used. The inset shows a Hill plot for Con A inhibition of F_1 membrane low K_m 5'-nucleotidase (Hill Coefficient, 2.2).

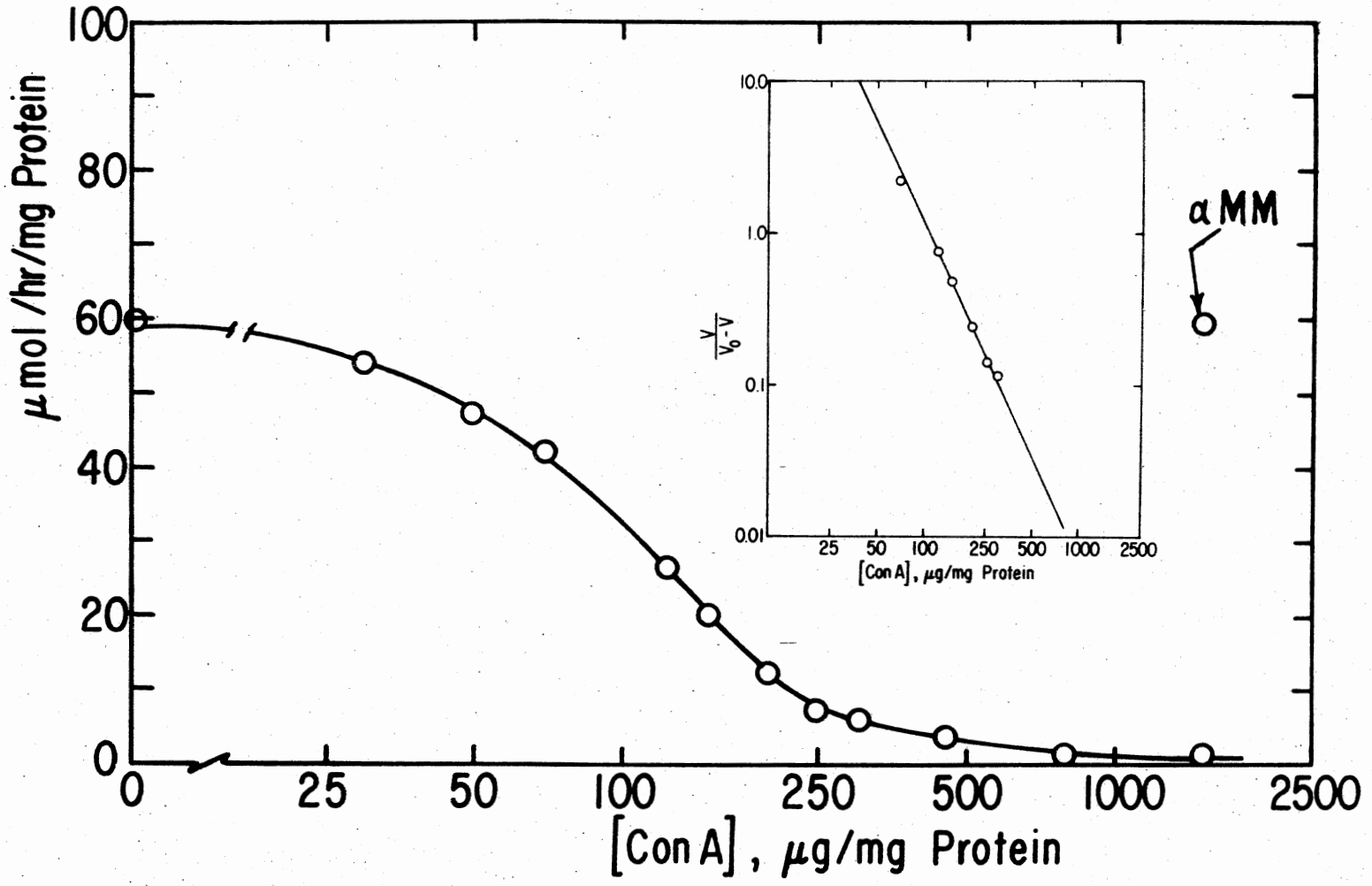

100
80
60
40
20
0
μmol /hr/mg Protein
0
25
50
100
250
500
1000
2500
[Con A], μg/mg Protein
αMM
10.0
1.0
0.1
0.01
V/V0-V
25 50 100 250 500 1000 2500
[Con A], μg/mg Protein

TABLE XIII

COOPERATIVITY IN THE CON A INHIBITION OF
NORMAL RAT MAMMARY MEMBRANE
5'-NUCLEOTIDASE

Membrane Fraction	Treatment	AMP (mM)	Hill Coefficient
F_1	----	0.1	2.3
F_1	----	1.0	1.4
F_2	----	0.1	1.9
F_1DF_3	----	1.0	1.4
F_1DF_3	DOC	0.1	2.1
F_1DF_3	DOC	1.0	2.1
F_1	DOC	1.0	2.2
F_1	TX-100	1.0	2.1

CHAPTER IV

DISCUSSION

Effects of Cytoskeletal Perturbants on the Cooperativity of the Inhibition of Ecto 5'-Nucleotidase by Con A

It is generally believed that the cell surface plays an important role in developmental processes and cancer; however, the forces controlling the organization and behavior of the macromolecular components of the cell surface are not well understood. Although considerable progress has been made in recent years in describing how cell surface molecules behave under a variety of conditions, the cell surface is so complex much more work at the molecular level is needed. Little attention has been given to the ecto-enzymes of different cellular systems until recent years. Their potential usefulness in monitoring cell surface membrane changes during development or neoplastic transformation is of considerable interest but has yet to be demonstrated clearly. The major difficulty is a lack of basic knowledge regarding the properties and functions of these enzymes.

The observation that 5'-nucleotidase and other cell surface enzymes can be perturbed by plant lectins offers an excellent method to understand the functions of these enzymes, the mode of action of the lectins, and the interaction of the enzyme with the membrane (60). It was previ-

ously demonstrated that the cooperativity of the Con A inhibition of 5'-nucleotidase may be a useful tool for investigating cell surface alterations (61,77). Carraway et al. (61) noted that Con A inhibition of the intact 13762 rat mammary ascites cell 5'-nucleotidase displayed no cooperativity, while extraction of the ascites cell surface envelopes with EDTA induced cooperativity. Since EDTA extraction has been shown to remove substantial amounts of cellular cytoskeletal elements (82), it followed that the pronounced cooperativity of the lectin-nucleotidase interaction was a result of the loss of these structures. Therefore, the simplest explanation would be that the nucleotidase is a transmembrane protein which interacts directly with the submembrane cytoskeletal elements, although an indirect interaction is also possible.

It was necessary to determine whether specific cytoskeletal-disrupting drugs and treatments could induce cooperativity, since it was suggested that intact cells displayed no cooperativity because of restraints imposed on the nucleotidase by the membrane (61,77). This particular study clearly indicates that in 13762 ascites cells the cooperativity parameter is sensitive to colchicine, the cytochalasins and dibucaine. BHK-21 fibroblasts also showed cooperativity when treated with cytochalasin D and colchicine, although induction of cooperativity was not as pronounced as in the 13762 ascites cells. To understand the full significance of these results it is necessary to know the mechanism of the change in cooperativity and the mechanism by which the drugs affect the nucleotidase. It was previously postulated that cooperativity occurs as a result of the Con A-induced clustering of enzyme molecules in the membrane (77). However, the results of Huggins et al. (100) show no correlation between cooperativity and the redistribution of the majority

of the Con A receptors. For example, the Con A receptors on MAT-A cells redistribute simply upon treatment with Con A, while no cooperativity is observed without drug treatment. In contrast, the MAT-C1 cells show no redistribution of their Con A receptors unless severe perturbation (e.g., hypotonic swelling) of the cells is employed (100), but cooperativity is readily induced simply by treatment with cytoskeletal-disrupting drugs. Thus, the two sublines are quite different in terms of the redistribution of their Con A receptors, but each behaves identically in the cooperativity of the Con A inhibition of their ecto 5'-nucleotidase. This similarity in the cooperative behavior of the Con A-enzyme interaction between the two sublines also contrasts with differences in cell surface glycoproteins and morphology (73,111).

The interpretation of these experiments should be approached with caution. First of all, nucleotidase represents a very small fraction of the total Con A receptors. Therefore, it may exhibit different behavior from the other more abundant Con A receptors. For example, the nucleotidase might be restricted in mobility by the cytoskeletal system in the presence of Con A in both MAT-A and MAT-C1 cell types unless released from these restraints by cytoskeletal-disrupting drugs. Secondly, the mobility of nucleotidase molecules required for cooperativity may be different from the mobility required to observe redistribution of Con A receptors by fluorescence microscopy. A shorter-range mobility might be involved in which nucleotidase molecules may have to travel only short distances in order to detect cooperativity, while Con A receptors must generally move greater distances to detect redistribution of bulk receptors on the cell surface by fluorescence methods. It may even be possible that cooperativity is due to a simple rotation of the enzyme in

the membrane, caused by a release of membrane restraints. In either case it appears that the interaction with cytochalasin- or colchicine-sensitive structures is different for the nucleotidase and for the majority of the Con A receptors.

The induction of cooperativity does not appear to result from changes in binding of Con A that has been determined by measuring total Con A binding (100). Cooperativity in Con A binding has been observed in some cells (112,113), but Con A binding in the 13762 ascites sublines did not exhibit cooperativity with or without cytoskeletal perturbants (100). Binding studies with the ascites cells showed a single class of binding sites. However, examination of Con A receptors after electrophoresis showed a variety of different Con A receptors (100). Therefore it seems that binding of Con A is not sensitive enough for detecting cellular responses to perturbation, especially since the nucleotidase shows different responses to cytoskeletal perturbants than the other Con A receptors.

The effects of colchicine and cytochalasin are usually attributed to specific perturbations of microtubules and microfilaments, respectively (27). However, nonspecific membrane effects for both drugs have been reported (114,115,116), but generally require higher drug concentrations. The results in the present study with the 13762 ascites cells indicate that the effects by these drugs are specific for cytoskeletal structures and seem to rule out any nonspecific membrane effects. First of all, the low concentrations of the drugs necessary for the effects points to a specific action on the cytoskeleton. Secondly, lumicolchicine which is a structural analog of colchicine that does not alter microtubule polymerization did not induce cooperativity. Finally, the order of effectiveness

of the different cytochalasins (E>D>B) is consistent with the order found for binding to the spectrin-actin cytoskeletal complex of the erythrocyte membrane (98) and for promoting morphological changes of 3T3 fibroblasts (99).

One of the most important observations in these studies is the fact that both cytochalasins and colchicine induce cooperativity of the inhibition process of nucleotidase. This contrasts with the different effects these drugs have on receptor mobilities in most cells, but is similar to the effects of these drugs on aminobutyrate transport in Chinese hamster ovary cells (104) and cap formation in pulmonary alveolar macrophages (117), in which both drugs showed essentially the same effect. Since the drug treatments did not alter the activity of the enzyme it is highly unlikely that the cooperativity changes are dependent on additional expressions of the enzyme by addition of new enzyme to the cell surface or on degradation of a specific fraction of enzyme molecules. This suggests that the cooperative effect is directly dependent on interactions of the enzyme with the membrane. The results with the rat mammary membrane fractions support the proposition that cooperativity is dependent on enzyme-membrane interactions. Two different fractions (F_1DF_1 and F_1DF_3) show different degrees of cooperativity. Solubilization of the membranes in DOC eliminates the cooperativity differences, suggesting they arise from interactions of the enzyme with the membrane.

The common effects of the cytochalasins and colchicine also suggests that these agents act on the cytoskeletal system rather than directly on the membrane or enzyme. It would seem highly unlikely that three very different drugs, cytochalasin, colchicine, and dibucaine, would produce a common effect on the cooperativity parameter by acting nonspecifically on

the enzyme or membrane, while a fourth agent, lumicolchicine, which is structurally related to colchicine, has no effect. However, if the drugs are presumed to act directly on the cytoskeletal system, then their common effect resulting from different sites of action must be explained. There appear to be two likely models for cytoskeletal organization. 1) Both microfilaments and microtubules are separately associated with the membrane such that perturbation of either results in release of restraints on nucleotidase mobility. 2) Microtubules are linked to microfilaments, which are attached to the membrane. Thus perturbation of microtubule structures would alter the microfilament network which would in turn cause a change in the membrane reflected in the behavior of the nucleotidase-Con A interaction. This second model is more consistent with structural evidence. Polymerized actin and microfilaments have been demonstrated in association with isolated plasma membranes (118). However, the association of microtubules with plasma membranes of mammalian cells is more questionable. Substantial amounts of tubulin polypeptide have not been found associated with plasma membrane envelopes from 13762 ascites cells, which do contain large amounts of actin and display no cooperativity in the Con A inhibition of 5'-nucleotidase (82). Furthermore, EDTA extraction of these membrane envelopes removes actin and other cytoskeletal components, inducing cooperativity (61). These results implicate actin-containing elements (microfilaments) as the link to the membrane. However, it should be kept in mind that the linkage between microfilaments and microtubules does not have to be direct.

The molecular mechanism of action of these drugs is still largely unknown. The cytochalasins may act by disrupting the linkages between actin filaments and the cytoplasmic surface of the membrane, possibly by

acting on actin binding protein (ABP). ABP has previously been postulated to be involved in linking actin filaments to the membrane (79). When sarcoma ascites cells were treated with hypotonic Zn^{++} decreased mobility of Con A receptors and an increased retention of ABP in Triton-extracted cell cytoskeletal residues were observed (82). These results correlate with the ability of Zn^{++} treatment to prevent induction of cooperativity of the nucleotidase inhibition in swollen cells. Cytochalasins have also been shown to prevent the formation of gels by purified ABP and F-actin (119), apparently without inhibiting actin polymerization.

A molecular mechanism for the disruption of microtubules by colchicine has been proposed (120). It has recently been observed that the microtubule assembly-disassembly "equilibrium" is really a steady state summation of two different reactions which occur at opposite ends of the microtubule (120). The assembly reaction is favored at one end and the disassembly reaction at the opposite end. Thus, it was postulated that tubulin subunits are constantly migrating from one end of the microtubule to the other, allowing for an orderly turnover of microtubules in the cell. Since assembly and disassembly sites are distant, assembly and disassembly signals that cause the net growth or decay of microtubules can possibly act by binding selectively to one end or the other and thus blocking tubulin exchange (120). From these observations, drug sensitivity of microtubules has been equated with dimer exchange rates on microtubules. Colchicine inhibits microtubule polymerization by adding as tubulin-colchicine complex(es) to the assembling end of the microtubule (120). It does not directly cause depolymerization (121). Although a mechanism for colchicine disruption of micro-

tubule assembly has been proposed it is still unclear how this perturbation is transmitted to the membrane.

One important finding is that theophylline and dibutyryl cyclic AMP are both able to prevent the cooperativity changes caused either by the cytochalasins or colchicine. Since both theophylline and dibutyryl cyclic AMP are known to increase intracellular cyclic AMP levels (104, 105), it would seem that the increased intracellular cyclic AMP is involved in preventing the effects of the cytoskeletal-disrupting drugs, perhaps by stabilizing the cytoskeletal elements. Cyclic AMP has been implicated in stabilization of microtubules (122), although the mode of its action is unclear. Puck (104) has proposed that cyclic AMP plays a necessary role in the formation and maintenance of the overall cytoskeletal structures. Cyclic AMP might act via a cyclic AMP dependent protein kinase which could regulate cytoskeletal integrity by phosphorylating key proteins involved in assembly or disassembly of microfilaments or microtubules. Some indirect evidence in support of this proposition was given by Rosenberg et al. (123) who showed that treatment of purified platelet ABP with alkaline phosphatase inhibits its ability to form a complex with F-actin. Thus cyclic AMP can be implicated in the interaction of ABP with actin filaments, and the effects of theophylline and dibutyryl cyclic AMP in preventing the induction of cooperativity by cytochalasin can be explained.

The induction of cooperativity by increased intracellular Ca^{++} is also consistent with an involvement of cytoskeletal elements. Calcium is known to activate actomyosin ATPase and induce contraction (101). It will inhibit gelation of cytoplasmic extracts of mammalian cells (124), a process which apparently requires actin filament formation and cross-

linking (125). It has also been reported that Ca^{++} may regulate microtubule polymerization in animal cells (126) in a process mediated by the calcium-dependent regulatory protein (127). This protein has also been shown to affect Ca^{++} transport, Ca^{++}-ATPase, cyclic AMP phosphodiesterase and actomyosin (128,129) as well as microtubules. Therefore, perturbation of any of these cytoskeletal or regulatory systems could potentially affect the membrane-nucleotidase association.

It was necessary to investigate the cooperativity response in other cell types and membranes in order to determine how widespread the phenomenon was and how it correlated with other specific cellular responses and properties. Toward this end we have studied the BHK-21 fibroblast, bovine MFGM, and normal lymphocytes isolated from rat spleen. The BHK-21 fibroblast showed a response to both colchicine and cytochalasin D, although both responses were diminished compared to the drug effects on the 13762 ascites cells. Colchicine provided a more pronounced induction of cooperativity than did cytochalasin D.

Since the Con A inhibition of bovine MFGM nucleotidase is not cooperative (76), it is necessary to determine the components responsible for this restriction on the enzyme. The bovine MFGM does contain fibrillar material attached to the previous cytoplasmic surface (106), suggesting that this material might be involved in restriction of nucleotidase. The results showing that neither colchicine nor cytochalasin D were able to induce cooperativity are consistent with previous observations that showed tubulin and actin to be absent from MFGM (106,107). However, the extraction with glycine-EDTA-mercaptoethanol, which solubilizes cytoskeletal proteins from 13762 envelopes and induces cooperativity, also induces cooperativity in MFGM. The fibrillar coat material

in MFGM has been shown to resemble, at least superficially in the electron microscope (130), that material found in association with Sarcoma-180 envelopes (79). This material is resistant to extraction with low or high ionic strength buffers and detergents (130), behavior similar to that of associated cytoskeletal material of Sarcoma envelopes (79). The surface coat material of MFGM is primarily composed of polypeptides with molecular weights of 155,000 and 67,000 (130), which is different from the polypeptide composition of the material found associated with Sarcoma envelopes (79). The extraction studies with glycine-EDTA-mercaptoethanol indicate that the 155,000 dalton component can be substantially removed from the MFGM under conditions which induce cooperativity. Therefore, it might be suggested that this 155,000 molecular weight polypeptide plays a part in controlling membrane functions in MFGM and is also responsible for the restriction of 5'-nucleotidase in this system. Although it has been suggested that xanthine oxidase is the 155,000 dalton component of MFGM (131), it is possible that this major electrophoretic band might also contain other polypeptides. It is also possible that glycine-EDTA-mercaptoethanol extracts a minor polypeptide which is responsible for both the restriction of nucleotidase and the binding of the 155,000 dalton polypeptide to the membrane.

The lymphocyte is an interesting cell to study since the intact lymphocyte exhibits cooperativity. However, purification of the lymphocyte nucleotidase leads to a loss of the cooperativity observed in intact cells (75). These results are clearly opposite to the results found with other cell and membrane systems previously described in these studies. Dornand et al. (75) have suggested that cooperativity depends on the presence of an interaction with other lectin receptors. However, it is

apparent from the results with drugs which raise intracellular cyclic AMP levels that this explanation for cooperativity in lymphocytes is not adequate. The fact that the inhibition results from direct interaction of lectin and enzyme is clear from studies on the purified enzyme (66). Both 1.0 mM theophylline and 1.0 mM dibutyryl cyclic AMP were successful in shutting down the cooperativity observed in untreated cells. Cytochalasin D was unable to eliminate the cooperative effect. These results might suggest that the cytoskeletal structures in lymphocytes are altered, possibly due to decreased cyclic AMP levels. It is interesting to note that theophylline and dibutyryl cyclic AMP were required at concentrations of 1.0 mM to produce their effects, while only 0.1 mM theophylline or dibutyryl cyclic AMP was required for reversal of the effects of the cytoskeletal drugs in the 13762 ascites cells. It is also possible that the increased cyclic AMP levels might affect interactions of the other lectin receptors with nucleotidase so that no cooperativity is observed. Thus, it is apparent from the results with BHK-21 fibroblasts, bovine MFGM, rat lymphocytes, and the 13762 ascites cells that the cooperativity of the Con A inhibition of 5'-nucleotidase is a fundamental parameter responsive to changes in the plasma membrane. The significance of this monitor of cell surface alterations stems from the results showing that it is observable in other cell types and is related to physiologically significant events.

The mechanism by which changes in the cytoskeletal system affects the membrane nucleotidase is an important facet of these observations. An interesting finding has recently been presented by Mannherz and Rohr (132), concerning the interaction of 5'-nucleotidase with the microfilament system. In this report evidence is presented that 5'-nucleotidase

is able to reverse the inhibitory action of actin on Dnase I. From the results, it appeared that 5'-nucleotidase interacts with actin leading to the formation of a filamentous network and the release of DNase I. The filamentous network resembles in its appearance F-actin after incubation with cytochalasin B (133). The assumption that 5'-nucleotidase interacts with actin is strengthened by the observation that this enzyme also accelerates the rate of actin polymerization (134). It was proposed that since 5'-nucleotidase is a widely distributed plasma membrane constituent of eukaryotic cells it might therefore act as an anchorage protein for intracellular filamentous forms of actin, modifying its availability for cellular motile responses (132).

It is also possible that disruption of the cytoskeleton, specifically the microfilaments, leads to a more general membrane perturbation in the area of the filament-membrane linkage, which is transmitted to the nucleotidase through other protein-protein or protein-lipid interactions in the membrane.

These studies on the nature and requirements of the cooperativity indicate a need to understand the association of nucleotidase with the membrane and the influence of that association on the properties of the enzyme. For these reasons, a different membrane system has been studied, plasma membranes isolated from lactating rat mammary gland.

The Influence of the Membrane on Properties of 5'-Nucleotidase

Rat mammary gland microsomes can be separated by flotation on a discontinuous sucrose density gradient into two fractions, designated F_1 and F_2. Both fractions contain plasma membrane material (92) and are

enriched in 5'-nucleotidase, (Na^+, K^+)-ATPase, cholesterol, sialic acid, and galactosyltransferase (88). Since galactosyltransferase is enriched in the Golgi it appears that the membrane preparations contain Golgi along with plasma membrane. Treatment of the F_1 fraction with digitonin and reflotation on a discontinuous sucrose density gradient yields a membrane fraction with a higher density (36-40% interface), designated F_1DF_3. This material is enriched in nucleotidase, sialic acid and cholesterol but contains no galactosyltransferase (88). The portion of the digitonin-treated fraction which retains the original density upon recentrifugation on the gradient (0.9-32% interface) is designated F_1DF_1. F_1DF_1 is enriched in galactosyltransferase and depleted in the plasma membrane "markers", nucleotidase, sialic acid, and cholesterol. The F_1DF_3 and F_1DF_1 fractions are therefore considered to be purified plasma membrane and Golgi fragments, respectively (109). Treatment of the F_2 fraction with digitonin and reflotation on the gradient also yields a membrane fraction with a higher density (36-40% interface), designated F_2DF_3. This F_2DF_3 fraction is enriched in nucleotidase, sialic acid, cholesterol, and also galactosyltransferase, suggesting it is a plasma membrane which contains galactosyltransferase (109).

Kinetics of the nucleotidase in the various membrane fractions indicates two forms of the enzyme in F_1. These two forms are segregated in the F_1DF_1 and F_1DF_3 fractions. Therefore, the question arises as to the nature and the cause of the differences between the two enzyme forms. Two possibilities are readily evident. First, the two forms might represent two different enzyme molecules (isoenzymes) which are separately synthesized and incorporated into the different membrane types. This possibility seems highly unlikely since the high K_m enzyme can be con-

verted to a form with properties closely resembling the low K_m enzyme by treatment with two different detergents. Because the conversion is accomplished by employing two different detergents (deoxycholate and Triton X-100) with different solubilizing properties, the effects are not likely due to a direct alteration of the enzyme by the detergent. In addition to the conversion of the high K_m enzyme to the low K_m form, two other properties of the enzyme are coordinately altered by detergents. These two properties are the temperature dependence of the nucleotidase activity and the cooperativity of the Con A inhibition.

A second possibility exists which is a more likely explanation for the differences between the two forms. In light of the results it appears that there is only one type of enzyme molecule. Its properties are dependent on its association with the membrane. According to this proposal, the increased K_m and decreased cooperativity observed in the F_1DF_3 fraction would result from restrictions placed on the enzyme by its association with other membrane components. The observation of a second transition temperature in the Arrhenius plots of the F_1DF_3 fraction enzyme is also indicative of another form of the enzyme. This higher transition temperature is eliminated by solubilization of the membrane with detergents, indicating that this enzyme property is dependent upon membrane interactions that are disrupted by the detergents. The transition temperature near 20°C is not influenced by detergents and shows no correspondence to changes in the other enzyme properties, suggesting that it is probably a transition inherent to the enzyme and is not related to any enzyme interactions with the membrane. This is a further indication that the detergents are not altering the enzyme itself since the lower transition near 20°C remains unchanged throughout all treatments, even though

changes in the K_m and respose to Con A are observed. Thus, the changes in the properties of the F_1DF_3 enzyme induced by detergent treatment are due to the release of membrane restraints and not to significant alterations in enzyme structure.

In light of these results it does not appear necessary to postulate the existance of isoenzymes to explain the differences between the two enzyme forms. This should not be taken to mean that there are absolutely no molecular differences between the two enzyme forms. Since the nucleotidase is a glycoprotein, possible variations in carbohydrate content between the two forms could give them different properties without significantly altering the K_m values or the cooperativity parameter. However, these subtle differences in enzyme structure would not be significant enough to account for the differences that are observed.

The molecular basis for the difference in the properties of the two forms remains unclear. It is interesting to note that the two purified membrane fractions (F_1DF_3 and F_2DF_3) which have characteristics of plasma membranes (high nucleotidase, cholesterol and sialic acid) possess different enzyme forms. However, the F_2DF_3 membrane does contain galactosyltransferase which is not present in F_1DF_3. This suggests that these two plasma membrane fractions might come from different regions of the cell surface (i.e., the apical, basal or lateral regions of the cell). The nucleotidase and density differences of these fractions indicate that they must have differences in protein or lipid compositions. Experiments utilizing DPH as a fluorescent probe of the various membranes do not show significant differences in microviscosities which can be related to enzyme behavior. These results are supported by the observations on membrane cholesterol, which is a primary modulator of membrane fluidity

(135). The F_1DF_3 and F_2DF_3 fractions contain different enzyme forms, but both fractions possess high amounts of cholesterol. In addition the F_1DF_1 and F_2DF_3 fractions contain the same low K_m enzyme form, yet the F_2DF_3 is enriched in cholesterol while F_1DF_1 has very little cholesterol content. Thus, there appears to be no relationship between cholesterol levels in the membrane fractions and the properties of the enzyme. These results suggest that the observed behavior of the enzyme in these membranes is dependent on specific enzyme-protein or enzyme-lipid interactions rather than on the bulk membrane lipid phase. Widnell and Unkeless (64) observed that sphingomyelin was associated with nucleotidase isolated from rat liver, suggesting a specific enzyme-lipid interaction. It remains to be seen whether such a specific association exists in the mammary membranes.

In previous studies it has been shown that the cooperativity of the nucleotidase inhibition by Con A is dependent on the "state" of the membrane (61). It was further postulated that the cooperativity was dependent on the multivalency of Con A and the mobility of the enzyme in the membrane (77). The results presented in this report are consistent with these hypotheses. The difference in the enzyme forms of the mammary membrane fractions is also reflected in the cooperativity of the Con A inhibition, suggesting that the same restraint from the membrane which alters the K_m also causes a change in the cooperativity of the inhibition. Therefore, it is assumed that decreased K_m and increased cooperativity correlate with a release of restraints in the membrane imposed by specific lipid-enzyme or protein-enzyme interactions. The results with the ascites cells, MFGM, and fibroblasts also indicate that release or disruption of cytoskeletal restraints induces cooperativity.

No specific correlation was observed between the induction of cooperativity and drug induced changes of Con A receptor redistributions or cell morphology previously reported (100). The morphology of MAT-A cells was grossly altered by cytochalasins or dibucaine but not substantially by colchicine. MAT-C1 cells exhibit more minor alterations in morphology as a result of these drug treatments. These results indicate that the inhibition of 5'-nucleotidase, which is a Con A receptor, is a different process from the redistribution of the bulk of the Con A receptors. It is more sensitive to certain membrane perturbations and more quantitative. Therefore, the results demonstrate the usefulness of the Con A inhibition of ecto-5'-nucleotidase as a monitor of cell surface alterations resulting from defined experimental perturbations. This should lead to a better understanding of membrane structure-function relationships that are important to cell behavior.

A SELECTED BIBLIOGRAPHY

(1) Hughes, R. C. (1976) Membrane Glycoproteins, Butterworths, London.

(2) Marchesi, V. T. (1976) Membranes and Neoplasia: New Approaches and Strategies, A. R. Liss, Inc., New York.

(3) Curtis, A. S. G. (1967) The Cell Surface: Its Molecular Role in Morphogenesis, Academic Press, New York.

(4) Singer, S. J. (1971) in Structure and Function of Biological Membranes, L. I. Rothfield, ed., Academic Press, New York, 145-222.

(5) Guidotti, G. (1972) Ann. Rev. Biochem., 41, 731-752.

(6) Singer, S. J., and Nicolson, G. L. (1972) Science, 175, 720-731.

(7) Bretscher, M. S. (1973) Science, 181, 622-629.

(8) Singer, S. J. (1974) Ann. Rev. Biochem., 43, 805-833.

(9) Lowden, J. A., Moscarello, M. A., and Morecki, R. (1966) Can. J. Biochem., 44, 567-577.

(10) Parsons, D. F., and Yano, Y. (1967) Biochim. Biophys. Acta, 135, 362-364.

(11) Danielli, J. F., and Davson, H. (1935) J. Cell. Comp. Physiol., 5, 495-508.

(12) Davson, H., and Danielli, J. F. (1952) The Permeability of Natural Membranes, 2nd edition, Cambridge University Press, London.

(13) Robertson, J. D. (1959) Biochem. Soc. Symp., 16, 3-29.

(14) Lenard, J., and Singer, S. J. (1966) Proc. Natl. Acad. Sci., U.S.A., 56, 1828-1835.

(15) Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., and van Deenen, L. L. M. (1973) Biochim. Biophys. Acta, 323, 178-193.

(16) Helenius, A., and Simons, K. (1975) Biochim. Biophys. Acta, 415, 29-80.

(17) Bretscher, M. S. (1971) J. Mol. Biol., 59, 351-357.

(18) Triplett, R. B., and Carraway, K. L. (1972) Biochemistry, 11, 2897-2903.

(19) Green, D. E., and MacLennan, D. H. (1969) Bioscience, 19, 213-222.

(20) Juliano, R. L., Rothstein, A., and LaVoy, L. (1971) Life Sciences, 10, 1105-1113.

(21) Bretscher, M. S. (1971) Nature New Biol., 231, 229-232.

(22) Roelofsen, B. and van Deenen, L. L. M. (1973) Eur. J. Biochem., 40, 245-257.

(23) Schairer, H. U., and Overath, P. (1969) J. Mol. Biol., 44, 209-214.

(24) Singer, S. J. (1974) Adv. Immunol., 19, 1-68.

(25) Changeux, J. P., Thiery, J., Tung, Y., and Kittel, C. (1967) Proc. Natl. Acad. Sci., U.S.A., 57, 335-341.

(26) Rothfield, L. I., ed. (1971) Structure and Function of Biological Membranes, Academic Press, New York.

(27) Nicolson, G. L. (1976) Biochim. Biophys. Acta, 457, 57-108.

(28) de Petris, S. (1978) in Methods in Membrane Biology, Vol. 9, E. D. Korn, ed., Plenum Press, New York, 1-202.

(29) Dyson, R. D. (1974) Cell Biology: A Molecular Approach, Allyn and Bacon, Inc., Boston, 14-31.

(30) Spooner, B. S., Yamada, K. M., and Wessels, N. K. (1971), J. Cell Biol., 49, 595-613.

(31) Goldman, R. D., Lazarides, E., Pollack, R., and Weber, K. (1975) Exp. Cell Res., 90, 333-350.

(32) Puszkin, S., and Berl, S. (1972) Biochim. Biophys. Acta, 256, 695-709.

(33) Pollard, T. D., and Weihing, R. R. (1974) CRC Crit. Rev. Biochem., 2, 1-65.

(34) Pollack, R., Osborn, M., and Weber, K. (1975) Proc. Natl. Acad. Sci., U.S.A., 72, 994-998.

(35) Rash, J. E., McDonald, T. F., Sachs, H. G., and Ebert, J. D. (1972) Nature New Biol., 237, 160.

(36) Olmsted, J. B., and Borisy, G. G. (1973) Ann. Rev. Biochem., 42, 507-540.

(37) Weber, K., Pollack, R., and Bibring, T. (1975) Proc. Natl. Acad. Sci., U.S.A., 72, 459-463.

(38) de Petris, S. (1975) J. Cell Biol., 65, 123-146.

(39) Wessels, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T., and Yamada, K. M. (1971) Science, 171, 135-143.

(40) Lis, H., and Sharon, N. (1973) Ann. Rev. Biochem., 42, 541-574.

(41) Andersson, J., and Melchers, F. (1976) in Concanavalin A as a Tool, H. Bittiger and H. P. Schnebli, eds., John Wiley and Sons, London, 505-522.

(42) Shoham, J., Inbar, M., and Sachs, L. (1970) Nature, 227, 1244-1246.

(43) Edelman, G. M., Cunningham, B. A., Reeke, G. N., Jr., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972) Proc. Natl. Acad. Sci., U.S.A., 69, 2580-2584.

(44) Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1971) Proc. Natl. Acad. Sci., U.S.A., 68, 1130-1134.

(45) Hardman, K. D., and Ainsworth, C. F. (1972) Biochemistry, 11, 4910-4919.

(46) Nicolson, G. L. (1976) in Concanavalin A as a Tool, H. Bittiger and H. P. Schnebli, eds., John Wiley and Sons, New York, 3-16.

(47) Shoham, J., and Sachs, L. (1974) Exp. Cell Res., 85, 8-14.

(48) O'Dell, D. S., Ortolani, G. and Monroy, A. (1973) Exp. Cell Res., 83, 408-411.

(49) Nicolson, G. L., and Lacorbiere, M. (1973) Proc. Natl. Acad. Sci., U.S.A., 70, 1672-1676.

(50) Wallach, D. F. H. (1975) in Membrane Biology of Neoplastic Cells, D. F. H. Wallach, ed., Elsevier Scientific Publishing Co., Amsterdam, 435-481.

(51) Unanue, E. R., Perkins, W. D., and Karnovsky, M. J. (1972) J. Exp. Med., 136, 885-906.

(52) Comoglio, P. M., and Guglielmone, R. (1972) FEBS Letters, 27, 256-258.

(53) Inbar, M., Ben-Bassat, H., and Sachs, L. (1973) Int. J. Cancer, 12, 93.

(54) Yahara, I., and Edelman, G. M. (1973) Nature, 236, 152-155.

(55) Taylor, R. B., Duffus, W. P. H., Raff, M. C., and de Petris, S. (1971) Nature New Biol., 233, 225-229.

(56) de Petris, S. (1974) Nature, 250, 54-56.

(57) Storrie, B., and Edelson, P. J. (1977) Cell, 11, 707-717.

(58) Podolsky, D. K., Weiser, M. M., LaMont, J. T., and Isselbacher, K. J. (1974) Proc. Natl. Acad. Sci., U.S.A., 71, 904-908.

(59) Jarrett, L., and Smith, R. M. J. (1974) J. Biol. Chem., 249, 5195-5199.

(60) Carraway, C. A. C., and Carraway, K. L. (1976) J. Supramol. Struct., 4, 121-126.

(61) Carraway, K. L., Fogle, D. D., Chesnut, R. W., Huggins, J. W., and Carraway, C. A. C. (1976) J. Biol. Chem., 251, 6173-6178.

(62) Reis, J. (1934) Bull. Soc. Chim. Biol. 16, 385-399.

(63) Bodansky, O., and Schwartz, M. K. (1968) Adv. Clin. Chem., 11, 277-328.

(64) Widnell, C. C., and Unkeless, J. C. (1968) Proc. Natl. Acad. Sci., U.S.A., 61, 1050-1057.

(65) Evans, W. H., and Gurd, J. W. (1973) Biochem. J., 133, 189-199.

(66) Dornand, J., Bonnafous, J.-C., and Mani, J.-C. (1978) Eur. J. Biochem., 87, 459-465.

(67) DePierre, J. W., and Karnovsky, M. L. (1974) J. Biol. Chem., 249, 7111-7120.

(68) Arch, J. R. S., and Newsholme, E. A. (1978) in Essays in Biochemistry, Vol. 14, P. N. Campbell and W. N. Aldridge, eds., Academic Press, London, 82-123.

(69) Fleit, H., Conklyn, M., Stebbins, R. D., and Silber, R. (1975) J. Biol. Chem., 250, 8889-8892.

(70) Goldberg, D. M. (1973) Digestion, 8, 87-99.

(71) Kim, U., Baumler, A., Carruthers, C., and Beilat, K. (1975) Proc. Natl. Acad. Sci., U.S.A., 72, 1012-1016.

(72) Ip, C., and Dao, T. (1978) Cancer Res., 38, 723-728.

(73) Carraway, K. L., Huggins, J. W., Sherblom, A. P., Chesnut, R. W., Buck, R. L., Howard, S. P., Ownby, C. L., and Carraway, C. A. C. (1978) in Glycoproteins and Glycolipids in Disease Processes, E. F. Walborg, Jr., ed., American Chemical Society, Washington, 432-446.

(74) Riordan, J. R., and Slavik, M. (1974) Biochim. Biophys. Acta, 373, 356-360.

(75) Dornand, J., Bonnafous, J.-C., and Mani, J.-C. (1978) Biochem. Biophys. Res. Commun., 82, 685-692.

(76) Snow, L. D. (1976) Ph.D. Thesis, Oklahoma State University, Stillwater, Oklahoma.

(77) Carraway, C. A. C., Jett, G., and Carraway, K. L. (1975) Biochem. Biophys. Res. Commun., 67, 1301-1306.

(78) Hill, A. V. (1913) Biochem. J., 7, 471-480.

(79) Moore, P. B., Ownby, C. L., and Carraway, K. L. (1978) Exp. Cell Res., 115, 331-342.

(80) Koshland, D. E., Jr., and Neet, K. E. (1968) Ann. Rev. Biochem., 37, 359-410.

(81) Nichol, L. W., Jackson, W. J. H., and Winzor, D. J. (1967) Biochemistry, 6, 2449-2456.

(82) Moore, P. B., Anderson, D. R., Huggins, J. W., and Carraway, K. L. (1976) Biochem. Biophys. Res. Commun., 72, 288-294.

(83) Beadling, L., and Rothfield, L. I. (1978) Proc. Natl. Acad. Sci., U.S.A., 75, 3669-3672.

(84) Low, M. G., and Finean, J. B. (1978) Biochim. Biophys. Acta, 508, 565-570.

(85) Dipple, I., Elliot, K. R. F., and Houslay, M. D. (1978) FEBS Letters, 89, 153-156.

(86) DePierre, J. W., and Karnovsky, M. L. (1973) J. Cell Biol., 56, 275-303.

(87) Huang, C. M., and Keenan, T. W. (1972) Biochim. Biophys. Acta, 274, 246-257.

(88) Huggins, J. W., and Carraway, K. L. (1976) J. Supramol. Struct., 5, 59-63.

(89) Mizel, S. B., and Wilson, L. (1972) Biochemistry, 11, 2573-2578.

(90) Ipata, D. L. (1967) Anal. Biochem., 20, 30-36.

(91) Kobylka, D., and Carraway, K. L. (1972) Biochim. Biophys. Acta, 288, 282-295.

(92) Shin, B. C., Ebner, K. E., Hudson, B. G., and Carraway, K. L. (1975) Cancer Res., 35, 1135-1140.

(93) King, J., and Laemmli, U. K. (1971) J. Mol. Biol., 62, 465-471.

(94) Adams, D. A. (1977) Ph.D. Thesis, Oklahoma State University, Stillwater, Oklahoma.

(95) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.

(96) Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., and Creswell, K. M. (1974) Fed. Proc., 33, 158-166.

(97) Tannenbaum, J., Tanenbaum , S. W., and Godman, G. C. (1977) J. Cell Physiol., 91, 239-248.

(98) Lin, D. C., and Lin, S. (1978) J. Biol. Chem., 253, 1415-1419.

(99) Lin, S., Lin, D. C., and Flanagan, M. D. (1978) Proc. Natl. Acad. Sci., U.S.A., 75, 329-333.

(100) Huggins, J. W., Trenbeath, T. P., Chesnut, R. W., and Carraway, K. L. (1979) submitted for publication.

(101) Durham, A. C. H. (1974) Cell, 2, 123-136.

(102) Lazarides, E., and Weber, K. (1974) Proc. Natl. Acad. Sci., U.S.A., 71, 2268-2272.

(103) Fuller, G. M., and Brinkley, B. R. (1976) J. Supramol. Struct., 5, 497-514.

(104) Puck, T. T. (1977) Proc. Natl. Acad. Sci., U.S.A., 74, 4491-4495.

(105) Turtle, J. R., and Kipnis, D. M. (1967) Biochem. Biophys. Res. Commun., 28, 797-802.

(106) Patton, S., and Keenan, T. W. (1975) Biochim. Biophys. Acta, 415, 273-309.

(107) Keenan, T. W., Frendenstein, C., and Franke, W. W. (1977) Cytobiologie, 14, 259-278.

(108) Mather, I. H., and Keenan, T. W. (1975) J. Memb. Biol., 21, 65-85.

(109) Huggins, J. W., Chesnut, R. W., Trenbeath, T. P., Carraway, C. A. C., and Carraway, K. L., manuscript in preparation.

(110) Carraway, K. L., Doss, R. C., and Carraway, C. A. C. (1978) Fed. Proc., 37, 1790 Abs.

(111) Sherblom, A. P., Huggins, J. W., and Carraway, K. L. (1979) submitted for publication.

(112) Schmidt-Ulrich, R., and Wallach, D. F. H. (1976) Biochem. Biophys. Res. Commun., 69, 1011-1018.

(113) Wright, J. A., and Ceri, H. (1977) FEBS Letters, 78, 124-126.

(114) Graff, J. C., Wolheuter, R. M., and Plagemann, P. G. W. (1977) J. Biol. Chem., 252, 4185-4190.

(115) Jacob, H., Amoden, T., and White, J. (1972) Proc. Natl. Acad. Sci., U.S.A., 69, 471-474.

(116) Plagemann, P. G. W., and Richey, D. P. (1974) Biochim. Biophys. Acta, 344, 263-305.

(117) Williams, D. A., Boxer, L. A., Oliver, J. M., and Baehuer, R. L. (1977) Nature, 267, 255-257.

(118) Korn, E. D. (1978) Proc. Natl. Acad. Sci., U.S.A., 75, 588-599.

(119) Hartwig, J. H., and Stossel, T. P. (1976) J. Cell. Biol., 71, 295-303.

(120) Margolis, R. L., and Wilson, L. (1978) Cell, 13, 1-8.

(121) Margolis, R. L., and Wilson, L. (1977) Proc. Natl. Acad. Sci., U.S.A., 74, 3466-3470.

(122) Rubin, R. W., and Weiss, G. D. (1975) J. Cell Biol., 64, 42-53.

(123) Rosenberg, S., Stracher, A., Detwiler, T. C., and Lucas, R. C. (1978) Fed. Proc., 37, 1790 Abs.

(124) Mimura, N., and Asano, A. (1978) Nature, 272, 273-276.

(125) Stossel, T. P., and Hartwig, J. H. (1976) J. Cell Biol., 68, 602-619.

(126) Fuller, G. M., Ellison, J. J., McGill, M., Sordahl, L. A., and Brinkley, B. R. (1975) in Microtubules and Microtubule Inhibitors, M. Borgers and M. de Brabander, eds., North-Holland Publishing Co., Amsterdam, 379-390.

(127) Marcum, J. M., Dedman, J. R., Brinkley, B. R., and Means, A. R. (1978) Proc. Natl. Acad. Sci., U.S.A., 75, 3771-3775.

(128) Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., and Means, A. R. (1977) J. Biol. Chem., 252, 8415-8422.

(129) Hinds, T. R., Larven, F. L., and Vincenzi, F. F. (1978) Biochem. Biophys. Res. Commun., 81, 455-461.

(130) Freudenstein, C., Keenan, T. W., Eigel, W. N., Sasaki, M., Stadler, J., and Franke, W. W. (1979) Exp. Cell Res., 118, 277-294.

(131) Mather, I. H., Weber, K., and Keenan, T. W. (1977) J. Dairy Sci., 60, 394-402.

(132) Mannherz, H. G., and Rohr, G. (1978) FEBS Letters, 95, 284-289.

(133) Spudich, J. A. (1973) Cold Spring Harb. Symp. Quant. Biol., 37, 585-593.

(134) Rohr, G., and Mannherz, H. G. (1979) FEBS Letters, 99, 351-356.

(135) Razin, S., and Rottem, S. (1978) Trends in Biochem. Sci., 3, 51-55.

VITA[2]

Robert Courtland Doss

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE UTILIZATION OF ECTO 5'-NUCLEOTIDASE AS A MONITOR OF CELL SURFACE ALTERATIONS

Major Field: Biochemistry

Biographical:

Personal Data: Born in Bartlesville, Oklahoma, August 29, 1953, the son of Mr. and Mrs. Richard C. Doss.

Education: Graduated from Sooner High School, Bartlesville, Oklahoma, in May, 1971; received Bachelor of Science degree in Biochemistry from Oklahoma State University in May, 1975; completed requirements for the Doctor of Philosophy degree in Biochemistry at Oklahoma State University in July, 1979.

Professional Experience: Undergraduate research assistant, September, 1972 to May, 1975, Department of Biochemistry, Oklahoma State University; Chemistry teaching assistant, October, 1974 to May, 1975, Department of Chemistry, Oklahoma State University; Research assistant, May, 1975 to August, 1975, Phillips Petroleum Company Physics and Analytical Section of the Research and Development Department; Research Assistant, August, 1975 to July, 1979, Oklahoma State University Biochemistry Department.

Honorary Societies: Phi Eta Sigma, Phi Lambda Upsilon, Alpha Epsilon Delta and Phi Kappa Phi.