THE UNIVERSITY OF MANITOBA

CALCIUM TRANSPORT AND ITS REGULATORY ROLE IN CELLULAR METABOLISM OF ACHLYA.

by

LINDA EILEEN CAMERON

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LINDA EILEEN CAMERON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To David, Carolyn and my mother

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ABSTRACT

The calcium transport system of germinated sporangiospores of Achlya was characterized by kinetic analysis, and shown to be dependent on pH, temperature and substrate concentration, but only partially inhibited by metabolic antagonists. Hg^{++}, organic mercurials, I_2, and Ag^{+} activated calcium uptake, and after pH and temperature analysis, these compounds showed differential effects. Kinetic analysis, pH and temperature studies, and effects of mercurial inhibitors all indicated a two component calcium transport system. Cold osmotic shock treatment by the method of Nossal and Heppel (1969) reduced calcium uptake, released only 0.1% of cell protein, and released a specific calcium binding glycopeptide into the surrounding medium while the osmotic shocked cells remained intact and viable. That the glycopeptide was located at the cell membrane surface was demonstrated by its ionic sensitivity, and ease of release by mild physical treatment. Kinetic analysis of calcium uptake in osmotically shocked cells demonstrated that one of two possible components of the calcium transport system of whole cells remained intact. Kinetic data of glycopeptide calcium binding activity agreed relatively well with the other 'component' of the calcium transport system of intact cells. By Scatchard plot analysis it was determined that the glycopeptide bound 7 - 14 moles of Ca^{++} per mole of glycopeptide.

By chemical and physical characterization, the molecular weight of the glycopeptide was determined to be 5,960 daltons. The composition of the glycopeptide was 35% protein and 65% carbohydrate.

The carbohydrate component contains 80% glucose, 19% ribose or fucose, and the remaining 1% is uncharacterized uronic acids. The peptide component consists of 50% glutamic acid and six other amino acids; glycine, aspartic acid, alanine, lysine, serine, and cystine. Various agents, notably bivalent cations, ruthenium red and chelators, interfered with glycopeptide calcium binding activity. Kinetic studies after enzymatic degradation of the glycopeptide indicated that calcium was bound by the glycopeptide at two sites; high affinity sites of a postulated protein nature (glutamic and aspartic acid) and low affinity sites, probably carbohydrate.

The plant hormones, cytokinins and auxin were also bound by the glycopeptide. Cytokinin inhibited calcium binding by the glycopeptide, and transport in whole cells, but enhanced uptake in osmotically shocked cells. Auxin enhanced uptake in both intact and osmotically shocked cells, and in the binding of calcium by the glycopeptide. In addition, cytokinin regulated calcium uptake in osmotically shocked cells. From results obtained in the interaction of cytokinin, auxin and Ca^{++} on the glycopeptide, it was concluded that both these plant hormones regulated movement of calcium at the cell surface as signals of cell metabolism.

Mercurial enhancement in conjunction with the activation effect of iodine and Ag^{+} on calcium binding by the glycopeptide indicated that sulfhydryl groups are involved in calcium binding possibly by interconversion of oxidized (maximal binding) and reduced (minimal binding) states. It has been suggested that hormone action involved the regulation of sulfhydryl interconversion (Stevenson, 1974). However, sulfhydryl interconversion can only be postulated, due to inherent problems of working with Hg^{++} in conjunction with cytokinins and Ca^{++}. On the basis of classical kinetic analysis, Hg^{++} appeared to desensitize the calcium transport system, producing maximum operation of the calcium transport

system and eliminating the regulation of the transport system by Mg^{++} and plant hormones that would occur under normal physiological conditions.

Nutritionally, calcium was required throughout the growth cycle of Achlya. Citrate inhibited growth immediately upon addition at any stage of the cycle, and thus was alleviated specifically by Ca^{++}. Ca^{++} increased cell growth as the concentration increased to an optimum of about 0.1 mM. An attempt to localize cell calcium was unsuccessful, but it was shown that 60% of cellularly attached calcium could be released by excess carrier.

Macromolecular synthesis was blocked when citrate, an impermeant anion was added to cells in culture. This blockade was reversed specifically by calcium. Calcium was shown to be closely associated with protein synthesis. An attempt to correlate overall calcium action with in vitro protein synthesis was unsuccessful. Calcium action, if linked to protein synthesis at all, was at the level of amino acid transport.

As a slight digression, it was of interest to investigate the tight coupling protein and RNA synthesis found during the study of calcium effect on macromolecule synthesis. Cycloheximide, normally a relatively specific inhibitor of protein synthesis in eucaryotes, stopped both RNA and protein synthesis in Achlya at low concentration. Further studies in which glutamate dehydrogenase was induced, showed tigher coupling of RNA and protein synthesis.

All active transport systems were inhibited by the removal of reversibly bound glycopeptide calcium and by inhibition of further calcium uptake. This was demonstrated by kinetic inhibition studies of citrate and ruthenium red on the transport of amino acids, nucleoside, nucleobase and Na^{+}. In addition, studies of reduced facilitated diff-

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Cytokinin action on macromolecule synthesis and transport paralled calcium action. Data of cytokinin regulation of calcium movement at the cell surface indicated that plant hormones, as the cell's physiological signals, regulate movement of calcium (second messenger concept) which in turn, regulates cell metabolism. The possibility that cytokinin and calcium also interact at endogenous sites is discussed.

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ABBREVIATIONS

AMP	- adenosine 5'-monophosphate
ATP	- adenosine 5'-triphosphate
BIS	- N,N'-methylene bisacrylamide
CAM	- chloramphenicol
cAMP	- cyclic adenosine 3':5'-monophosphate
CCCP	- carbonylcyanide-ρ-chlorophenylhydrazone
DMSO	- dimethylsulfoxide
2,4-DNP	- 2,4-dinitrophenol
DTT	- dithiothreitiol
EDTA	- ethylene diamine tetraacetic acid
EGTA	- ethylene glycol bis (β-aminoethyl ether)-N',N'-tetra-acetic acid
$I_{0.5}$	- effector concentration giving half inhibition reaction velocity
IAA	- indole-3-acetic acid
IAAld	- indole acetaldehyde
IAOx	- indoleacetaloxine
6ipAde	- N^6-(Δ^2-isopentenyl)adenine
IpyA	- indole pyruvic acid
mRNA	- messenger ribonucleic acid
NADH	- reduced nicotinamide adenine dinucleotide
$NADP^+$	- nicotinamide adenine dinucleotide phosphate
PEP	- phospho enol pyruvate
PHMB	- ρ-hydroxy mercuribenzoate (Na salt)

POPOP	- 1,4 -Bis- [2(5-phenyloxazolyl)] -benzene
PPO	- 2,5-diphenyl oxazole
RNase	- ribonuclease
rRNA	- ribosomal ribonucleic acid
$S_{0.5}$	- substrate concentration giving half maximal reaction velocity
TEMED	- N,N,N',N'-tetramethylethylene diamine
thimerosal	- [(O-carboxy phenyl)thio] ethyl mercuri (Na salt)
TNA_2	- tryptamine
TOL	- tryptophol
Tris	- tris (hydroxymethyl)aminomethane
tRNA	- transfer ribonucleic acid

INTRODUCTION

INTRODUCTION

Cellular metabolism is regulated at many levels: transcription, translation, allosteric regulation of enzymes, and metabolic transport. The interactions that occur between and at the various levels of regulation can sometimes be so complex that a study of cell metabolic regulation is almost impossible.

The cumulative results reported in this study are an attempt to understand calcium regulation of cell physiology in a simple eucaryote, the water mould Achlya. The study of the regulation of cell physiology in this organism has been made possible due to related studies by LéJohn and associates (LéJohn, 1971; Smaluck, 1971; Stevenson, 1974; Singh, 1974; Goh, 1975) characterizing enzymatic allosteric regulation, enzyme induction, and active transport of metabolites; nucleosides, amino acids and sugars.

Studies on the characterization of the calcium transport system are presented in the first section of the Results. Kinetic analysis of the calcium transport system in conjunction with the differential mercurial activating effect on calcium transport showed that it was important to chemically and physically characterize the transport system. The first step in the analysis of a complex transport system is to attempt separation of possible binding proteins as already had been shown for ion transport in bacteria (see Oxender, 1972b). After successfully isolating a calcium binding glycopeptide from the surrounding milieu of osmotic shocked cells, the glyco-

peptide was chemically and physically characterized as to composition (65% carbohydrate and 35% protein) and molecular weight (5,960 daltons). However, the intrinsic calcium transport component remaining in the osmotically shocked cells was only kinetically characterized. Because of the unusual nature of the two component calcium transport system, regulatory mechanisms for calcium movement were studied next. The consideration of calcium regulation was aided by concurrent studies by Stevenson (1974) who showed that adenine and N^6-(substituted) adenine compounds binding was enhanced by mercurials, iodine, and Ag^+, similar to calcium activation by these compounds. Demonstration of cytokinin regulation of calcium in concert with auxin and Mg^{++} or Mn^{++} was successful.

As a result of finding a complex calcium transport system hormonally regulated in Achlya, further studies of the importance of calcium in cell physiology were instigated as discussed in section II of Results. Not only was calcium found to be required for sporangium formation, as shown by Griffin (1966) but removal of calcium at any time during the asexual ontogenic cycle curtailed growth. After an examination of macromolecule synthesis, calcium regulation appeared to be at the level of translation; by further studies, as presented in the third section of Results, calcium was found to exert the major part of its regulation at the level of the plasma membrane.

A study of calcium effect on transport showed that only energy dependent transport processes were regulated by calcium. Drawing a full circle in this study, calcium effect on energy dependent transport was shown to be indirect, controlling transport from the calcium transport system.

HISTORICAL

TRANSPORT PROTEINS

Membrane Structure

The idea that the cell is surrounded by a discrete membrane appeared in the late 19th and early 20th century. In 1855, Nageli first proposed that a membrane surrounded the protoplasm of cells and by 1924, after Chambers had demonstrated the presence of a protoplasmic surface film in starfish eggs, little doubt remained about the existence of a plasma membrane in cells (see Finean, 1972). Recent reviews on early work of membrane structure can be found in Branton and Park (1968), Smith (1962), and Finean (1972).

Overton (1899) was the first to suggest that cholesterol and other lipids are part of the chemical constitution of the membrane. In 1935, Danielli and Davson proposed the first model of membrane structure which is that a lipid bilayer formed the core of the membrane with hydrophobic tails buried in the centre and hydrophilic groups forming the outer surface. Globular proteins were aligned along the two hydrophilic surfaces of the lipid bilayer. Later, this model was modified to allow for protein-lined pores to extend through a lipid bilayer to account for

facilitated permeability. Robertson (1963) proposed that all cellular membranes, including the plasma membrane and cell organelle membrane, were similar in structure and supported the model of Danielli and Davson (1935). Robertson's model not only suggested similarity of structure but allowed for variation in the chemical composition and morphology of the cell membrane. In recent years by using improved experimental techniques such as nuclear magnetic resonance, optical rotatory dispersion, circular dichroism, X-ray diffraction (Wilkins et al, 1971), electron microscope freeze etching (Branton, 1969), and chemical modification procedures (Finean et al, 1971), it has been shown that the membrane is a lipid bilayer that is interrupted by protein that either extends completely or partially through the membrane.

Membranes can be classified into three categories depending on the amount of protein associated with the lipid bilayer. One of the simplest membranes is myelin. Myelin is composed of mainly lipid with very little protein present. Only a small amount of enzyme activity is associated with myelin membrane and its major role is to act as an insulator of the nerve fibers. The next membrane type is characterized by plasma membrane which has approximately a one to one molar ratio of protein to lipid. Plasma membrane is involved in a variety of enzymatic processes and metabolite transport, in addition to acting as a permeability barrier. The third type of membrane is characterized by the inner membrane of the

mitochondrion and the bacterial cell membrane. Approximately 75% of the membrane is protein. The function is similar to plasma membrane but has the added roles of oxidative phosphorylation, electron transport chain, and synthesis of nucleic acids.

There are three possible models to explain integration of polypeptide chains into the membrane. The protein molecule can extend through the lipid bilayer with the intrinsic part of the molecule being hydrophobic in nature while the carbohydrate-containing (if present) neutral and slightly basic NH_2-terminal peptide portion would be on the outer membrane surface, and the COOH- acidic terminal peptide would be located on the inner surface (Bretcher, 1973). The other possible models of protein integration into the membrane are that the polypeptide could be attached to the membrane outer surface either without being inserted into the lipid bilayer (Harrison et al, 1971a) or partially inserted into the lipid bilayer (Morawiecki, 1964; Winzler, 1969). All three models for protein integration into the cellular membrane may occur varying with functional role of membrane proteins and membrane itself. In the model suggested by Harrison et al (1971) either a hydrophilic peptide segment of the glycopeptide would interact with the phosphogroups of the phospholipid or possibly a hydrophobic peptide segment could set in a groove on the membrane surface interacting by a hydrophobic bond with membrane lipid. This model is in agreement with the membrane structure model first proposed by Danielli and Davson in 1935. Evidence supporting this type of

glycoprotein organization in the membrane has mainly been shown in viruses such as Sindbis virus (Harrison et al, 1971). The model suggested independently by both Morawiecki (1964) and Winzler (1969) is similar to Harrison's model except the polypeptide chain has a hydrophobic nature and extends into the lipid bilayer. In general, the majority of membrane proteins are bound to lipid by a combination of both electrostatic and hydrophobic bonds. These bonds are also involved in lipid-lipid and protein-protein pairs in the membrane. Divalent cations are instrumental in overcoming the electrostatic repulsion between the lipid and protein of the membrane at physiological pH. Divalent cations also stabilize the membrane by forming salt bridges between the acidic groups. Numerous surface membrane proteins can be separated from the membrane by changing the ionic strength, pH, or by adding a chelating agent. These surface proteins are usually acidic in nature and require a divalent cation such as Ca^{++} or Mg^{++} to restore the released proteins back to the membrane. In some cases other membrane proteins are required for reconstitution of membrane, for example, the protein nectin in addition to Mg^{++} in Streptococcus faecalis is required before ATPase can be added back to the membrane (Baron & Abram, 1971).

The presence of proteins dispersed in a sea of lipid had led to an extensive study of the structure and function of these proteins. Well studied examples of membrane protein are the red blood cell membrane proteins (Bretcher, 1973). One third of the proteins are

represented by two high molecular weight proteins (molecular weight in excess of 200,000 daltons) and one quarter of the protein is represented by the transport protein, component 'a', which has a molecular weight about 100,000 daltons. There are also nine other components varying in molecular weight from 90,000 daltons to 15,000 daltons of which a number are glycoproteins. Numerous other component proteins are known to be present but quantitatively diffucult to examine. Experimental procedures using proteolytic enzymes and chemical labelling have shown that component 'a' is located at the cell surface and that it extends from the outside to the inside of the membrane. Only 30% of the component 'a' molecules is exposed on the upper and lower surface of the membrane. The segment of the component 'a' located on the membrane surface contains approximately 10% carbohydrate which may be covalently bound to the protein. Component 'a' may function to transport anions such as Cl^- and HCO_3^- across the membrane since that is the major physiological function of the red blood cell membrane. In the red blood cell no proteins are known to be completely buried in the membrane while almost all the proteins are located on the inner surface of the membrane. Tektin A or more commonly called spectrin (the two high molecular weight proteins α and α' that comprise one third of membrane protein) may function as a contractile system (Mazia & Ruby, 1968; Marchesi et al, 1970; Hulla & Gratzer, 1972). Tektin A forms large filamentous aggregates in the presence of K^+ and Ca^{++}. The molecule most likely has an $\alpha\alpha'$ composition and may cover the

entire inner surface of the membrane. Although Tektin A has been suggested to be myosin-like, no evidence has been presented to support this theory.

Glycoproteins

Glycoproteins are of particular interest as the majority of all proteins are glycoproteins. The shape, molecular size (6,000 to 10^6 daltons), and amount of carbohydrate and protein are extensively varied in glycoproteins. Glycoproteins can be found in all biological tissues, for example, blood plasma, antarctic fish, collagen, brain, nerve endings, plants, fungi, bacteria, and viruses, and the functional role of glycoprotein is just as varied, for example; human blood serum determinants, antifreeze agents, antiviral agents (interferon), memory, transmission of nerve pulse, and clumping of cells (plant lectins). A number of recent reviews have been published on glycoproteins by Sharon (1974), Cook (1968), Spiro (1966, 1970), Hughes (1973), Marshall & Neuberger (1970), and Winterburn & Phelps (1972).

Chemistry

The chemical constitution is as varied as the location and biological activity of the glycoprotein (see Hughes, 1973). The

amino acid and carbohydrate composition of a wide variety of glycoproteins isolated from membrane such as marine sponges, human erythrocytes, rhodopsin, viral haemagglutinin, myelin, and mitochondria are listed in Hughes' review. The amino acid composition of glycoprotein is similar to that of other proteins. In the carbohydrate moiety only nine monosaccharides have been detected which are glucose, lactose, mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, arabinose, xylose, and neuraminic acid (one type called sialic acid). At the peripheral positions sialic acid, which is a sugar having both amino acetyl and carboxyl groups, serves the role of conferring ionic and hydrophilic property to the glycoprotein. Fucose may substitute for sialiac acid at the peripheral positions. Usually just one or the other is present in the glycoprotein. Carbohydrate can represent 1 to 85% of the molecular weight. The blood group glycopeptides contain 85% carbohydrate but their function is unknown. The carbohydrate portion can be complex since the monosaccharide combinations are numerous and branching, while amino acids only combine in rigorous set patterns. However, ther is usually a carbohydrate core rich in mannose and N-acetyl-glucosamine. Sharon (1974) suggested that the varied composition and structure of the carbohydrate moiety of the glycoprotein and constant protein pattern is inherent in the nature of their synthesis. Protein is a genetically linear determinant, while carbohydrate is added on after the synthesis of the poly-peptide by enzymes that are specific for a particular sugar unit

not the entire sugar oligosaccharide. The carbohydrate and peptides chains are covalently bonded. Only six amino acids are known to form linkages with carbohydrate: aspartic acid (asparagine), alanine, serine, threonine, hydroxylysine, and hydroxyproline. Four possible bond types can occur between carbohydrate and polypeptide: glycosylamine bonds, glycosidic bonds, ester bonds, and amide bonds (see Grant & Simkin, 1964). Glycosylamine bonds occur in a wide variety of glycoproteins. Aspartic acid or asparagine are linked to prosthetic carbohydrate, for example, N-acetylhexosamine, galactose, or xylose. The glycosylamine bond is comparatively stable to acid and alkali. O-β-Glycoside linkage involves the β-hydroxyl group of hydroxyamino acid such as threonine, serine and cysteine with the sugar. An example of an ester linkage ($-C\lessgtr^{O}_{O}$) is techoic acid. Alanine is linked by an ester bond to the 2-carbon of the glycerol residue. Another example is the formation of an ester linkage with the 6-hydroxyl group of N-acetylglucosamine. Bacterial cell walls have acid-stable amide bonds in the mucopeptide. An amide bond ($-C\lessgtr^{O}_{N}$) is formed between alanine and the carboxyl group of N-acetylmuramic acid.

Synthesis

The synthesis of the glycoprotein collagen has been extensively studied in recent years by Spiro (1971a, b, c). After the synthesis of the polypeptide segment probably on the rough endo-

plasmic reticulum, it is glycosylated as it passes through the cisternae of the rough endoplasmic reticulum and into the Golgi apparatus. The collagen polypeptide is first modified by the hydroxylation of a lysine residue at which position the carbohydrate component is added. Upon completion of glycosylation three dimensional structural arrangement of the collagen occurs. A number ofglycosyltransferases are involved in the addition of the carbohydrate component. The glycosyltransferases are specific for the carbohydrate. The first sugar to be added to the collagen polypeptide is galactose. Galactosyl transferase catalyzes the transfer of galactose from UDP-galactose to the hydroxyl group of the modified amino acid, lysine. The sequence of the polypeptide is important for the binding of the first carbohydrate but not subsequent carbohydrates. This catalytic action is also influenced by environmental factors such as amount of donor and acceptor molecules resulting in varying carbohydrate chain length.

Membrane glycoproteins present a special problem as they are located external to the lipid bilayer. Membrane glycoproteins may be synthesized on mRNA that has encoded in it the message for secreted glycoproteins to which only a special class of ribosomes would bind and be responsible for synthesis (Redman & Sabatini, 1966; Ganoza & Williams, 1969; Uenoyama & Ono, 1972; Tanaka & Ogata, 1972). Membrane glycoproteins would be a special class of secreted glycoproteins. As an alternative, membrane glycoproteins could be synthesized in a manner similar to cytoplasmic proteins,

then inserted into the membrane from the cytoplasm finding the correct orientation because of differential solubility characteristics of the membrane (Bretcher, 1972). In the case of the red blood cell glycoprotein, this would occur because the NH_2^- neutral terminal peptide segment could travel across the membrane to the outer membrane surface, the central hydrophobic peptide segment would remain stable in the lipid milieu, and the COOH- acidic terminal peptide segment would stick on the inner surface of the membrane.

Significance of Carbohydrate

Eylar (1966) suggested that the carbohydrate unit of the glycoprotein serves only as a handle for the transport of the protein across the plasma membrane. If this were true extracellular proteins would be glycosylated and cytoplasmic proteins would not. It is unlikely that this is the sole reason for the carbohydrate unit of the glycoprotein since a large number of glycoproteins are located both intracellularly and extracellularly, and also a number of non-glycosylated proteins are secreted from the cell. As this is not the only role for the carbohydrate moiety, several other theories have been suggested. In some cases it is possible to remove portions of the carbohydrate unit without interferring with the protein function or enzyme activity, for example; thrombin (Skaug & Christensen, 1971), glucoamylase (Pazur et al,

1970), and chloroperoxidase (Lee & Hager, 1970), but in other cases the carbohydrate unit is required for complete activity, for example; fibrinogen (Morgan et al, 1970), blood group specific glycoproteins (Morgan, 1967), and specific interaction of hormone with target cell (Suttajit et al, 1971; Van Hall et al, 1971). Ashwell and Morell proposed that the carbohydrate side chains serve as signal for metabolic control of the protein by the organism (see Sharon, 1974). Winterburn and Phillips (1972) had earlier suggested a similar role for the carbohydrate unit of the glycoprotein. The carbohydrate unit is required for specific recognition and anti-recognition inherent in the glycoprotein. Anti-recognition role may be the masking of antigenic determinant, and the covering of protein to prevent self-aggregation. This model, in summary, states that the glycoproteins located at the cell surface play an important role in cell to cell interaction, or that one cell has the ability to modify the other cell, and vice versa. Intracellular adhesion seemingly occurs by specific recognition of glycoprotein oligosaccharide on the surface of one cell with the corresponding specific glycosyltransferase on the surface of another cell (Roseman, 1970). The oligosaccharide units form rigid receptor-specific sites which may be associated with disulfide bridge formation and cation requirement (Cleland, 1970; Sundararajan & Rao, 1970; Greer et al, 1970). The carbohydrate unit of the glycoprotein also could serve as a

determinant of glycoprotein catabolism. When sialic acid is removed from the carbohydrate unit of serum α -acid glycoprotein, it is a signal for the removal of the glycoprotein from the blood stream. Cell surface glycosyltransferase molecules may be the binding sites for soluble glycoproteins such as serum α-acid glycoprotein as suggested by Pricer and Ashwell (1971). In agreement, Hudgin and co-workers (1974) suggested that the liver plasma membrane has an intrinsic sialyltransferase that can bind asialo-glycoproteins.

Glycoprotein Action

Glycoproteins are universal in cell plasma membrane and organelle membrane such as nuclei, mitochondria, and endoplasmic reticulum, or as Sharon (1974) so aptly states, "all cells come with a sugar coat". As the aquatic fungus Achlya apparently has a hormonal regulated surface glycopeptide intimately associated with calcium transport processes across the plasma membrane, it was of particular interest to investigate the numerous functions of membrane glycoproteins in other eucaryotic systems and also in procaryotic systems. Membrane glycoproteins can be associated with hormonal regulation, cell structure, enzyme catalyst activity, and transport processes. The glycoproteins of mammalian cell membranes are involved in evolutionary advanced physiological roles

such as cell-cell association, contact phenomena in controlling growth, histocompatibility-antigenic specificities, and nerve transmission.

Junctional complexes that exist between mammalian cells are of three structural types, tight junctions, intermediate junctions, and desmosomes and are functionally different but all allow free exchange of molecules and ions between the cells (Goodenough & Revel, 1970). Glycoproteins are present in the junction complexes. Junctions can be easily opened when treated with chelating agents such as EDTA and EGTA. Farquhar and Palade (1963) suggested that the sialic acid residues are cross-linked by calcium, since when EDTA treated, periodate is able to react with sialic acid which was not available for periodate staining prior to EDTA treatment. Whether this is a correct assumption is unknown, but that glycoproteins do interact with divalent cations across the junction gap has been confirmed by Goodenough and Revel (1970).

On the nonjunction plasma membrane surface, glycoproteins appear to be evenly distributed, for example; rat liver (Benedetti & Emmelot, 1967), human erythrocyte (Nicolson & Singer 1971; Nicolson et al, 1971), and mouse fibroblast culture cells (Nicolson, 1971). The major glycoprotein of the red blood cells is an extensively studied example of a mammalian membrane glycoprotein. Two thirds of the major glycoprotein of the red blood cell is carbohydrate, approximately 100 sugar residues while

the protein portion is only 87 amino acid residues long. This glycoprotein is numerous in the red blood cell membrane comprising 80% of the carbohydrate and 90% of the sialic acid. The carbohydrate branches (10 residues per branch) are attached to the protein at 10 places (Kathan & Winzler, 1963) near the amino terminal end with the remainder of the protein free (insoluble) extending across the membrane. Approximately 23 hydrophobic amino acids extend through the membrane with the COOH- acidic terminal segment on the inner surface of the membrane (Segrest et al, 1972). This glycoprotein contributes the negative charge to the red blood cell membrane and a number of antigenic properties. The function of the glycoprotein is unknown but Bretcher (1973) suggests that it keeps the red blood cells separated because of the negative charge it carries and that it may be a primitive form of cell wall giving the red blood cell rigidity. The rigidity could be produced by the cross-linking of the carbohydrate residues through H bonds.

Glycoprotein membrane markers may be arranged in different ways over the cell surface. Trypsin treatment causes clustering of the glycoproteins on the cell surface (Nicolson, 1971). Marchesi also saw a similar clustering of glycoproteins after treatment with trypsin (see Hughes, 1973). Freeze etching technique showed that intramembranous particles also clustered after trypsin treatment which indicated concomitant movement with glycoproteins. The intramembranous particles and glyco-

proteins are probably synonymous. Frye and Edinin (1970) have shown glycoproteins are easily able to move laterally on the membrane surface (cis-migration), but other reports disagree. Bretcher (1973) suggested that membrane protein are rigidly anchored in the membrane, thus being unable to cis-migrate.

Because the plasma membrane of mammalian system turns over rapidly, the release of glycoproteins from the cell surface as a natural phenomenon was investigated by Kappler et al (1973). Kappler and co-workers found that glycoproteins on the surface of chick embryo cells in culture were released as a normal metabolic process and that this process could be imitated by treatment of cells with trypsin. Trypsin released glycoproteins that are normally released since no enzymatic degradation of these proteins could be found. Rubin (1966) had previously proposed that cells in culture be viewed as a dynamic system, that is, the subunits of the cell membrane are being continually released into the immediate surroundings with the released membrane subunits in steady state equilibrium with the membrane. This steady state equilibrium would be required for the cells to proliferate. As cells in culture are usually transformed, this release of glycoprotein may allow continual cell proliferation. A recent report published by Lipkin and Knecht (1974) agrees with Rubin's proposal. For the first time a soluble protein factor produced by a contact-inhibited melanocytic cell line can reversibly restore contact inhibition to malignant hamster

melanocytes. The regulation of normal cell growth and division occurs at the cell surface involving membrane proteins, but when cells become transformed regulating surface proteins are lost causing uncontrolled proliferation.

The role of membrane glycoproteins in non-mammalian cells is unknown and until recently the presence of glycoproteins in the cell membrane of micro-organisms had not been demonstrated. Antigenic determinants are associated with bacterial cell wall glycoproteins but it appears that the glycoproteins of the bacterial cell membrane may have a different function from that of mammalian cells. In M. lysodeikticus and Streptococcus pyogenes glycoproteins were extracted from the cell membrane that could be precipitated at low concentrations of Ca^{++} and Mg^{++}. When examined microscopically myelin-like figures are discerned. Aggregation produced by divalent cations could be prevented by trypsin which suggested peptide-peptide linkage (Okuda & Weinbaum, 1968; Weinbaum et al, 1970). In fungi, as for the case of bacteria, the presence of glycoproteins in the cell membrane is hard to demonstrate because the cell wall is so rich in carbohydrate. A number of secreted enzymes are glycoproteins or have glycoproteins associated with them. Aspergillus and Rhizopus excrete the enzymes, α-amylase, glucose oxidase and glucamylase, all of which are glycoproteins (Puzar et al, 1969; Letoublin et al, 1971). Lipase, when excreted from Rhizopus arrhizus, has a small

molecular weight (8,500 daltons) glycopeptide associated with it. The function of this small glycopeptide is unknown, but Semeriva and co-workers (1969) suggested that it may facilitate the movement of lipase through the membrane. Several enzymes that are important in cell wall biosynthesis are known to be glycoproteins and may be associated with either the cell wall or the cell membrane. The enzyme invertase is a glycoprotein of which 15% is carbohydrate in Neurospora (Meachum et al, 1971), and 53% is carbohydrate in yeast (Neuman & Lampen, 1967). Metzenberg (1963) suggested that the enzyme invertase is located between the cell membrane and the cell wall. Invertase contains the carbohydrate mannan which may be involved in fixing the enzyme to the inner surface of the outer cell wall. Villemez (1970) has isolated an insoluble low molecular weight glycoprotein (11,500 daltons) from the particulate fraction (membrane) of the bean plant, Phaseolus aureus which appears to be a carrier protein that mediates the polymerization of glucose from GDP-glucose.

Glycoproteins have been isolated from the membrane of the sponges, Microciona prolifera, Haliclona occulata (Margoliesh et al, 1965), and Microciona parllena (Henkart, 1971). Sponge cell aggregates can be separated into single cells if washed in a Ca^{++} and Mg^{++} free solution. A specific aggregating glycoprotein appeared to be removed when divalent cations are absent from the incubating buffer (Margoliesh et al, 1965; Henkart, 1971). Henkart (1971) demonstrated that the released glycopeptide was self-

aggregating and bound to the surface membrane in the presence of bivalent cation (20 mM $CaCl_2$). What the exact function calcium serves is unknown but calcium may either play a role in cell aggregation along with glycoprotein or may be required for assembly of glycoprotein into the membrane. As a note of interest, a carbohydrate binding protein is present on the surface of cohesive cells of the slime mold Dictyostelium discoideum but absent on vegetative cells that bind N-acetyl-D-galactosamine, D-galactose, and L-fucose. As this sugar binding protein can agglutinate red blood cells it may function to bind cells of Dictyostelium discoideum together when cells aggregate to form a spore (Rosen et al, 1973).

Calcium Binding Proteins

Calcium binds to a variety of cell constituents such as, protein (Siegel, 1974), phospholipid (Piccinini et al, 1970), gangliosides (Gielen, 1966), nucleic acids (Carr & Chang, 1971), polyglucose matrix (Reed, 1973), and ATP (Chau-Wong & Seeman, 1971). Of particular interest is calcium binding to protein and carbohydrate.

The majority of calcium binding proteins are acidic in nature containing high levels of aspartic acid, glutamic acid, lysine, glycine, and low levels of tyrosine, tryptophan and sulfur containing amino acids. Table I lists the amino acid composition

Table I. Composition of Acidic Calcium Binding Proteins

Amino Acid	Moles Amino Acid Per Mole of Protein				
		Muscle		Avian intestinal	Brain
	Calsequestrin (1)	Troponin C (2)	Hake parvalbumin (3)	mucosa border (6)	CBP-I (6)
aspartic acid	18.9	14.2	11.5	14.0	15.3
glutamic acid	18.4	21.3	9.2	15.6	15.3
glycine	3.8	7.7	11.2	7.1	10.8
alanine	6.6	7.8	17.5	7.4	9.6
valine	6.9	4.3	3.7	2.8	7.9
leucine	8.9	5.5	7.4	11.9	10.8
isoleucine	5.1	5.5	6.6	4.6	6.5
serine	3.6	4.1	4.8	4.2	10.4
threonine	2.8	3.4	4.6	4.1	11.6
lysine	6.6	7.0	11.4	9.7	15.5
histidine	1.5	0.7	0.9	1.6	2.8
arginine	1.8	4.2	0.9	2.5	3.0
phenylalanine	6.1	5.7	8.8	5.4	7.1
tyrosine	1.5	1.1	-	4.1	1.4
tryptophan	-	-	-	-	0.8
proline	5.1	1.2	-	1.5	0.3
methionine	1.5	5.6	0.9	2.0	1.4
cysteine	0.8	0.6	0.9	1.5	2.0

Table I. Composition of Acidic Calcium Binding Proteins

Amino Acid	Mole Amino Acid Per Mole of Protein	
	bone sialoprotein (7)	elastin (8)
aspartic acid	17.4	0.7
glutamic acid	22.9	1.8
glycine	12.2	32.2
alanine	4.0	21.7
valine	3.1	13.7
leucine	2.9	6.6
isoleucine	2.5	2.7
serine	8.0	1.0
threonine	11.1	1.0
lysine	2.9	0.4
histidine	1.2	0.1
arginine	1.2	0.7
phenylalanine	2.3	3.4
tyrosine	1.2	0.6
tryptophan	1.1	-
proline	4.9	12.8
methionine	-	-
cysteine	1.3	-

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Table I. Continued

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of several calcium binding proteins.

A conformational change is produced in the protein molecule when calcium is bound (Murray & Kay, 1972; Van Eerd & Kawasaki, 1972), but the nature of calcium binding to the protein has only been demonstrated unequivocally for fish parvalbumin and thermolysin. The structure of fish parvalbumins has been extensively characterized by both Pechère and Kretsinger's group and it is one of the best molecular studies of the interaction of calcium with protein yet published (Benzonana et al, 1972; Nockolds et al, 1972). Fish parvalbumin can bind two moles of calcium per mole of protein. At the binding site, calcium forms a tetrahedral coordination through the carboxyl groups of three aspartic acid residues and one glutamic acid residue which lie in the sequence Gly-Asp-Ser-Asp-Gly-Asp-Gly-Lys-Ile-Gly-Val-Asp-Glu-. Another similar site for calcium binding is found for the peptidase enzyme, thermolysin of Bacillus thermoproteolyticus. Thermolysin binds three moles of calcium per mole of protein which confers stability to the enzyme. Two of the three calcium ions form octahedral coordinate binding with three aspartic acid and two glutamic acid residues (Matthews et al, 1972).

A slightly different mode of calcium binding is found in the calcium binding protein, elastin. Elastin has a high content of the neutral amino acid, glycine (65%). Urry (1971) suggested that the high glycine content favors formation of β-turns similar to the ionophores, valinomycin

and gramicidin. Calcium may be co-ordinated to oxygen atoms of the neutral amino acids. In conclusion, it is likely that all high affinity binding sites of calcium binding proteins bind calcium via oxygen coordination (see Siegel, 1973), although, in some cases, other factors may be involved. The acidic phosphoprotein (CBP II) of the brain binds calcium only if intact, and if phosphate is removed, calcium binding is inhibited.

The nature of calcium binding by carbohydrate molecules or the glyco- moiety of glycoproteins is unknown, but it is believed to occur by ionic binding to acidic groups of the carbohydrate units (Farquhar & Palade, 1963), for example; the terminal sialic residues of the glycoproteins in junction complexes are cross-linked by calcium bridges between the acidic groups.

Calcium binding proteins are involved in numerous basic metabolic processes such as, intestinal calcium absorption, muscle action, bone formation, and energy state of the cell. In general, the calcium binding proteins are associated with calcium transport systems that play an important part of these metabolic processes. In Table II a number of the better studied examples of calcium binding proteins associated with transport systems are characterized.

Binding Proteins Associated With Transport Systems

Transport across the membrane is proposed to involve 'carrier' mediated transport systems that are composed of one or

Table II. Characterization of Calcium Binding Proteins

Name	Source	Molecular Weight	High Affinity Binding		Function*	Literature Reference
			K_d (M)	Calcium Bound		
soluble calcium binding glycoprotein	mitochondria	42,000	1×10^{-7}	3	associated with calcium transport in the mitochondria	Sottacasa, G., G. Sandri, E. Panfili, B. de Bernard, P. Gazzotti, F. D. Vasington & E. Carafoli (1972) Biochem. Biophys. Res. Comm. 47 808-813.
insoluble calcium binding glycoprotein	mitochondria	67,000	1×10^{-7}	2	associated with calcium transport in the mitochondria (possibleprecipitate of soluble calcium binding glycoprotein of the mitochondria)	Gomez-Puyou A., M. T. de Gomez-Puyou, G. Becker & A. L. Lehninger (1972) Biochem. Biophys. Res. Comm. 47 814-819.
calcium binding glycoprotein	cartilage	2,000,000	1×10^{-7}	2	serve as initiation sites for calcification	Pal, S., P. T. Doganges & M. Schubert (1966) J. Biol. Chem. 241 4261-4266.

Continued
Table II. Characterization of Calcium Binding Proteins

Name	Source	Molecular Weight	High Affinity Binding K_d (M)	High Affinity Binding Calcium Bound	Function*	Literature Reference
calcium binding sialoprotein	bone	21,800-25,000	-	-	transport of calcium from bone cells	Williams, P. A. & A. R. Peacock (1967) Biochem. J. 105 1177-1185 and Chipperfield, A. R. (1970) Biochem. J. 118 36P.
elastin	tendon	74,000	1.4×10^{-6}	2	calcium-phosphate calcification of elastin	Urry, D. W. (1971) Proc. Nat. Acad. Sci. 68 810-814.
Calsequestrin	sarcoplasmic reticulum	44,000	4×10^{-5}	43	calcium storage in muscle tissue	MacLennan, D. H. & P. T. S. Wong (1971) Proc. Nat. Acad. Sci. 68 1231-1235.
Troponin C	skeletal muscle	24,000	1.9×10^{-7}	1	contributes calcium sensitivity to muscle	Greaser, M. L. & J. Gergely (1971) J. Biol. Chem. 246 4226-4233.
Paralbumins	fish white muscle	11,000	1×10^{-7}	2	troponin-like role in lower vertebrates	Benzonana, G., J. P. Capony & J. F. Perchère (1972) Biochem. Biophys. Acta. 278 110-116.

Continued
Table II. Characterization of Calcium Binding Proteins

Name	Source	Molecular Weight	High Affinity Binding K_d (M)	High Affinity Binding Calcium Bound	Function*	Literature Reference
vitamin D inducible calcium binding protein	Avian duodenal mucosa	24,000	3.8×10^{-6}	1	associated with intestinal absorption of calcium	Wasserman, R. H., R. A. Corradino & A. N. Taylor (1968) J. Biol. Chem. 243 3978-3986.
Brain CBP-II acidic phosphoprotein	brain, adrenal medulla	11,500	2.5×10^{-5}	1	CDR of phosphodiesterase	Wolff, D. F., J. Heubner & F. L. Siegel (1972) J. Neurochem. 19 2855- and Brooks, J. & F. L. Siegel (1973) J. Biol. Chem. 248 4189-4193.
Brain CBP-I	brain	13,000	-	-	associated with calcium uptake in nerve tissue	Wolff, D. J. & F. L. Siegel (1972) Arch. Biochem. Biophys. 150 578-584.

*The function of calcium binding protein is usually not definitely known and the most likely function is listed here.

more protein components. In 1964, Malamy and Horecker were the first to demonstrate the release of metabolite binding proteins from bacterial cell membrane. Since that time, binding proteins of organic and inorganic metabolites have been isolated after treatment of cells either by salt exchange (Nieman & Willis, 1971), or by osmotic shock treatment (Heppel. 1969). The release of these binding proteins is accompanied by a reduction of transport for that entity into the cell. The binding proteins released are believed to be located on the membrane surface and to form an association with membrane transport systems. It is unlikely that all metabolite transport systems have binding proteins, but the binding proteins can add specificity to the transport system and can either increase its maximum velocity or affinity. The majority of the published literature is on the release of binding proteins from bacteria, and only a few recent reports on the release of binding proteins from other organisms can be found, Neurospora (Wiley, 1970), yeast (Patching & Rose, 1971; Schwenche et al, 1971), higher plants (Amar & Reinhold, 1973; Nieman & Willis, 1971), and Achlya (LeJohn et al, 1973).

The gram negative bacteria, E. coli and Salmonella typhimurium, upon cold osmotic shock treatment release binding proteins believed to be involved in specific transport systems. The cold osmotic shock procedure as described by Heppel (1969) involves two steps.

In the first step, the bacteria are suspended in 0.5 M sucrose containing EDTA, and in the second step, after the removal of sucrose, the bacteria are rapidly suspended in ice-cold dilute $MgCl_2$ (0.5 mM). Membrane-bound binding proteins are selectively released during the second step. Approximate 24 binding proteins have been isolated from E. coli by osmotic shock treatment, for example, the binding proteins for phosphate (see Oxender, 1972a), sulfate (Pardee, 1968), leucine (Oxender et al, 1968), and D-galactose (Anraku, 1967) have been isolated and purified. Their respective molecular weights are 42,000, 32,000, 24,000 -36,000, and 35,000 daltons, and one mole of substrate was estimated to bind to one mole of protein in each case. The kinetics of substrate binding by the released protein and the transport of substrate in the intact cell are similar. Genetic studies have also been carried out on the reported binding proteins and a correlation between negative mutants and no release of the specific binding protein can be drawn. Heppel (1967) suggested that the binding proteins are free or loosely bound in the 'periplasmic space'. As indirect evidence; high ionic strength sensitivity, rapid binding which is easily reversible, and release of protein by mild treatment, indicate that these proteins are located on the cell surface but no binding has yet been demonstrated to the cell wall or membrane by the binding proteins. Attempts to reactivate transport systems for inorganic sulfate and leucine by adding back the shock fluid to the osmotically shocked cells have failed, but Anraku (1967) has reported partial restoration of D-galactose transport (rate and extent) by adding the galactose binding protein to osmotically shocked cells.

In higher plants, investigation of the link between substrate binding and transport was first studied by kinetic technique. During sulfate transport in barley, both binding and absorption, were specifically inhibited by selenate but not by monovalent anions. Therefore, the specificity of binding matched the specificity of transport (Epstein, 1956). Similar findings were reported by Pettersson (1966) for sulfate binding in sunflower roots, by Persson (1969) for sulfate binding in wheat, by Pettersson (1971) for phosphate binding in sunflower roots, and by Cohen (1962a, b) for rubidium binding in Chlorella pyrenoidosa. These reports linked binding of ions to transport through the membrane but did not establish whether transport protein was identical with binding protein. Only recently, initial investigations on releasable binding proteins have been reported for higher plants. The released proteins have not been characterized and labelled binding to the crude shock fluid has not been shown, but the still viable treated cells have a reduced uptake of substrate(s), and, in some cases, if calcium or crude shock fluid is added back to the treated cells, partial ability to take up metabolites occurs.

Nieman and Willis (1971) were able to cause the release of membrane protein from carrot discs by treatment with NaCl. As the active transport of glucose and phosphate was reduced, a link between released proteins and transport was suggested by Nieman and Willis (1971). If calcium was added prior to treatment of the cells with NaCl no protein was released and phosphate and glucose uptake remained intact. Calcium could play an important role in attachment of released proteins. An

attempt to reactivate transport processes by addition of supernatant fluid proved futile, but over a period of time normal active transport resumed. In a recent report by Amar and Reinhold (1973), leaf strips of Phaseolus vulgaris after cold osmotic shock treatment patterned after Heppel (1969) showed a reduced ability to take up the proline analogue, α-aminoisobutyric acid, and 3.5% of the protein was released. Uptake capacity was regained if cells were immersed in 1 mM $CaSO_4$ (75% of the control in 30 min.) or in shock fluid, but this data was not always reproducible. Amar and Reinhold (1973) could find no protective capacity for calcium during the cold osmotic shock treatment as Nieman and Willis (1971) had earlier shown during increased ionic strength treatment. No binding of labelled α-aminoisobutyric acid to the shock fluid could be obtained, but Amar and Reinhold suggested that this was due to the high K_m value (0.45 mM) making it almost experimently impossible to do by equilibrium dialysis because of the high concentration of protein required. Amar and Reinhold (1973) suggested that the protein released is involved in the specific transport system of this amino acid because osmotic shock procedure interferred with influx. Only a small percentage of the membrane protein was released. Release of protein is by a mild treatment, and the integrity of the membrane is maintained because of the retention of viability.

The cold osmotic treatment of Heppel was used to release binding proteins and to reduce uptake capacity of fungal cells. In yeast, Schwencke's group (1971) demonstrated that cold osmotic shock treatment of Saccharomyces chevalieri reduced capacity of the cells to

take up L-proline, released 7% of the cell surface protein, and the shocked cells retain their viability. No binding protein for L-proline was demonstrated in the shock fluid but Schwencke and co-workers suggested that an essential component of the L-proline transport system was released into the medium because of reduced capacity of the osmotic shocked cells to take up L-proline. Also, Patching and Rose (1971) showed that in the yeast, Saccharomyces cerevisiae, after cold osmotic shock treatment, the cells had a reduced capacity to accumulate non-metabolizable solutes, D-glucosamine-hydrochloride and 2-aminoisobutyrate; had lost 6 to 8% of their cell protein; and the cells remained viable. In agreement with Schwencke's group (1971), Patching and Rose (1971) inferred that transport proteins are released because of the reduced capacity to accumulate D-glucosamine-hydrochloride and 2-aminoisobutyrate.

Using potassium chloride extracts instead of cold osmotic shock extracts Stuart and DeBusk (1971) isolated two arginine-binding fractions from ungerminated conidia of Neurospora. Since both fractions were found to be absent in the mutant strain $Pm^{-}B^{37}$ which lacks the conidial basic amino acid transport system, the arginine binding fractions isolated may be related to the arginine transport system. In one of the most detailed studies of eucaryotic cells, Wiley (1970) has described the release of a recoverable tryptophan binding protein from the cold osmotic shock fluid of Neurospora crassa without any appreciable loss of cell viability. From substantial experimental evidence, Wiley (1970) suggested that the released tryptophan binding protein is part of the previously characterized

neutral amino acid transport system that transports tryptophan, leucine, methionine, and phenylalanine (Wiley & Matchett, 1966). Since the binding protein could be released by mild treatment, it is probably located on the cell surface. The binding of tryptophan by the released binding protein was specific and correlated to the binding of tryptophan by the intact tryptophan transport system, that is, the K_d for binding and the apparent K_m for cellular transport were similar, 8.0×10^{-5} M and 5.0×10^{-5} M respectively. Wiley (1970) also showed that mutants (mtr locus) (Stadler, 1965) for a component of the *in vivo* tryptophan system released a tryptophan binding protein that had a reduced capacity to bind tryptophan. This report by Wiley (1970) is the first report of characterization of a specific binding protein releasable by osmotic shock treatment in a eucaryotic cell, and the implications that this may exist in other eucaryotes suggest that transport binding proteins may be common to all cells.

Sulfhydryls in Membrane Structure and Transport

It is well documented that sulfhydryl groups do play an important role at the active site of enzymes including both transport proteins and cytoplasmic proteins (Rothstein, 1970). In addition to the role sulfhydryl groups play at the active centres of enzymes, sulfhydryl groups are important in maintaining the integrity of the membrane. Szent-Györgyi and McLaughlin (1972) have proposed a model of sulfhydryl reaction where the SH- group catalyzes a charge transfer between an

amino group and a carbonyl group. In the absence of SH-group the reaction took place, but when SH-group containing compounds such as cysteine, glutathione, or thyglycollic acid were added, the reaction was accelerated. For example, the reaction between either a protein molecule or ethylenediamine and carbonyl (glyoxal, glyceraldehyde, and dihydroxyacetone) was specifically catalyzed to varying degrees by cysteine or glutathione.

Reagents that bind sulfhydryl groups interfered with membrane function (Scott et al, 1970; Sutherland et al, 1967; Carter & Martin, 1969a; Rothstein, 1970). Numerous types of sulfhydryl reacting agents exist but those that form mercaptides are of major interest for studying biological membranes (Rothstein, 1970; Seidel, 1972). $HgCl_2$ and organic mercuricals, such as, pCMBS, pCMPS, and thimerosal interact with membrane sulfhydryl groups by mercaptide formation which is easily reversible by the addition of reducing agents, cysteine or DDT. Organic mercurials are bulky molecules that react mainly at the membrane surface with a simple relationship of binding and inhibition, but when incubation time is increased organic mercurials can diffuse into the membrane. $HgCl_2$ is specific for sulfhydryl groups but at high $HgCl_2$ concentration can also interact with phosphoryl and carboxyl groups. By incorporation of Hg^{++} into organic compounds the specificity for sulfhydryl groups can be increased. Organic mercurials react with only one ligand and individual reactivity varies depending on size and solubility in water and lipid.

One way that sulfhydryl agents disrupt membrane function is by causing a general change in membrane structure (Robinson, 1966; Godin & Schrier, 1972). Some erythrocyte ghost membrane proteins are released by sulfhydryl reacting compounds (Smith & Verpoorle, 1970; Godin & Shrier, 1972). In an extension of these studies Carter (1973) demonstrated that the highly specific non-penetrating sulfhydryl binding agent pCMBS can cause temperature independent release of 40% of the red cell ghost membrane proteins and one minor glycoprotein. The major glycoprotein and component 'a' of the red blood cell are not released by pCMBS treatment since they are intrinsic membrane proteins. The proteins released are probably the proteins attached to the inner surface of the membrane which also can be released by changes in ionic strength (Fairbanks et al, 1971) or dilute alkali and β-mercaptoethanol (Steck, 1972). Sulfhydryl groups that interact with pCMBS are believed to contribute to general membrane structure by interconnecting protein to membrane via hydrophobic bonds as earlier suggested by Cecil and Thomas (1965) and Heitmann (1968). The mechanism of pCMBS action is unknown but part of pCMBS activity may be due to the induction of a net negative charge on the inner surface of the red blood cell resulting in loss of loosely attached membrane proteins. The proteins can not be added back in the presence of DTT, but pretreatment with DTT prior to incubation with pCMBS does prevent the loss of surface membrane proteins. In red blood cell ghosts pCMBS causes K^+ leakage. It was found that simultaneous inclusion of calcium with pCMBS in red blood cell ghosts prevented the induced K+ efflux (Colombe & Macey, 1974). This could be

interpreted as calcium preventing the induced negative charge of erythrocyte membrane or calcium binding to SH- groups preventing access to pCMBS. In agreement with the later, Weed et al (1962), Tolberg and Maoey (1972), and Palek et al (1971) also had suggested that calcium interacts directly with SH- groups on the membrane.

Sulfhydryl reacting agents can act as chemical probes to study the role of proteins in membrane structure and function which has already been demonstrated for sugar and cation transport, ATPase activity, and hormone binding in a number of systems (see Rothstein, 1970). Generally, mercurial compounds have a negative effect on membrane function resulting in loss of activity. However in the mitochondria, ATPase activity is enhanced by the surface reacting organic mercurial reagent, pCMPS, when low mercurial binding occurs (less than or equal to 20 nmoles/mg protein). Enhanced ATPase activity is associated with the accumulation of K^+ in the presence of ATP. In addition, pCMPS does not induce passive permeability to H^+ or other cations but does prevent removal of inorganic phosphate from the mitochondrial matrix and induces passive permeability to Cl^-. Other less polar mercurials, such as NEM react with both surface and internal sulfhydryl groups of the mitochondria inducing passive permeability to both anions and cations, and ATPase activity is strongly inhibited. Brierfley and co-workers (1971) suggested that surface reacting sulfhydryl reagents bound to membrane proteins and enhanced cation accumulation of ATPase activity by making transport sites more readily available for cations. However at high concentration of pCMPS, this mercurial easily reacts with internal sulfhydryl groups. The membrane integrity is destroyed

resulting in loss of both energy supply and structural form so that passive diffusion of cations and anions occur.

TRANSPORT SYSTEMS

The plasma membrane must contain transport systems for the survival of the cell. The transport systems are made up of intrinsic protein molecule(s) and in some cases binding proteins which span the plasma membrane to provide passage for the essential metabolites; energy, carbon, nitrogen, and ions which are related to macromolecule synthesis, growth, and cell division (see Stein, 1972). Transport systems allow specific uptake of metabolites usually against a concentration gradient that can be differentially regulated by exogeneous and endogeneous cellular constituents. However, some hydrophobic molecules can enter the cell by diffusion, but the vast majority of cell metabolites are transported via 'carrier' mediated processes. The uptake of metabolites on the outer surface and release into cytoplasm is presumably regulated by a conformational cycle of the 'carrier' protein(s) produced by either a direct or an indirect energy source.

Types of Transport Systems

Active uptake of substrate by a 'carrier' mediated transport system can be kinetically characterized by initial linear uptake of substrate which becomes saturable after a short period of time;

specificity for a particular substrate or group of substrates susceptible to competitive inhibition; pH and temperature dependency; and the ability of metabolic poisons to interfere with transport either directly or indirectly by disrupting ion counterflow and membrane conformation. How metabolism is connected to active transport is varied. Ion co-transport down an electropotential gradient generated by metabolic extrusion of ions, conformation cycling as a result of electrical charge changes, ATP hydrolysis-linked, respiration-linked, and vectorial phosphorylation are proposed mechanisms.

For a number of metabolites to be transported by the plasma membrane a reduced affinity for metabolite on the inner membrane as compared to the outer membrane is maintained by a concentration or electrochemical gradient across the membrane. This type of facilitated transport is called secondary active transport. Evidence now indicates that ions have the ability to maintain this difference of affinity (Jennings, 1974). The majority of proposed models for co-transport of metabolites involve a protein 'carrier' that transports the organic solute and complexes with the activating cation. Na^+ and K^+ are intimately involved with carbohydrate and amino acid transport systems in animal cells (Schultz & Curren, 1970; Heinz, 1967, 1972). The solute molecule binds to the protein 'carrier' only if Na^+ is present at a high concentration and this results in parallel transport of solute and Na^+ across the cell membrane, with the anti-parallel movement of K^+. The energy for transport of amino acids and sugars is supplied by movement of Na^+ down it's electrochemical potential

gradient. When the intracellular concentration of Na^+ becomes too high or K^+ is increased, transport of organic solute is inhibited. This problem is overcome by the cell having an active Na^+ ion pump (Baker, 1966) which pumps Na^+ out of the cell against the electropotential gradient. The energy for transport of Na^+ out of the cells involves ATP expenditure at a membrane Na^+ -K^+ ATPase site (Skou, 1965). This model is not the only one possible (see Heinz, Geck, and Wilbrandt, 1972), but it does represent the basic ideas involved in all models.

The concept of the model applies equally well to micro-organisms as it does to animal systems, except for one major difference, the important activation cations are K^+ and H^+, not K^+ and Na^+ (Eddy, Ingle, Backen, & Nowaki 1970; Eddy & Nowaki, 1971). Micro-organisms like higher plants have rigid cell walls making the organism less sensitive to osmotic regulation that is so important for animal cells. Animal cells use the energetic Na^+ extrusion pump to maintain osmotic equilibrium, while demands of metabolism (enzymatic activity) dictate ion transport in rigid cell wall organisms. The major differences between energetic ion pumps in animal and rigid cell wall organisms are; any available cation (H^+, Na^+, and Mg^{++}) can be extruded in exchange for K^+ uptake in cell wall organisms, and diffusion fluxes in cell wall organisms are minimal (5%) as compared to 75% for K^+ flux in nerve and muscle cells (Slayman, 1970).

Accumulated evidence from membrane potential difference studies using the giant algae Nitella supports the model where H^+ active transport outward and K^+ and Cl^- active transport inward across the plasma membrane maintain an electropotential gradient

that is responsible for metabolic transport. At first it was believed that K^+ alone maintained the electropotential gradient, but the maintenance of the potential gradient by K^+ only occurred in the absence of calcium and small amounts of Ca^{++} desensitized the membrane to K^+ (Kitasato, 1968). Calcium (1 mM) made the membrane more sensitive to H^+. It is likely that it is H^+ that carries the majority of current across the membrane. The internal milieu of the cell is maintained at an alkaline pH and this process requires energy. The electrogenic H^+ pump proposed by Slayman (1970) could maintain a high internal pH by extruding cations. ATP is the most likely candidate for the energy source of H^+ extrusion since cation transport is coupled to ATP formation and utilization in the green algae, Nitella, Tolypella, and Hydrodictyon (Raven et al, 1969).

Substantial proof has been presented by Slayman and Slayman (1970) that an electrogenic H^+ pump is located in the plasma membrane of Neurospora crassa that is responsible for maintaining a potential gradient across the plasma membrane which allows secondary active transport. At pH 6.0, the cell wall of Neurospora crassa has a fixed negative charge. Inhibition by metabolic inhibitors such as, cyanide, carbon monoxide, azide, anoxia, 2,4-DNP, and low temperature give proportionate changes in the membrane potential efflux of H^+ and ATP levels. Perhaps, most conclusive is that the efflux of H^+ varies in parallel with membrane potential. K^+ had at first appeared to be prominent in maintaining the potential gradient but the membrane was easily desensitized to K^+ by Ca^{++} as is the case for green

algae. It appears likely that K^+ is taken up by an active exchange of K^+ for H^+. In Fusarium oxysporium K^+ uptake is balanced by a loss of Mg^{++} as well as H^+, and there is concomitant gain of organic acids especially succinic acid. In yeast, K^+ is also taken up, with an accompanying active extrusion of H^+ (Rothstein, 1964). The neutral amino acid transport system in the yeast, Saccharomyces carlsbergensis, is linked to H^+ transport and in special situations to K^+ transport (Eddy & Nowacki, 1971). No direct metabolic energy is required for the transport of glycine or phenylalanine if there is a favorable distribution of K^+ and H^+ across the plasmalemma. Eddy and Nowacki (1971) have shown that for energy deficient conditions, one to two H^+ are co-transported for every glycine molecule and one to two K^+ are extruded. Transport of neutral amino acids under normal metabolic conditions is represented by the transport of one glycine molecule plus one hydrogen molecule inward while one hydrogen molecule is being pumped out by a energy requiring H^+ pump. Eddy and Nowacki (1971) suggested that the amino acid transport system and the H^+ pump can be interconnected in two ways; either structurally associated in the plasmalemma as previously suggested by Conway and O'Malley (1946) and Rothstein and Enns (1946), or structurally separated in the plasmalemma but still dependent upon one another; one glycine molecule and one hydrogen molecule could be transported by the neutral amino acid transport system, while the H^+ pump extrudes one hydrogen ion energetically either in the presence of K^+ or K^+ transport through the plasmalemma could be associated with the H^+ pump.

Preliminary reports which concern Na^+ or K^+ uptake association with amino acid transport in yeast have been published by Conway and Duggan (1958), Eddy and Ingle (1962), and Surdin et al (1965).

Like algae and fungi, bacteria also release acid into the surrounding milieu (Shultz et al, 1963). There is no direct evidence that ATP is required for acid release or whether it produces an electropotential gradient across the plasma membrane, but circumstantial evidence does support the presence of an electrogenic H^+ pump in the plasma membrane of bacteria that maintains a potential gradient (Harold et al, 1970). Streptococcus faecalis lacks a cytochrome system and obtains nearly all its energy from glycolysis with the release of lactate and hydrogen ions into the surrounding medium (Smith & Sherman, 1942). If K^+ is added to bacterial cells growing in low K^+ concentration, H^+ efflux is promoted with a ratio of one to one for H^+:K^+:ATP (Zarlengo & Schultz, 1966). Since ATP is synthesized by glycolysis, uncoupling agents of oxidative phosphorylation have little effect on ATP synthesis and metabolism, but do inhibit Na^+ exit (Na^+ loaded cells), Rb^+ exit, amino acid uptake, and phosphate uptake (Harold et al, 1970). However, metabolic uncouplers do depolarise the membrane. The metabolic uncouplers CCCP and 2,4-DNP are known to carry H^+ ions selectively through the membrane (Hopfer et al, 1968) which would allow diffusion uptake of H^+ that had been electrogenically pumped out destroying the potential gradient of the plasma membrane. A number of reports which concern the involvement of K^+ or Na^+ in amino acid transport by bacteria that

have a cytochrome system have been published by Wong et al (1969), Mora and Snell (1963), Harold and Boarada (1968a, b) Gale (1953), Mitchell (1963) and Frank and Hopkins (1969). E. coli grows poorly on glutamate as the carbon or carbon and nitrogen source, but if sodium is added glutamate is rapidly taken up and growth is stimulated. When analogues of glutamate were used, Na^+ increased their competitive inhibition effect. No definite conclusion of the role of Na^+ in bacterial amino acid transport could be reached but Frank and Hopkins (1969) suggested that Na^+ was taken up with glutamate by using a Na^+ extrusion pump as the energy source similar to mammalian cells.

Ion coupled transport of metabolites in plants has not been shown in any conclusive way as yet due to the difficulty of using plant tissue for ion transport studies. In plasma membranes that favor 'downhill' movement of ions, ion transport still does not take place. A possible model of co-transport was suggested by Robertson (1968) where the driving force need not be located at the plasma membrane, but could be located across the mitochondria or chloroplast membrane. However, no experimental proof for Robertson's model exists. There is some evidence which does support ion regulation of forward transport in conducting vessels of the xylem (Greenway, 1967; Johansen et al, 1970; and Pitman, 1971).

In summary, the H^+ or Na^+ electrogenic pump exists in the plasma membrane to (1) maintain a potential across the membrane for metabolite uptake and efflux of inorganic and organic acids; (2) maintain K^+ level in the cytoplasm near its normal steady state level for enzyme activity; and (3) to produce a high resting potential

essential for membrane structure. By the maintenance of the membrane structure, the electrogenic pump allows the correct conformation cycle of the transport 'carrier' protein(s) which makes metabolite transport a selective process and as a consequence controls enzyme activity.

Another type of facilitated transport of metabolites across the plasma membrane is via an ionophore. Ionophores specifically carry ions such as Na^+, K^+, and Ca^{++} across the lipid membrane. The ionophore molecule's interior is hydrophilic and the exterior is hydrophobic allowing lipid solubility while still retaining ion transport capacity. A dimer ionophore, gramacidin A can be oriented in a lipid bilayer so as to allow selective passive transport of K^+ across the membrane (Urry, 1972a). Ionophore channels can be interconverted by application of an electrical field across the plasma membrane. One conformation form would represent the conducting form, and the other conformation would represent the non-conducting form (Urry, 1972b). Urry has found interconvertable ionophores such as gramacidin A with substantially different dipole moments that can span a biological membrane. Such a theory of membrane transport could be used to explain nerve and cardiac cell membrane, that is, transport induced by a change in electrical charge.

An ionophore molecule may also enhance uptake across the plasma membrane by traversing the membrane and so permit free diffusion of metabolite. The 'carrier model' for ion transport by ionophores was proposed by McLaughlin in 1972. The anionic ionophore dinitrophenol (2,4-DNP) binds to the surface of the lipid bilayer and

changes the inter-membrane charge density thereby allowing free diffusion of metabolite through the lipid bilayer.

Primary active transport systems are directly coupled to cell energy supply via ATP expenditure. The energy from ATP hydrolysis is used directly to convert the transport 'carrier' protein(s) from high to low affinity forms, causing transport of metabolite across the membrane and release into the cytoplasm. The (Na^+ - K^+)-stimulated, ouabain inhibited ATPase (Whittam, 1967; Stein, 1967; Bonting, 1970; Caldwell, 1970; Skou, 1971), and the Ca^{++}-stimulated ATPase (Martonosi, 1972; MacLennan & Wong, 1971) of higher organisms are membrane bound ATPase enzyme systems that use ATP energy to transport cations across animal plasma membranes. The ATPase enzyme complex is physically linked to the active transport of these cations.

The discovery of a Na^+ - K^+ stimulated ATPase (ouabain inhibited) in animals led to a search for a similar system in plants, but ouabain was found not to inhibit ion absorption in plants (Dodds & Ellis, 1966). However, on the basis of arsenate and oligomycin inhibition of ion transport in plant roots, ATP appears to be the energy source (Higinbotham, 1959; Hodges, 1966; Jacoby & Plessner, 1970). Numerous reports have been published which concern the involvement of ion-stimulated ATPase ion transport in plant tissue by Atkinson and Polya (1967), Brown et al (1965), Dodds and Ellis (1966), Kylin and Gee (1970), Neuman and Gruener (1967), Sexton and Sutcliffe (1969), and Fisher and Hodges (1969), but none could show that ATPase had a direct relationship to ion transport. However, Fisher and co-workers (1970) recently showed that Rb^+ and K^+ stimulated ATPase in

oat root plasma membrane had sufficient activity to account for observed rates of K^+ and Rb^+ transport across the plasma membrane. In excised corn root tissue (Zea mays L.) a (Mg^{++} - K^+)-stimulated ATPase has been isolated from the microsome fraction (Leonard & Hanson, 1972a). Leonard and Hanson (1972) showed that ion transport closely paralleled ATPase activity with both responding similarily to inhibitors of RNA and protein synthesis. Also in homogenates of oat roots, Avena sativa cv. Goodfield, Leonard and co-workers (1973) have found five membrane associated ATPase activities; one associated with the mitochondria, one with the plasma membrane (sensitive to KCl), and the other three unidentified.

Active transport processes (primary) can also be directly linked to other membrane bound energy sources in the cell, such as the bacterial electron transport system which is located in the plasma membrane. In bacteria, active accumulation of amino acid is coupled to electron transport (Lombardi & Kaback, 1972) which does not involve either the generation or the utilization of higher energy phosphate compounds. In E. coli, membrane vesicle amino acid transport is dependent on D-lactate and the point of coupling between respiration and amino acid transport is located between the primary dehydrogenase and cytochrome b_1 of the electron transport chain. Experimental evidence of Lombardi and Kaback (1972) supports this conclusion since D-lactate amino acid transport is antagonized by anaerobiosis and electron transfer inhibitors such as, cyanide, 2-heptyl-4-hydroxyquinoline-N-oxide, and amytal. ρ-Chloromer-

curibenzoate (ρ-CMB) inhibition can be reversed by dithiothreitol, but transport is not stimulated by ATP or ADP in the presence of D-lactate or other electron donors, and E. coli membrane vesicles do not carry out oxidative phosphorylation. Kaback proposed a provisional model of respiration-linked amino acid transport carrier. The carrier proteins are electron transfer intermediates between D-lactic dehydrogenase and cytochrome b_1 which undergo cyclic sulfhydryl-disulfide conversions. Amino acids are only transported when the carrier is oxidized (disulfide). Lactate reduces the electron carrier model and causes the transmission of amino acid across the membrane to the inner surface and release into the cytoplasm. Experimental testing of this model has so far supported the basic concept of amino acid-respiration linked transport (see Lombardi & Kaback, 1972). It is not essential that the location of respiration-linked amino acid transport be located between D-lactate dehydrogenase and cytochrome b_1. In Staphylococcus aureus membrane vesicles, amino acid transport is coupled to α-glycerol phosphate dehydrogenase (Short et al, 1972) in a similar manner as D-lactic dehydrogenase-linked amino acid transport of E. coli. In contrast to Streptococcus faecalis which lacks a cytochrome system, E. coli and S. aureus do not have ion-dependent transport of amino acids. Although K^+ inhibits the transport of proline and reduces the transport of serine and aspartic acid in E. coli membrane vesicles, K^+ is not specifically required for amino acid transport. In addition, membrane vesicles do not accumulate Rb^+ (K^+ analogue), Mg^{++}, sulfate, or phosphate ions under conditions where amino acids are actively

accumulated.

Another type of active transport is the direct energy coupling via vectorial phosphorylation (Kaback, 1970). In bacteria, sugars are transported across the membrane by carriers that are coupled with phosphoenolpyruvate (PEP)-dependent phosphorylation. In this process, the sugar becomes phosphorylated and as a result has a much reduced affinity for the carrier which results in sugar accumulation inside the membrane.

The energy expenditure for active transport for some cases may not be associated with inward transport of metabolites but could occur after release of metabolites into the cytoplasm to allow carrier protein to go back to the original conformation (Rotman & Ellis, 1972). Direct energy is then required only for the reverse process. Rotman and Ellis (1972) proposed that the substrate binding protein was the actual carrier. Galactose binding in E. coli induces a conformation change in the binding protein which may allow the binding to a high molecular weight effector in the plasma membrane. The galactose-binding protein complex would cause translocation of the binding protein and a reduced affinity for the substrate galactose which would cause release of galactose into the cytoplasm. Energy expenditure would allow binding protein to return to its original conformation and the process would be repeated.

Activity of Transport Systems

In addition to kinetic characterization of transport systems, reconstitution of the isolated components into a structurally normal

and enzymatic functioning membrane has proved essential to the study of metabolite transport to show the requirements for an active enzyme or transport system (see Razin, 1972). Only a few studies have yet been reported that have been able to restore original activity to the reconstituted membrane transport systems.

Membrane proteins (loosely associated) that can be easily released from the membrane by changes in the pH, ionic strength and by addition of a chelating or sulfhydryl agent do not require lipid for enzymatic activity. Numerous ATPases are released by any of the above treatments using Streptococcus faecalis (Baron & Abrams, 1971), Micrococcus lysodeikticus (Munoz et al, 1969), Bacillus megaterium (Ishida & Mizushima, 1969; Mirsky & Barlow, 1971), as test materials. All require Ca^{++} or Mg^{++} for activity.

Other membrane proteins can be released when the lipid is solubilized by the method of Fleischer et al (1967). Reassembly of these enzymes with phospholipid is required before the enzyme activity can be restored. What role the lipid plays, whether as a cofactor or involved in the enzymatic process, is questionable since the amount of lipid required is too large and non-specific. The lipid is likely required for proper conformation of the membrane proteins so that orderly enzymatic reactions are carried out (Razin, 1972). A few examples of membrane proteins that are dependent on lipid for enzyme activity are $(Na^{+} - K^{+})$-ATPase of bovine brain (Noguchi & Freed, 1971), rat kidney and brain (Fenster & Copenhaven Jr., 1967), and ATPase and calcium uptake of sarcoplasmic reticulum

(Martonosi et al, 1968). After phospholipase C digestion of the sarcoplasmic reticulum, membrane ATPase is released with calcium transport activity.

The calcium transport system of the sacroplasmic reticulum (SR) is a good example of a reconstituted system. In the sarcoplasmic reticulum the major membrane function is the transport of calcium in both directions through the membrane (Yu & Masoro, 1970), in fact, the majority of proteins are involved in calcium transport. Martonosi (1968a) was the first to demonstrate that the (Mg^{++} - Ca^{++})-dependent ATPase activity could be restored by reconstitution of the SR membrane, but calcium accumulation in the reconstituted membrane vesicles was only 25 to 30% of the original activity. Using purified SR ATPase particles, MacLennan and co-workers were also able to reconstitute functionally active sarcoplasmic reticulum vesicles (MacLennan, 1970; MacLennan et al, 1971). The SR ATPase particles consisting of enzyme protein (102,00 daltons), phospholipid, and neutral lipid probably accounted for the majority of the SR membrane. Although ATPase activity in these purified sacroplasmic reticulum vesicles was restored to originality, again calcium was found to be bound only in small amounts in the presence of ATP and oxalate (MacLennan & Wong, 1971). The low calcium transport could have been due to poor sealing of membrane vesicles or a small molecule essential to binding and transport of calcium was not present. The latter of these two possibilities appeared to be correct since calsequestrin, a calcium sequestering protein, located on the inner surface of SR membrane is lost during reconstitution of the purified ATPase membrane vesicles, and 40 Å particles shown by freeze etching

technique for normal SR vesicles are missing from the surface of the purified ATPase membrane vesicles (Martonosi, 1968b). Recently, the calcium pump activity of the sarcoplasmic reticulum has been restored to the purified ATPase molecule by Racker (1972), and Warren et al (1974). The purified enzyme complex of dioleoyl lecithin- ATPase is unable to accumulate calcium but upon addition of excess SR lipids in the presence of cholate which is subsequently removed, the system can accumulate calcium at a rate comparable to native sarcoplasmic reticulum (Warren et al, 1974). The active calcium pump contains essentially a single membrane protein and exogeneous lipid which makes it likely that the ATPase molecule is responsible for both ATPase activity and calcium transport. The lipid component surrounding the calcium transport protein is important in membrane integrity preventing calcium leakage and probably acts as a cofactor since it does not appear to move laterally with other lipids in the membrane bilayer but it forms a shell around the calcium transporting ATPase. But this is not to say that the calcium sequestering protein located on the inner surface of the membrane is not additionally important in calcium transport. Calsequestrin acts as a storage site for physiological control of calcium compartmentalization in the muscle tissue which allows continual release and uptake of calcium in the sarcoplasmic reticulum by it's intimate association with (Mg^{++} + Ca^{++})-dependent ATPase of the sarcoplasmic reticulum.

Regulation of Transport Systems

Transport systems are regulated at two cellular levels; the first is at the enzyme level, and the second involves genetic expression. At the genetic level of regulation, transport systems of micro-organisms, plants, and mammalian membranes are either constitutive or inductive. In bacteria, sugar transport is mainly inducible except for glucose. When amino acids are used as either carbon or nitrogen sources, such as glutamate and tryptophan, the systems are often inducible. Also, high affinity 'scavenger' amino acid transport systems located in the plasma membrane of Neurospora (Pall, 1969; 1971), Saccharomyces cerviserae (Grenson, 1973; Benko et al, 1967), and soybean roots (King & Oleniuk, 1973) are induced under starvation conditions. At the enzymatic level of regulation, transport is controlled by cell metabolism, and in addition, cell metabolism is controlled by transport (see Jennings, 1974). Transport systems can be affected by a wide variety of cell constituents, such as, intracellular and extracellular levels of substrate, and effectors that can interact allosterically with the transport system.

The transport of solutes is closely linked to biochemical process, and regardless of cellular regulation, a particular reaction results which can produce a morphogenetic change. The transport systems in fungi may play a key function in switching morhogenetic

development. Smith and Galbraith (1971) showed that fungal conidiation was closely linked to transport since the level of certain nutrients, such as sugar in the medium, play an important role in determining conidiation.

Ion Transport

Plant Systems

The mechanism of ion transport at a molecular level may be the same for animal, plant, fungi, algae, and bacteria, but each system contains unique features. Ions are readily transported across the cell wall of plants, while movement across the plasma membrane requires metabolic energy. As early as 1952, Osterhout described ion transport in plants as being 'carrier' mediated transport. The uptake of ions is an active, saturable process (Epstein, 1956; Sutcliffe, 1953) that has been kinetically characterized for a large number of mono- and di-valent cations; K^+, Na^+, Rb^+, Cs^+, NH_4^+, Ca^{++}, Mg^{++}, Zn^{++}, Cu^{++}, Fe^{+++}, and anions; Cl^-, $H_2BO_3^-$, SO_4^{2-}, $H_2PO_4^-$ (see reviews by Epstein, 1973; Nissen, 1973 a, b, c). These ions are taken up by a wide variety of plant tissues, for example, roots, barley, corn, wheatgrass, ryegrass, clover, mung bean; leaf tissue; sugarcane, corn bean, mangrove and stem tissue. There is a general acceptance that ions are taken up by plant tissues, but disagreement arises when the mechanism is discussed. Determination of the mechanism of ion transport in plants has involved kinetics of ion uptake in conjunction with competitive inhibition of uptake, and electropotential differences across the membrane.

A wide variety of models have been proposed to explain the

mechanism of ion transport in plants (Oertli, 1967; Hiatt, 1968; Pitman, 1969; Laties, 1969; Thellier, 1970; MacRobbie, 1971; Gerson & Poole, 1971; Epstein, 1973; Nissen, 1973c). The models of Laties, Epstein, and Nissen are of particular interest, since other transport models are taken into consideration and recent data on plant ion transport has been reviewed while still presenting three different points of view.

The first model is the dual mechanism model of ion transport which was suggested by Epstein in 1973. The transport of ions in plants involves two distinct mechanisms. Type 1, operates at low ion concentration, and type 2, operates at high ion concentration. Type 2 mechanism occurs only after the saturation of type 1 mechanism. By a study of kinetic transport properties the two mechanisms have been separated and characterized. Mechanism 1 is characterized by its high affinity for ions, such as K^+, Rb^+, and Cl^- at concentrations less than 1 mM and Michaelis-Menten kinetics which has a wide V_{max} range (0.2 - 1.0 mM). The apparent K_m value for K^+ and Cl^- in excised barley roots is 0.02 mM, and at saturation the 'accumulation ratio' is 3 - 4 (Epstein, 1972). Mechanism 1 is selective since closely related ions compete, and low temperature, respiration poisons, and oxidative phosphorylation uncouplers inhibit transport. Mechanism 2 is considered polyvalent ion transport which has a spectrum of active sites, each with a slightly different affinity for the substrate ion. In summary, Epstein's model for ion transport in plants consists of active transport carriers which mediate the movement of ions across the plasma membrane. Ions are transported by a dual mechanism that can be either additive or in parallel. Other

models that are in agreement with Epstein's model of ion transport can be found by Laties (1969) and Lüttge (1971).

The second model is the series mechanism model of ions transport which was suggested by Laties in 1969. Like Epstein's model for ion transport, Laties (1969) agreed that dual transport kinetics existed for roots of the corn, Zea mays, but suggested that type 1 mechanism and type 2 mechanism operate in series with mechanism 2 behind mechanism 1. Both Laties and Epstein agree that type 1 mechanism of ion transport is located in the plasmalemma, but Laties considers the type 2 mechanism to be located in the tonoplast which is the membrane that intervenes between the cytoplasm and the central vacuole. At low salt concentration, type 1 mechanism at the plasma membrane operates, and at high external salt concentration, type 2 mechanism is in operation accompanied by a considerable diffusion component across the plasma membrane.

The third model is the multiphasic mechanism model of ion transport which was suggested by Nissen in 1971. In contrast to Epstein's separate site or mechanism model of ion transport in plants, Nissen (1971) suggested that there is one 'carrier' mediated transport process located at the plasma membrane with different kinetic phases that are determined by the external concentration of ion. Each phase obeys Michaelis-Menten kinetics and the phases are separated by discontinuous transitions. There is no parallel uptake of ions, and very little diffusion. In 1973, Nissen revised the multiphasic model of ion transport by combining it with Laties (1969) series model, and called it the multiphasic series model of ion transport.

The model was expanded to allow multiphasic transport across both the plasmalemma and the tonoplast. Similarities of uptake patterns (discontinuous transitions) of various ions have been shown for different plant tissues during varying conditions which may reflect the change in the membrane state. In the range of 5×10^{-4} M to 1×10^{-2} M the uptake similarities in number of phases and transition points has been indicated for PO_4^{3-} and SO_4^{2-}, alkaline cations (K^+), and halide ions (Cl^-).

In summary, Nissen suggests that multiphasic uptake mechanisms are widespread in plants and may also be found in other systems, for example, bacterial transport of amino acids glutamic acid, alanine, and valine (Yabu, 1970; Reid et al, 1970). However, caution must be exercised to allow for variations within the mechanism of ion transport itself. Even among plant cells, complex kinetics dictate that differences occur (Epstein, 1972). As both Nissen and Epstein, with the same data to review, are able to propose two completely different models, there is a need for further in depth study, physical isolation and reconstitution of transport systems before the mechanism of ion transport in plants can be completely understood.

Calcium uptake in plant tissue has usually been studied in relation to its effect on transport of other ions, but there are a few kinetic studies of calcium transport. In general, calcium uptake has been found to be low or neglible, for example; in excised barley roots (Moore et al, 1961), in excised wheat roots (Johnson & Jackson, 1964), and in excised soybean root (Leggett & Goblert,

1967). However, Handley and Overstreet (1961) observed that excised maize roots readily absorbed calcium with temperature dependence at kinetic rates comparable to the rate of uptake for other actively absorbed cations. Maas (1969) suggested that calcium transport in excised maize roots is metabolically mediated, since both dinitrophenol and low temperature (membrane remained intact) antagonized calcium uptake. Calcium kinetic uptake is influenced by counter-ion, pH, concentration of the ambient solution, and the presence of other cations, while Li^{+} and Na^{+} have only slight effects.

Calcium uptake is antagonized by direct competitive inhibition by related cations such as Sr^{++}, Ba^{++}, and Mn^{++}, but how other ions function to activate or inhibit calcium accumulation is unknown. K^{+} and H^{+} interfere with Ca^{++} absorption, and the reduction in Ca^{++} uptake is associated with an increase in K^{+} uptake (Maas, 1969). Other examples of ion effect on calcium uptake can be found first in the wheat seedling (nitrogen-depleted) where nitrate stimulates the uptake of Ca^{++} and to a lesser extent Sr^{++} (Anderson & Jackson, 1972), and second in the maize plant, Zea mays where Ca^{++} uptake is stimulated by K^{+} and Mg^{++}, but only at certain ratios of Ca^{++}: K^{+}: Mg^{++}, while at other ratios K^{+} and Mg^{++} are inhibitory (Falade, 1973).

In cotton roots, calcium is transported by multiphasic kinetic mechanism over a period of 14 days. There are two phases, and growth is associated with the lower phase. The transport kinetics of calcium is a Michaelis-Menten hyperbola up to a concentration of 0.02 mM, which is followed by a plateau in the concentration ranges;

0.25 to 1.0 mM and 1.5 to 3.0 mM with marked increases in between.

The influence of calcium on ion uptake in plants is well established, but the mechanism involved is unknown. In 1944, Viets described the well known phenomenon in plant roots, 'Viets effect' which shows that calcium is essential to the mechanism of K^+ accumulation. In general, the presence of calcium (0.5 mM) in the medium is essential for ion transport systems to have normal unimpaired functioning (see Epstein, 1961). Calcium maintains the membrane integrity, since in the absence of calcium the membrane becomes leaky to ions and the selectivity of ion transport is abolished. Calcium also maintains the proper conformation of the ion binding site to give specificity to the transport system, since in the absence of calcium other non-specific ions bind to the transport system and antagonize calcium uptake by competitive inhibition (Rains & Epstein, 1967). When calcium is present, K^+ absorption by excised barley roots is indifferent to Na^+, but if calcium is removed K^+ uptake is competitively inhibited by Na^+ (Epstein, 1973). Absorption of both anions and cations is much more pH dependent in the absence of calcium. Frequently, the effect of calcium on ion transport is interconnected with other ions such as H^+ that are present, (Jacobson et al, 1950; Jacobson et al, 1960; Rains et al, 1964; Marschner et al, 1966).

The uptake phase patterns of other ions can be significantly different in the presence and absence of calcium (Nissen, 1974). The uptake of Na^+, in the presence of K^+, is an example of an ion

transport system that has different phase patterns in the presence and the absence of calcium (Nissen, 1973). In another study, Elzam and Epstein (1965) showed biphasic transport of Cl^- below 0.2 mM only in the presence of calcium. The chloride uptake has a higher affinity and a lower value for maximum velocity when the calcium concentration is increased from 0.5 mM to 10 mM which indicates a tightening of the membrane structure (Elzam et al, 1964). Sulfate uptake patterns are also affected by calcium. Nissen (1974) showed that the removal of calcium by chelating agents abolished the lower phase of sulfate uptake, and this could be restored if calcium was added back. High pH (8.2) also reduces the number of phases especially at low sulfate concentrations.

As early as 1920, True had demonstrated that calcium enhanced uptake in plants, but the mechanism of calcium activation is still unknown. If excised plant tissue is prewashed in the presence of calcium prior to ion transport, four effects on transport occur which are as follows. (a) A high affinity K^+ absorbing mechanism is induced that is temperature dependent and sensitive to metabolic inhibitors and cycloheximide (Rains & Floyd, 1970). (b) Na^+ uptake decreases (Rains & Floyd, 1970; LaHaye & Epstein, 1971). (c) A rapid rise in negative potential occurs (Pitman et al, 1970). (d) K^+ and phosphorous uptake are enhanced as a result of an increase in the maximum velocity of transport with no change in transport affinity (Leonard & Hanson, 1972a). Calcium activation of ion transport may be linked indirectly either through the effect of an electropotential gradient across the plasma membrane (Pitman et al, 1970),

or directly by interacting with a membrane bound ATPase (Leonard & Hanson, 1972a; Jones & Lunt, 1967). The latter mechanism is supported by Leonard & Hanson (1972a) who showed that both RNA and protein synthesis inhibitors antagonized calcium prewashing effect. The calcium washing process may activate an existing membrane transport mechanism, such as K^+-stimulated (Ca^{++} - Mg^{++})-dependent ATPase, by a process which involves protein synthesis. In agreement, Albersheim and co-workers have reported the presence of a Ca^{++}-stimulated ATPase that may function as an extrusion pump in sycamore and bean tissue cultures (Fisher & Albersheim, 1973; Fisher et al, 1970).

Rates of ion accumulation and protein synthesis vary in a similar manner in plants (Steward & Mott, 1970) and both protein and K^+ content are maximum at the same time. No fixed quantitative correlation between protein synthesis and K^+ transport has as yet been drawn. Inhibitors of protein synthesis reduce ion absorption (Sutcliffe, 1960; MacDonald et al, 1966). Sutcliffe (1960) suggested that ion absorption is directly affected by D-threo -chloramphenicol (CAM) inhibition of protein synthesis. In isolated maize mitochondria CAM inhibits calcium absorption (MacDonald et al, 1966). Problems may arise when using CAM as a specific inhibitor of protein synthesis since ATP synthesis which links CAM action to the energy supply is also interfered with. Recently Sutcliffe (1972) eliminated the problem of specific inhibition by using a variety of inhibitors such as amino acid analogues, nucleic acid antagonists,

and cycloheximide, all of which inhibit ion absorption in plants. Cycloheximide at low concentration, 0.1 μM, does not uncouple oxidative phosphorylation or interfere with growth but still inhibited K^+ uptake in excised radish roots (see Sutcliffe, 1972).

The mechanism of inhibition by the various protein synthesis antagonists is unknown, but three possible mechanisms that could explain the inhibitory effect of protein synthesis antagonists are as follows: (i) direct interaction with 'carrier' mediated transport system, (ii) protein synthesis and transport are tightly associated; inhibition of one produces an immediate effect on the other, and (iii) interferes with the protein synthesis 'carrier' protein(s) (ATPase) essential to ion transport. Steward and Lyndin (1965) postulated a complex scheme where ion transport is interrelated with the metabolic processes of the cell, all of which serve some role in regulation of ion transport. The transport systems are in turn regulated by environmental conditions and the morphological stage of the cell.

Fungal Systems

One of the better characterized ion transport systems is that of K^+. K^+ is actively transported by a single specific transport system in the following fungi: Neurospora crassa (Slayman & Slayman, 1970; Glynn et al, 1971), Dendryphiella salina (Allaway & Jennings, 1970), Fusarium oxysporium (Shere & Jacobson, 1970), and Neocosmospora vasinfecta (Budd, 1969). In Neurospora crassa

the single carrier K^+ transport system (secondary energy supplied by H^+ extrusion pump) has the property of a two site carrier at high pH 8.0, and has a carrier site for K^+ and a modifier site for H^+ that is an allosteric modulator. At low pH (pH 4 - 6) simple hyperbolic Michaelis-Menten kinetics occurs as a function of external K^+.

Another ion transport system that has been adequately characterized by kinetics of transport, metabolic and competitive inhibition, and genetic mutation is the sulfur transport system of Neurospora. Sulfur is taken up by two transport systems called sulfate I and sulfate II (Roberts & Marzluf, 1971). Sulfate I is a low affinity (K_m = 200 μM) transport system which is found in both germinated conidia and ungerminated conidia. Sulfate II is a high affinity transport system (K_m = 10 μM) found only in growing cells which accounts for 75% of the sulfur transport.

In yeast, there appears to be only one transport system for all divalent cations. By competitive saturation kinetics the divalent transport system shows the following affinity specificities: Mg^{++}, Co^{++}, Zn^{++} > Mn^{++} > Ni^{++} > Ca^{++} > Sr^{++} (Fuhrmann & Rothstein, 1968). Either 2 K^+ or 2 Na^+ (Na^+ loaded cells) are exchangeable for uptake of each divalent cation to maintain electrical balance. The divalent cation transport system does not transport monovalent cations. Divalent cation transport in yeast is affected by K^+, phosphate, and glucose. In the absence of K^+, phosphate, or glucose, divalent cations are bound only to the membrane surface since the ions are readily exchangeable (Rothstein, 1972). When phosphate is

added non-exchangeable cation transport occurs. The effect produced by phosphate is a direct effect which depends upon transport of phosphate into the yeast cell, while the K^+ and glucose effects are indirect, respectively, enhancing phosphate uptake and producing an energy-rich system. Like monovalent cation transport systems, the divalent cation transport system is important for maintaining the required levels of cations for intracellular metabolic processes.

The majority of published reports of calcium uptake in fungi show negative results. In Fusarium oxysporum K^+ and Na^+ are accumulated without appreciable accumulation of Ca^{++} (Shere & Jacobson, 1970). Rothstein (1955) showed resting cells of yeast do not take up calcium, and only small amounts of calcium are bound to the outside of the cell membrane. No accumulation of calcium occurs in any of the following fungi, but Mg^{++} uptake can be demonstrated: Ophiobolus graminins var., Neurospora crassa, Aspergillus niger, nor Pythium irregulare (Olsen, 1971). However transport system for calcium must exist since calcium is required for cell division and mitosis in lower plants (Sorokin & Sommer, 1940; Herwitt, 1963; Moss et al, 1971).

A K^+ transport system is essential to cell metabolism since K^+ is required for growth, protein synthesis, ribosome metabolism, osmotic content, and certain enzyme activities (Lubin & Ennis, 1964). Ions are of particular importance in the regulation of osmotic pressure, since electrolytes make up a large part of the osmotic content. During growth there must be increased turgor pressure which is mainly reflected in a rise in intracellular K^+. K^+ uptake

is balanced by a loose association with efflux of H^+, since a tight association is not required due to the rigid cell wall which maintains the appropriate osmotic pressure gradient. Cation transport can also play a role in acid tolerance phenomena of some cells, for example; in the presence of high K^+, H^+ efflux can compensate for an acidic environment.

As K^+ uptake is closely interconnected with cell metabolism, the K^+ transport system is regulated by a number of different factors. Na^+ inhibited growth of fungi is largely due to the induced K^+ efflux from the hyphae. Calcium has the general effect of preventing ion loss from the fungi, Ophiobolus graminis and Neurospora crassa, probably by maintaining the membrane structure. Consequently, calcium prevents the toxic effect of Na^+ by preventing K^+ efflux (Jones & Jennings, 1965; 1973). Jennings (1973) suggested a mechanism for calcium effect on K^+ transport by combining Slayman's model of an electropotential gradient across the membrane generated by an active H^+ extrusion pump that allows active accumulation of K^+ into the cell with the passive flux of ions that occurs to a small degree across the plasmalemma. The interconnection between active transport and passive fluxes can be represented as two electromotive forces in parallel; an electrogenic potential and a diffusion potential. Na^+ would increase efflux of K^+ via diffusion fluxes but in the presence of Ca^{++} which increases the diffusion resistance the effect of Na^+ on diffusion potential is eliminated. K^+ then would move down the electrogenic potential gradient that is insensitive to the K^+/Na^+ ratio. Hunter and Segel (1973b) reported

a similar relationship between calcium and electrogenic potential gradient across the membrane. Membrane bound calcium apparently is essential for the proper operation of the potential gradient across the plasma membrane generated by an electrogenic H^+ extrusion pump.

Bacterial Systems

In bacteria, ion transport systems have not only been characterized by kinetics of uptake, competitive inhibition, and genetic mutations, but releasable ion binding proteins for sulfate and phosphate that are connected with the transport system have been purified. The binding proteins bind the substrate with a K_d value similar to the apparent K_m of ion transport in normal cells. K^+, sulfate, and phosphate transport systems are the three major ion transport systems in bacteria. In E. coli there are at least 3 saturable K^+ transport systems with apparent K_m values of 1.5, 0.5, and 0.001 mM respectively (see Oxender, 1972a, b). Sulfate transport in S. typhimurium is by a complex system being regulated by at least 3 cistrons. Genetic characterization of the sulfate transport system shows that in addition to structural genes there are genes that regulate transport. Phosphate is taken up in E. coli by two transport systems that are regulated by the level of phosphate in the cell.

Mammalian Systems

One ion transport system of animal tissue that is of particular interest is the (Na^+ - K^+)-dependent ATPase transport system since it maintains the electropotential gradient across the plasma membrane that is responsible for the secondary active transport of metabolites in animal tissue. The (Na^+ - K^+)-dependent ATPase maintains a high level of intracellular K^+ and a low level of Na^+ (Skou, 1971) by actively transporting Na^+ out of the cell while actively transporting K^+ into the cell. The ATPase is ouabain (cardiac glycoside) sensitive. The ATPase molecule has been isolated from bovine brain by detergent solubilization, and chemically and physically characterized. The (Na^+ - K^+)-dependent ATPase has a molecular weight of 94,000 daltons, and a composition of 50% protein, 25% phospholipid, and 2-3% carbohydrate. In addition, a γ-glutamyl derivative has been isolated which represents a phosphorylated intermediate of alkali metal ion-ATPase. Another major ion transport system, that is of interest in this study, is the calcium transport systems.

Calcium is required for a number of diverse functions in animal cells and tissue which includes bone formation, nerve impulse transmission, the action potential, blood clot formation, muscle contraction, membrane integrity, and membrane function. Because of the specific requirement of calcium for these functions, complex transport systems for calcium have evolved in each of these cases. The calcium transport systems coupled with calcium regulating hormones provide a defined homeostatic control of calcium levels in extracellular fluids of animal tissue.

Calcium transport in animals can be classified as follows: (a) carrier-mediated influx (diffusion) across the plasma membrane (Borle, 1973; Lamb & Lindsay, 1971); (b) (energy-substrate)-dependent uptake by the mitochondria (Cittadini et al, 1973; Perdue, 1971); (c) sequestration in specialized cells, such as, muscle sarcoplasmic reticulum (MacLennan & Wong, 1971) and cells involved in calcification or in chorioepithelial (Kashiwa, 1970; Rasmussen et al, 1972; Sutfin et al, 1971; Tanaka & DeLuca, 1973); (d) efflux via Ca^{++} sensitive ATPase in nonexcitable cells (Borle, 1973; Cohn et al, 1972; Cooper et al, 1972); and (e) efflux of calcium in excitable cells may be controlled by diffusion exchange of calcium for sodium (Blaustein & Hodgkin, 1969; Glitsch et al, 1970; Klotz, 1971). Calcium transport has been recently reviewed in animals by Borle (1973, 1974), Martonosi (1972), Wasserman (1972) and Moore (1972).

Calcium transport in the sarcoplasmic reticulum is via an active transport system that is mediated by a (Ca^{++} - Mg^{++})-dependent ATPase (MacLennan, 1970; MacLennan et al, 1971; MacLennan & Wong, 1971; Martonosi, 1968a, b, 1972; Warren et al, 1974). Calcium content of skeletal muscle tissue controls contraction and relaxation (Sandow, 1970). Calcium uptake by the (Mg^{++} - Ca^{++})-dependent ATPase into the sarcoplasmic reticulum causes relaxation of the muscle, while release of stored calcium from the sarcoplasmic reticulum causes contraction. The (Mg^{++} - Ca^{++})-dependent ATPase consists of a single protein of molecular weight 102,000 daltons in conjugate with 45,000 daltons of lipid. The enzyme catalyzes ATP hydrolysis, ATP-ADP exchange, and becomes phosphorylated in the presence of calcium.

Both acetyl-P and ATP are substrates for (Mg^{++} - Ca^{++})-ATPase activity of the sarcoplasmic reticulum and an acyl phosphate is probably an intermediate in this process (deMeis, 1971a). The energy substrates and calcium interact with the membrane bound ATPase in an allosteric manner. ATP activates a specific structure that is subsequently able to bind and transport calcium. For every ATP molecule split two Ca^{++} are transported into the cytoplasm.

Associated with the (Ca^{++} - Mg^{++})-ATPase of sarcoplasmic reticulum is a calcium sequestering protein which is attached by hydrophobic binding to SR vesicles (MacLennan & Wong, 1971). The calcium sequestering protein has a molecular weight of 44,000 daltons and consists entirely of protein with no phosphorous, sialic acid, or lipid. Fifty percent of the amino acid residues is glutamic and aspartic acid. This protein has been named 'Calsequesterin' and is believed to play a role in sequestering calcium (43 mole calcium/ mole protein) in the sarcoplasmic reticulum. Calsequesterin does not resemble other binding proteins attached to transport systems for the following reasons. First, a large number of substrate molecules are bound. Second, the apparent K_d is much lower than the apparent K_m of calcium transport, usually if binding protein is associated with transport the K_d value is equivalent to the apparent K_m value. Third, calsequesterin probably does not aid in transport of calcium. Fourth, calsequestrin is firmly bound to the membrane, usually transport binding proteins are loosely associated with membrane surface or free in the periplasmic space. Calsequesterin appears to be an unique protein molecule which is involved in storage of calcium when surrounding fluid contains a high concen-

tration of calcium (MacLennan & Wong, 1971).

In smooth muscle, calcium ions activate contractile elements as it does for all muscle fibers, but the sarcoplasmic reticulum is not always involved in movement of calcium. In the guinea pig ileum longitudinal smooth muscle, the regulation of calcium is mainly associated with the plasma membrane as it is in red blood cells (Hurwitz et al, 1973). There appears to be two types of smooth muscle. First, in the aorta of rabbit, the sarcoplasmic reticulum serves as reservoir of calcium similar to skeletal muscle. Second, in the longitudinal smooth muscle of guinea pig ileum the plasma membrane serves as reservoir of calcium. Microsome fractions of guinea pig ileum longitudinal muscle plasma membrane actively sequester calcium in the presence of Mg^{++}-ATP. The plasma membrane probably contains a (Mg^{++} - Ca^{++})-dependent ATPase and has a calcium sequestering protein that actively extrudes cytoplasmic calcium during relaxation. During contraction of smooth muscle the plasma membrane and extracellular space would be the source of calcium for contraction of muscle fibers of the cytoplasm.

In the intestinal mucosa, calcium is actively accumulated with an apparent K_m value of 1.25 to 2.0 mM (Omdahl & DeLuca, 1973), but the question of how calcium is transported across the intestinal mucosal border had long been a point of controversy (Borle, 1974). Vitamin D induces calcium transport and the enzymes alkaline phosphatase, pyrophosphatase, and K^{+}-activated ouabain insensitive (Ca^{++} - Mg^{++})-ATPase. The (Ca^{++} - Mg^{++})-ATPase is likely involved in calcium transport across the serasol membrane (side of membrane

next to the blood stream). Wass and Butterworth (1972) suggested that alkaline phosphatase is identical to, or is a component of the calcium-stimulated ATPase.

In addition, a calcium binding protein which has been isolated from the intestinal mucosa, flux level can be correlated with (a) intestinal calcium transport; (b) changes in flux of calcium and phosphorous; (c) vitamin D induced calcium transport; and (d) variations throughout regions of the small intestine. The calcium binding protein is present in the small intestine, colon, and kidney of chick, and also in the uterus (shell gland) of the laying hen (Fullmer & Wasserman, 1973). The intestinal calcium binding protein is a general phenomenon in mammalian systems. It has been found in rat, dog, chick, monkey, pig, human and guinea pig. One mole of calcium is bound per mole of protein with an apparent dissociation constant of 2.6×10^{-5} M. The exact role of calcium binding protein of mucosal membrane is unknown but it is generally accepted that the calcium binding protein plays a significant role in calcium uptake.

The mitochondria of mucosal cells is now attracting attention as the most probable control centre for active uptake of calcium, since it also contains a Ca^{++}-dependent ATPase transport system. In summary, calcium uptake may involve energy dependent accumulation by mitochondria of mucosal border cells possibly via calcium binding protein in plasma membrane of intestine border and Ca^{++}-ATPase of mitochondria, while release on serasol side is via another (Ca^{++}-Mg^{++})-dependent ATPase calcium transport system.

In the mitochondria, calcium is transported across the membrane by three mechanisms which are as follows: (a) high (apparent $K_m = 6 \times 10^{-6}$ M) and low (apparent $K_m = 6 \times 10^{-5}$ M) affinity binding sites on the mitochondria membrane which may be associated with an ATPase alkaline metal transport system (Rossi et al, 1965; Reynafarje & Lehninger, 1969; Lehninger, 1971); (b) ATP-driven and respiration-dependent uptake of calcium; and (c) transmembrane transport of calcium that is induced by discharge of the intra-mitochondrial K^+ and H^+ (Lehninger et al, 1967). Sr^{++}, Mn^{++}, and La^{+++} competitively inhibit calcium binding but Mg^{++} does not interfere. The exact location of the two calcium binding sites is unknown, but high affinity binding sites are known to be absent from the outer membrane and low affinity binding sites are scattered throughout outer and inner membrane (Carafoli et al, 1971). Carafoli and Rossi (1972) suggested that the low affinity calcium binding sites are of a phospholipid nature and the high affinity calcium binding sites are of a proteinaceous nature.

All three calcium transport mechanisms are inhibited by ruthenium red. Ruthenium red ($Ru(NH_4)_3\ (OH)Cl_2$) mode of action does not involve Ru^{+++} chelation of calcium as ruthenium red is a hexavalent cation (Watson et al, 1971), or energy conservation since at low ruthenium red concentrations the energy source is not inhibited while both calcium binding and transport are antagonized. However, ruthenium red which absorbs maximum at 530 nm interacts specifically with mucopolysaccharides. Mucopolysaccharides in some form, either mucoprotein or glycolipid, are probably located at the active site of calcium transport in the mitochondria. The likely

candidates for calcium binding to the mucopolysaccharide are sulfate and/or carboxyl groups (Moore, 1971). Ruthenium red can also promote the release of calcium from Ca^{++}-loaded mitochondria and reabsorption of Ca^{++} if ruthenium red is added to the medium before the process of calcium accumulation by the mitochondria has been completed which suggests a direct competition between calcium and ruthenium red (Rossi et al, 1973). However, if all the calcium is taken up from the medium prior to the addition of ruthenium red, the intracellular calcium can not be released by ruthenium red. The sites that are accessible to ruthenium red during calcium uptake are no longer accessible after calcium uptake.

The complex cation hexamine cobaltrichloride (HAC) $(Co(NH_3)_6)Cl_3$ reacts with negatively charged sites of sulfate-containing glycosaminoglycans (see Tashmukhamedov et al, 1972). HAC can completely inhibit mitochondrial calcium transport but has no effect on energy transformation processes of the mitochondria, except the calcium translocation induced respiration rate. Studies involving the use of HAC confirms the ruthenium red data showing that it is likely the anionic groups of mucopolysaccharides that are involved in calcium transport in the mitochondria.

The intracristal space or surface of inner membrane of the mitochondria contains a major calcium binding glycoprotein (20% of the total protein of this fraction) that is released by mitochondrial swelling in the presence of phosphate. The major soluble glycoprotein was isolated as a single discrete band on polyacrylamide gel by

Sottocasa and co-workers (1971), and was characterized by Sottocasa et al (1972). The non-protein constituents are sialic acid, galactosamine, glucosamine, glucose, xylose, and phospholipid. There are two classes of binding sites; a high affinity site with a K_d value of 1.0×10^{-7} M and a low affinity site with a K_d value of 1.0×10^{-5} M. The calcium binding was sensitive to inhibitors of respiration-linked calcium uptake and high affinity calcium binding found in intact mitochondria. It appears likely that the glycoprotein fraction contains the high affinity calcium binding sites on the mitochondria membrane and may be the specific calcium 'carrier' involved in translocation of calcium across mitochondrial membrane. An insoluble calcium binding glycoprotein has also been isolated from rat liver mitochondria by Lehninger and co-workers (Gomez-Puyou et al, 1972) which has similar properties to the soluble calcium binding glycoprotein of the mitochondria. The non-protein constituents are sialic acid, hexosamines, lyso-phosphatidyl choline, free fatty acid, and phosphoprotein phosphorous. The insoluble glycoprotein also has two classes of calcium binding sites; a high affinity site with a K_d value of 4.0×10^{-6} M and a low affinity site with a K_d value of 5.0×10^{-4} M. The insoluble calcium binding glycoprotein may be an insoluble aggregate of the soluble glycoprotein or it could occupy a different position in the mitochondria, either membranal or from the matrix.

In the blowfly flight muscle mitochondria, calcium stimulates the oxidation of α-glycerol-phosphate dehydrogenase which is located on the outer surface of the inner mitochondrial membrane (Hansford & Chappell, 1967; Donnellan et al, 1970). The blowly flight muscle mitochondria differs from other mitochondria since no high affinity

calcium binding sites are found, and ruthenium red does not interfere with calcium-stimulated α-glycerol-phosphate dehydrogenase activity (Carafoli & Sacktor, 1972). However, ruthenium red does inhibit the respiration dependent uptake of calcium in the presence of phosphorous.

In the red blood cell, which is relatively impermeable to calcium, calcium is transported by a Ca^{++}-activated Mg^{++}-dependent ATPase (Ca^{++}-ATPase) that is specifically inhibited by ruthenium red. The calcium pump extrudes calcium to maintain a low intracellular level of calcium (4×10^{-5} M), a value which is considerably below the serum calcium level (approximately 1.25×10^{-3} M) (Schatzman & Vincenzi, 1969). As would be expected, only intracellular calcium activates the ATPase transport system with a maximum activation at a concentration of 10^{-6} M. Neither ouabain nor oligomycin, which inhibit (Na^{+} - K^{+})-dependent ATPase systems, inhibit the calcium transport system (Watson et al, 1971).

Schatzmann and Rossi (1971) suggested there were two calcium ATPase fractions that could be differentiated by independence or dependence on either Na^{+} and/or K^{+} for activity. What the significance of the two Ca^{++}-ATPase fractions is unknown, since ruthenium red inhibits both fractions to the same degree (Watson et al, 1971), and the monovalent cation independent Ca^{++}-ATPase alone was sufficient for calcium transport. One Ca^{++}-ATPase may be a calcium transport ATPase system and the other Ca^{++}-ATPase may be a contractile ATPase. It is established that the Ca^{++}-ATPase system transports calcium through the erythrocyte plasma membrane, but the involvement of Ca^{++}-

ATPase with a system of actomysin-like fibers in the plasma membrane has only been postulated by Ohnishi (1962), Rosenthal et al (1970), and Watson et al (1971). The actin-like fibers could maintain the flexibility and biconcave shape of the cell. As an added note of interest, the red blood cell membrane protein, Tektin A which covers the entire inner surface of the membrane, may also have myosin-like properties (Hulla & Gratzer, 1972).

Recently, Bond and Clough (1973) showed that Ca^{++} activated Mg^{++}-dependent ATPase of the human red blood cell requires one or more soluble proteins of the hemolysate for maximum activity. The protein binds to a saturable site on the Ca^{++}-ATPase and allosterically regulated calcium transport by increasing the maximum velocity. Both activator protein and Ca^{++}-ATPase were inactivated in a similar manner when treated with the sulfhydryl reagent NEM, and heat. The hemolysate has no effect on Mg^{++}-dependent ATPase and (Mg^{++}- K^{+}- Na^{+})-dependent ATPase also found in the red blood cell membrane.

In the guinea pig placenta, movement of calcium up a concentration gradient is apparently by a (Ca^{++} - Mg^{++})-dependent ATPase ion transport system (Shami & Radde, 1971). The (Ca^{++} - Mg^{++})-dependent ATPase is similar to that found in other animal tissues, that is, the ATPase does not require either Na^{+} or K^{+}, and is not inhibited by ouabain. Calcium preferentially stimulates the ATPase since the maximum velocity in the presence of calcium is higher than in the presence of Mg^{++}. EDTA inhibition of the ATPase is greater than just chelation of calcium, and the Ca^{++} - EDTA complex may produce the inhibitory effect.

Bone cells, osteoblast, osteoclasts, and oesteocytes are intimately associated with the calcified tissues bone and cartilage. In bone cells, calcium particles are found which contain 6.8% calcium, 3.4% phosphate, 52% protein, and 34% phospholipid of the total dry weight. The intracellular calcium exchanges with the extracellular calcium, but the mode of action is controversial. Calcium uptake by isolated bone cells apparently is an energetic process which depends on phosphate in the extracellular medium (Stern & Austin, 1970; Nichols et al, 1971; Dziak & Brand, 1974). However, calcium uptake by mammalian cells is usually a passive process. For example, in red blood cells, cultured kidney and HeLa cells, and in nerve cells, calcium is extruded via an energetic Ca^{++} -ATPase transport system. The apparent active calcium uptake across the bone cell plasma membrane appears to be energetically regulated at the mitochondrial level by the Ca^{++}-dependent transport system of the mitochondria which allows passive influx across the plasma membrane similar to other mammalian systems. An active efflux pump for free calcium in bone cells may exist similar to other active calcium extrusion systems in mammalian cells.

The developing skeleton of chick embryo obtains calcium from the shell. The 'calcium containing bodies' are transported from the shell through the embryo by a saturable process (Terepka et al, 1971). Wasserman (1972) suggested that calcium transport is via a Ca^{++} - dependent ATPase which can be inhibited by sulfhydryl reagents and metabolic antagonists. A vitamin D inducible calcium binding protein has been demonstrated in the avian shell gland membrane (Corradino

et al, 1968) which may be linked to the calcium transport system in avian shell gland since active transport of calcium across the shell gland can only be demonstrated when an egg is present (Schaer & Schaer, 1971). The inducible calcium binding protein may function in regulation of morphogenic differentiation.

Calcium transport in tissue culture cells has been characterized by Borle and co-workers (Borle, 1968, 1969a, b, c, 1970, 1971) who showed that calcium is accumulated by kidney and HeLa cells in culture across two membranes; the plasma membrane and the mitochondria membrane. Calcium influx across the plasma membrane occurs by facilitated diffusion down an electrochemical gradient while efflux is believed to be metabolically dependent. The hormone, PTH stimulates facilitated diffusion of calcium into cytoplasm and calcitonin inhibits calcium efflux in cells. The resultant hormonal action is identical, but the mode of action for PTH and calcitonin is completely different. As in other mammalian tissue, the calcium level in the cytoplasm is mainly controlled by the calcium transport of the mitochondria.

Calcium transport in chick embryo fibroblasts is dependent on $(Ca^{++} - Mg^{++})$-ATPase located in the plasma membrane (Perdue, 1971). Kinetic characterization demonstrated that transport of calcium by the ATPase system is by a specific system which is inhibited by Mn^{++}, mersalate, oligomycin, and hydroxylamine, and activated by Na^{+} and/or K^{+}, and oxalate. Chick embryo fibroblasts are interesting since the fibroblasts are able to move by ameboid motion. Microfilaments resembling actinomycin are found in chick embryo fibroblasts which constitutes the main structural element in the pseudopodia of the

ameboid moving cell. Yang and Morton (1971) showed that maintenance of the cell's characteristic spindle shape and cell's motility requires calcium. One conclusion that can be formulated is that the Ca^{++}-ATPase of the plasma membrane of chick embryo could regulate cell motility, in a manner similar to the Ca^{++} -ATPase system in the sarcoplasmic reticulum of the muscle cell.

Calcium confers stability to all biological membrane and so prevents ion leakage, and it also allosterically regulates other ion transport systems in mammalian, plants and microbial cells (Burger et al, 1968; Reynolds, 1971; Bolingbroke & Maizels, 1959; Hoffman, 1962). The red blood cell is a relatively simple system to study the effect of calcium on transport systems, since erythrocyte ghost cell can be easily obtained that are void of any cytoplasmic entities, and the erythrocyte ghost cells still retain much of the activity of normal red blood cells, such as, active uptake of ions, relative impermeability to cations, osmotic water flow, and functioning enzyme systems. Calcium confers stability to the erythrocyte membrane and prevents ion leakage because if calcium is added at low concentrations (0.01 to 0.1 mM) passive K^+ leakage is comparable to normal red blood cells, but if a high concentration of calcium is added (0.1 to 1.0 mM) leakage of K^+ is induced (Colombe & Macey, 1974).

Generally the maintenance of low intracellular levels of calcium is required in all animal systems since intracellular calcium ions depress the (Na^+ - K^+)-dependent ATPase of the membrane. In the red blood cell the calcium transport system maintains a low cytoplasmic calcium level which prevents inhibition of (Na^+ - K^+)-

dependent ATPase by intracellular calcium. As membrane bound (Na^+ - K^+)-dependent ATPase is important in maintaining electropotential gradient across the plasma membrane for transport of sugars, amino acids and ions, the active calcium extrusion transport system of the cell is important in the regulation of metabolic transport of all cell constituents because of the intracellular inhibitory effect on (Na^+ - K^+)-dependent ATPase system.

Because of the importance of the calcium transport system in metabolic transport, the calcium transport system is also regulated by a number of rigorous controls. In the mammalian system, the level of calcium throughout the body is controlled by hormones and they in turn are controlled by the level of calcium in the cell (see Borle, 1974). Calcium is compartmentalized mainly in the sarcoplasmic reticulum, myofibrils, and the mitochondria (Kaplan & Richman, 1973), and transport between the various compartments is controlled by the parathyroid hormone (PTH), calcitonin (CT), and cholecalciferol (vitamin D_3). Parathyroid hormone plays the principle role in calcium homeostatis by maintaining the level of calcium in the extracellular fluid and by reabsorption of calcium for bone metabolism. Parathyroid hormone secretion from the parathyroid gland is mediated by cAMP. High extracellular calcium inhibits PTH secretion, possibly by inhibiting calcium sensitive adenyl cyclase. This situation is unique since calcium generally enhances secretion activity of other endocrine glands (hormone secreting glands), as is the case for calcitonin where high extracellular levels of calcium lead to increased secretion of calcitonin from the thyroid gland. Calcitonin secretion is also

stimulated by gastro-intestinal hormones which share the same C-terminal tetrapeptide amide such as glucagon, and cAMP or theophylline. The gastro-intestinal hormones and calcium act by enhancing adenyl cyclase activity. Calcitonin activates PTH secretion, possibly by lowering the intracellular level of calcium which leads to the derepression of adenyl cyclase. The third hormone that regulates calcium level in mammalian cells is 25-hydroxycholecalciferol, which is the active component of vitamin D. Calcium uptake across the mucosal membrane of the intestine is controlled by vitamin D.

Since all mammalian cells require that the intracellular level of calcium be maintained between 10^{-5} and 10^{-7} M, the cytoplasmic level of calcium must be maintained either by the active calcium extrusion pump in the plasma membrane, such as found in the red blood cell, or by absorption of calcium into intracellular compartments, such as the mitochondria. In some specialized cases, other cell plasmalemma control calcium level of cytoplasm, as in the sarcoplasmic reticulum of skeletal muscle cells (MacLennan & Wong, 1971) and the plasma membrane of red blood cells (Schatzman & Vincenzi, 1968). Recently, Diculescu and Popescu (1973) suggested that mitochondria of skeletal muscle may also be involved in regulation of cytoplasmic calcium level. In the majority of animal cells control of calcium cell influx and efflux is intracellular at the mitochondria level which acts as a buffer system and trap of intracellular calcium (Borle, 1973; Lehninger, 1970). As a buffer system, the mitochondria regulates calcium homeostasis, calcium transport, and hormone effect through

three parameters that regulate mitochondria calcium; influx, efflux, and sequestration of calcium (Borle, 1973). Borle's model of regulation for calcium cellular level shows that control of calcium is at a single point, the mitochondria, as compared to the two step model for control of cellular calcium level. Walser (1968) and Wasserman and Taylor (1969) postulated in the two step model, that calcium cellular level is controlled by the carrier mediated uptake and metabolically active efflux of calcium across the plasma membrane. However, not all experimental data supports the role of the plasma membrane as the point of regulation of the calcium level in mammalian tissue (Borle, 1972; Chausner et al, 1972; MacManus & Whitfield, 1970). Borle (1973) suggested that the controversy can be reconciled if the mitochondrion is the controlling point of cellular calcium level. Borle proposed the following model of calcium regulation that correlates calcium and hormone interaction at the physiological level with regulation of calcium at the cell molecular level. In the target cell, PTH stimulates the plasma membrane adenyl cyclase and as a consequence increases the intracellular level of cAMP. Cyclic AMP, in turn, increases the cytoplasmic level of calcium by causing the release of stored calcium from the mitochondria. PTH and cAMP also stimulate influx and efflux of calcium across the plasma membrane (Borle, 1972a; Rasmussen et al, 1972), but Borle suggested that this action is secondary to mobilization of intracellular calcium by cAMP. Calcitonin increases calcium uptake by the mitochondria and cell but calcitonin also causes sequestration of calcium as calcium phosphate in mitochondria which results in a lowering of the cytoplasmic calcium (see Borle, 1974). In the absence of ATP, calcitonin lowers

the cytoplasmic level of calcium in the red blood cell by enhancement of calcium efflux (Parkinson & Radde, 1970), but this later effect may only occur because erythrocytes do not contain mitochondria.

In the intestinal mucosal cells the mode of vitamin D action is similar to calcitonin in other target tissues. Vitamin D activates calcium uptake in the mitochondria which causes a passive uptake of calcium into the mucosal border cytoplasm, therefore, the level of regulation of calcium uptake is the mitochondrion. However, there is another level of regulation of the calcium across the mucosal membrane which is at the serasol membrane (next to the blood stream) which contains a Ca^{++} -ATPase system that actively extrudes cytoplasmic calcium. The glucocorticoid hormones, such as, cortisone inhibit calcium absorption by inhibition of calcium uptake by the mitochondria, and inhibit Ca^{++} -ATPase of the serasol membrane and alkaline phosphatase, but the glucocorticoids have no effect on the level of mucosal border calcium binding protein.

Phosphate also influences cellular calcium metabolism (Borle, 1972b Rasmussen, 1973). Extracellular phosphate reduces cytoplasmic activity of calcium, but this may be due to the inhibitory effect on PTH and the enhancement effect of calcitonin (Borle, 1973). Phosphate also lowers mitochondrial calcium activity.

Amino Acid Transport

Amino acids are transported by multiple transport systems that can be specific for a single amino acid or a group of amino acids with

similar chemical characteristics, such as, neutral, aromatic, acidic, and basic amino acids. A particular amino acid can be transported by several specific transport systems. The multiple amino acid transport systems are essential to the survival of the cell by allowing uptake of amino acids to be selectively controlled to comply with stage of growth cycle, extracellular amino acid supply, and intracellular amino acid requirement. If all amino acids were transported by a general transport system differential regulation would be impossible.

Plant Systems

Amino acid uptake by a variety of plants, wheat, barley, sunflower, cucumber, radish, and other plant tissues has been demonstrated by Webster (1954), Rheinhold and Powell (1958), Kursanav (1956), and for a general review see Higinbotham, (1973). In 1956, Birt and Hird were the first to associate amino acid uptake with a possible uptake process showing that metabolic inhibitors abolished histidine uptake in carrot tissue slices.

Kinetic characterization of amino acid uptake by higher plants shows that amino acid uptake systems usually have a high apparent K_m for transport. It is possible that amino acid transport in higher plants may operate by a mechanism different from that of heterothallic bacteria and fungi which have high specific affinity transport systems for amino acid transport. However, a few exceptions do occur, the

'scavenger' plant transport system transports amino acid with a high affinity (King & Oleniuk, 1973). The high affinity amino acid transport is required during starvation conditions which allows uptake of amino acids that are present in small amounts and recovery of leaking amino acids from the intracellular spaces. Known amino acid transport systems of plants are summarized in Table III. In general, the specificity of plant transport systems had not been characterized to any degree, but it seems likely there may be a general transport system that transports all amino acids (see Nissen, 1974), except under starvation conditions when additional 'scavenger' transport systems are induced.

TABLE III AMINO ACID TRANSPORT IN PLANTS

AMINO ACID(S)	PLANT TISSUE	INHIBITORS	ACTIVATORS	KINETICS	LITERATURE REFERENCE
glutamic acid	sunflower hypocotyl			slightly curved line on a rate plot in the range 2.0-87.0 mM (possibly biphasic)	Reinhold & Poweell, 1958
all common amino acids	carrot root cells	CN, DNP, iodoacetate, fluoride, and metabolic inhibitors	'washing'	monobasic (1-50 mM) common carrier for all amino acids, stereo-specific for L-amino acids -saturated at high amino acid concentration	Birt & Hird, 1958
α-aminoisobutyric acid (AIB)*	barley leaf tissue	alanine competitive		biphasic, pH dependent K_m = 0.6 and 9.5 mM -saturation kinetics in the range of 2.5 to 6 mM	Reinhold, Shtarkshall, & Garrpt. 1970

*AIB - non-metabolizable amino acid analogue of glycine.

Table III. Continued

AMINO ACID(S)	PLANT TISSUE	INHIBITORS	ACTIVATORS	KINETICS	LITERATURE REFERENCE
α-aminoisobutyric acid (AIB)	barley leaf tissue		'washing in the presence of 0.1 mM $CaSO_4$ (increased 4 fold)	linear increase in AIB uptake for 5 h then levelled off -other kinetics as above	Shtarkshall, Reinhold, & Harel, 1970
glycine; phenylalanine & alanine	wheat, barley sunflower, cucumber, & radish	competitive with 21 amino acids		multiphasic kinetics in the range of 0.01 to 10 mM -single amino acid transport system	Cseh & Böszörményi, 1964; see review by Nissen, 1974)
arginine, and lysine	sugarcane	metabolic inhibitors: CN, Azide, DNP EDTA cycloheximide		-separate active transport mechanisms K_m (arginine)= 0.1 mM K_m (lysine) = 2.4 mM	Maretzki & Thom, 1970

Table III. Continued

AMINO ACID(S)	PLANT TISSUE	INHIBITORS	ACTIVATORS	KINETICS	LITERATURE REFERENCE
alanine	soybean root cells	respiratory inhibitors: azide, 2,4-DNP		-low rates of uptake similar to Maretzki & Thom (1970) in non-nitrogen starved tissue -high uptake rates in nitrogen starved tissue $K_m = 2.55 \times 10^{-6} M$ (also low cytoplasm amino acid content)	King & Oleniuk, 1973

Fungal Systems

Amino acid uptake in fungi resembles the active transport kinetics of other micro-organisms more closely than amino acid transport in plants since high affinity amino acid transport systems are involved. Also amino acid transport in fungi differs from that of animal amino acid transport, for example; the yeast, Saccharomyces carlsbergensis can concentrate glycine 10^3 fold when the external concentration is 10 μM glycine, whereas a ratio of less than 30 is found in mammalian mouse ascite tumor cells (Eddy, Backen & Watson; 1970). The activity of fungal amino acid transport systems varies extensively throughout the growth cycle, therefore, it is important when discussing amino acid transport systems of fungi to mention the particular stage of growth. Specific active transport systems (at present five are known in Neurospora crassa) exist for groups of amino acids with similar structure and for single amino acids. In addition, in fungi, a particular amino acid can be transported by more than one transport system. In Neurospora crassa, amino acid transport has been characterized by Pall (Pall 1969, 1970a, b, 1971). Amino acid transport system I (AAI) transports the neutral amino acids, tryptophan, leucine, and phenylalanine in young rapidly growing germinated conidia (Wiley & Matchett, 1966; Pall, 1969). In addition, several other amino acids have a reduced affinity for transport system I; alanine, histidine, serine, glycine, and methionine. Genetic mutant studies of the neutral amino acid system show that the 'mtr' locus located on the

linkage group IV controls tryptophan transport. A mutation at the 'mtr' locus reduces tryptophan transport 85 to 90% (Wiley & Matchett, 1968). Transport system II (AAII) is a high affinity transport system (postulated scavenger) that transports a variety of neutral and basic amino acids, arginine, phenylalanine, glycine, tryptophan, leucine, and others (Pall, 1969). The 'scavenger' amino acid transport system of Neurospora crassa is only induced in older, carbon-depleted cultures, and is virtually absent in young, rapidly growing cultures. A mutation in 'mtr' locus has no effect on this transport system (Pall, 1969). Transport system II can also be separated from transport system I by the fact that arginine has high affinity for transport system II, while having very little affinity for transport system I. The basic amino acids arginine and lysine, are taken up by transport system III. Ornithine and canavanine are also transported by transport system III (Pall, 1970a). Transport system III (AAIII) is active in young growing germinated conidia with activity decreasing as the mycelia age. Amino acid transport system IV (AAIV) catalyzes the transport of acidic amino acids, aspartic, and glutamic acid. Above pH 6.0, transport system IV is specific for acidic amino acids relative to other transport systems. Below pH 6.0 other amino acid transport systems also take up acidic amino acids. AA IV is similar to AAII because it is present only in older mycelial mats (starved for carbon, sulfur, or nitrogen) and has a scavenger role, but AAII has a lower affinity for acidic amino acids (Pall, 1970b). The fifth transport system (AAV) is specific for methionine and its analogue ethionine (Pall, 1971). AAV is only present in sulfur-starved mycelial mats. This

transport system probably serves as a scavenger for a sulfur-source. In ungerminated conidia there are several amino acid transport systems which are specific for the following groups of amino acids: (a) aromatic and neutral amino acids; (b) basic amino acids; and (c) non-specific amino acid system (DeBusk & DeBusk, 1965; Roess & DeBusk, 1968; Tisdale & DeBusk, 1970; Wolfinbarger & DeBusk, 1971; Wolfinbarger et al, 1971). The amino acid transport systems in ungerminated conidia are likely the same as those reported for growing cells since specificity is almost identical, although absolute rates of uptake are lower in ungerminated conidia than rates of uptake in rapidly growing cells. Detailed kinetic characterization of the amino acid transport systems of Neurospora are summarized in Table IV.

In yeast, specific transport systems for amino acids have been characterized by Grenson and co-workers (Grenson, 1973). Six amino acid transport systems have been reported in Saccharomyces cerevisiae which are specific for the following groups of amino acids: (a) lysine (Grenson, 1966), (b) arginine (Grenson et al, 1966), (c) histidine (Crabell & Grenson, 1970), (d) methionine (Gits & Grenson, 1967), (e) aspartic and glutamic acid (Joiris & Grenson, 1969), and (f) a general amino acid transport system (Grenson et al, 1966). The later system is thought to act as a scavenger similar to the scavenger amino acid transport system found in Neurospora crassa (Pall, 1969) and Penicillium chrysogenum (Benko et al, 1967).

The amino acid transport systems of Achlya have been kinetically characterized by Singh (1974). Nine amino acid transport systems are

TABLE IV. ACTIVE AMINO ACID TRANSPORT SYSTEMS IN FUNGI

Transport System Notation	Amino Acid Group	Fungi	Kinetic Characterization amino acid	K_m (M)	Literature Reference
AA I	neutral transport system: tryptophan, leucine, and phenylalanine	Neurospora crassa (germinated) conidia)	tryptophan leucine phenylalanine	5×10^{-8} 1.1×10^{-7} 1.4×10^{-7}	Wiley & Matchett, 1966; 1968 and Pall, 1969
AA II	neutral and basic transport system: tryptophan, leucine, arginine, phenylalanine	Neurospora crassa (germinated aged cell cultures)	tryptophan leucine phenylalanine	5×10^{-5} 4×10^{-6} 2×10^{-6}	Pall, 1969
AA III	basic transport system: arginine, lysine	Neurospora crassa (germinated conidia)	arginine lysine	2.4×10^{-6} 4.8×10^{-6}	Pall, 1970a
AA IV	acidic transport system: cysteic acid, aspartic acid, glutamic acid	Neurospora crassa (greater than 3 day cell mats)	cysteic aspartate glutamate	7×10^{-6} 1.3×10^{-5} 1.6×10^{-5}	Pall, 1970b
AA V	methionine and ethionine	Neurospora crassa (sulfur starved cell mats)	methionine ethionine	2.3×10^{-5} 1.0×10^{-4}	Pall, 1971

present which are specific for the following groups of amino acids: (a) glutamate and aspartate; (b) leucine, isoleucine, and valine; (c) phenylalanine, tyrosine, and tryptophan; (d) histidine, lysine, and arginine; (e) cysteine; (f) methionine; (g) serine and threonine; (h) proline, and (i) asparagine and glutamine. Methionine is probably transported by two systems, of which one is specific only for methionine.

Bacterial Systems

Specific amino acid transport systems in bacteria have been characterized by initial rate kinetic studies, competition inhibition studies, genetic studies of transport mutants, temperature optimum, and physical isolation of component proteins of transport systems (Piperno & Oxender, 1966). Uptake patterns are simple first rate Michaelis-Menten kinetics with apparent K_m values ranging from 0.1 to 40 μM. The specific amino acid transport systems of bacteria have been described in detail in a recent review by Oxender (1972b) with special emphasis on initial rate kinetic studies. Amino acid transport in bacteria is different from amino acid transport in animals, plants, and fungi. Bacteria do not contain mitochondria, therefore, respiration (electron transport) mechanism is located in the plasma membrane and is the dominant energy source for amino acid transport.

Isolated membrane vesicles of E. coli have at least nine specific amino acid uptake systems which are as follows: (a) proline, (b) lysine, (c) glycine-alanine, (d) serine-threonine, (e) glutamic and aspartic acid, (f) phenylalanine-tyrosine-tryptophan, (g) histidine, (h) leucine-isoleucine-valine, and (i) cysteine (Lombardi & Kaback, 1972). The amino acid transport in membrane vesicles represents approximately 70% found in intact cells (Brown, 1970), but the specific transport systems for both intact cell and membrane vesicles are almost identical. However, some amino acid binding proteins such as leucine-isoleucine-valine and histidine binding proteins are released. Binding proteins are important for high affinity transport of amino acid since there is a reduced amino acid affinity in membrane vesicles. Intact E. coli cells have specific transport systems for glutamine, asparagine, methionine and arginine in addition to those systems found in bacterial membrane vesicles (Willis & Woolfock, see Lombardi & Kaback, 1972; Piperno & Oxender, 1966; Wilson & Holden, 1969). E. coli has two systems involved in lysine and arginine transport, one specific for lysine or arginine, and the other specific for lysine, arginine and ornithine (Rosen, 1971).

Mammalian Systems

In animal cells amino acid transport systems are firmly attached to the cell membrane. A general review of amino acid transport in animal systems has been published by Heinz (1972). A detailed

account of animal amino acid transport systems will not presented. However, amino acid transport in animals can be separated into specific carrier-mediated transport systems as for most micro-organisms. Ehrlich cells transport glycine-alanine-serine by a specific transport system that is Na^+ dependent (Eddy, Backen & Watson, 1970). Neutral amino acids with large branched hydrocarbon chains valine-leucine-isoleucine are transported by a general Na^+-independent transport system (see Oxender, 1972b), and apparently glutamic acid is transported by this neutral amino acid transport system.

Regulation of Transport

Feedback control mechanisms of transport by ions, other effectors, or substrates are known to control transport mechanisms either at the genetic or enzymatic level. This is especially characteristic of bacterial systems where a number of inducible transport systems exist for amino acids, sugars, and ions (see Heinz, 1967). The control of inducible transport systems functions at the genetic level, that is, induction and repression of the structure 'carrier' protein(s). Usually the transport genes occur in the same operon as the genes that control further metabolism of transported substrate, a classic example is the lactose operon of *E. coli*. However, there are some exceptions to the rule, sulfate transport in *Salmonella typhimurium* is controlled by three different cistrons (Dreyfuss, 1966). Induction of amino acid transport systems in fungi, *Neurospora* and yeast, *Saccharomyces* occurs under starvation conditions or in aging cell

cultures, and the induced transport systems are of high affinity and are of a general nature (Grenson et al, 1970; Pall, 1969, 1971). In addition, induction of an amino acid transport system that has broad amino acid affinity and a relatively low apparent K_m occurs in Penicillium chyrsogenum under nitrogen starvation conditions (Benko et al, 1967).

A more general phenomenon is the regulation of amino acid uptake by feedback control at the enzymatic level. Feedback by substrate interferes directly with the transport system. In Streptomyces hydrogenans the accumulation of the non-metabolizable amino acid α-aminoisobutyrate immediately slows down further transport of this amino acid (Ring & Heinz, 1966). The effect of substrate feedback control mechanisms in yeast are specific. When yeast cells are preloaded with histidine, the subsequent transport of amino acids occurs except histidine transport is reduced due to substrate feedback inhibition (Crabeel & Grenson, 1970; Grenson, 1973). Regulation of amino acid transport by amino acids already present in cytoplasmic pools permits selective uptake of only the required amino acids. Extrusion of amino acids also occurs to regulate the size of the amino acid pools.

Whether protein synthesis and amino acid transport are two separate processes has been debated in recent years. Amino acid transport and protein synthesis can be interconnected in three ways (i) Amino acids are transported into the cell where they are compartmentalized into two or more distinct pools with a small rapidly

equilibrated pool being the precursor for protein synthesis. (ii) Extracellular amino acids are transported across the cell membrane where they are directly incorporated into protein, instead of going into intracellular pools (Hendler, 1962; Rosenberg et al, 1963; Hider et al, 1969). (iii) A complex formed between amino acid and membrane transport system is the source of amino acids for protein synthesis whether the amino acid source is the extracellular medium or an intracellular amino acid pool (Adamson et al, 1972). The third model takes into consideration the evidence that intracellular amino acids are the main source of amino acids for protein synthesis (Righette et al, 1971), and the contradictory statement that extracellular amino acids are the main source of amino acids for protein synthesis (Hider et al, 1969, 1971). In Adamson's model the choice of amino acid source would depend on relative affinities of membrane transport sites for a particular amino acid, and internal and external environment and concentrations.

In a comparative study of incorporation of extracellular and intracellular proline into protein of embryonic chick cartilage, Adamson and co-workers (1972) were able to support their model for amino acid transport and protein synthesis interconnection by experimental data. Ouabain was able to inhibit the incorporation of intracellular proline into protein equally well as incorporation of extracellular amino acids into protein, and α-aminoisobutyric acid (non-metabolizable amino acid) inhibited the incorporation of intracellular proline into protein. In agreement with an early report by Fritz & Knobil (1964) that had shown that insulin enhanced the trans-

port of L-amino acids, Adamson and co-workers (1972) showed that serum (insulin) enhanced the formation of the amino acid transport complex. Insulin preferentially stimulated the incorporation of extracellular proline into protein, but if no extracellular proline was present insulin could stimulate intracellular proline into protein.

Amino acid transport and protein synthesis apparently are separable in bacteria since protein synthesis inhibitors do not interfere with amino acid transport (see Oxender, 1972b), but in yeast, fungi, and mammalian cells, inhibitors of protein and RNA synthesis appear to antagonize amino acid transport (Ring, 1970). In yeast and fungi, when the inhibition of protein synthesis affects amino acid transport, this may be due to a direct feedback inhibition by cellular amino acids (transinhibition). This effect has been observed in yeast (Grenson et al, 1968), Penicillium chrysogenum (Hackette et al, 1970), and Streptomyces hydrogenans (Ring, 1970). Transinhibition could also be due to interference with amino acid carrier protein synthesis (Elas & Rosenberg, 1967; Holden & Utech, 1967; Gross & Ring, 1969) when RNA synthesis is also involved.

In characterizing the association between protein synthesis and amino acid transport, the involvement of calcium in both processes is of particular interest. The direct requirement of Ca^{++} for protein synthesis has been considered in a number of systems. It is generally accepted that protein synthesis requires Na^{+}, K^{+} (Pestka, 1971), and Mg^{++} (Conway, 1964), and it now seems likely that

calcium is also involved in protein synthesis. Protein synthesis in E. coli specifically requires Mg^{++} although Mn^{++} or Ca^{++} can substitute with reduced efficiency (Tissières et al, 1960). However in the polyuridylic (poly U) in vitro protein synthesizing system, calcium enhanced protein synthesis twice as much as Mg^{++} (Gorden & Lipmann, 1967). In the microsomal system of rat liver, when Mg^{++} concentration is low, calcium can substitute for Mg^{++} insufficiency with the same efficiency of protein synthesis although when poly U codon was used calcium could not substitute (Hershko et al, 1961). In general the role of calcium in protein synthesis may not be identical to Mg^{++} since Ca^{++} can only substitute for Mg^{++} in a few select cases. However, calcium can stimulate protein synthesis. Calcium promoted protein synthesis using the in vitro polyribosome system isolated from imbibed rice embryo, but if poly U codon was used protein synthesis was not promoted (McCarthy et al, 1971). But calcium did promote in vitro polypeptide synthesis after a preincubation of ribosomes with Mg^{++}, poly U, and phenylalanine-tRNA. McCarthy and co-workers (1971) found that both Ca^{++} and Mg^{++} could bind poly U and phenylalanine tRNA functionally to ribosomes, but calcium ions could not substitute for Mg^{++} in the initiation process of polypeptide synthesis. Calcium was able to support elongation of polypeptide synthesis in this eucaryotic system but not polypeptide initiation. Calcium also promoted protein synthesis in mammalian tissue such as, adrenal gland, in vitro (Farese, 1971), liver (Burton et al, 1969; Reboud et al, 1972; Agostini et al, 1972), parotide gland (Grand & Gross, 1969), parathyroid gland (Hamilton

& Cohn, 1969), and heart ventricles (Kaplan & Richman, 1973).

Several models for calcium enhancement of protein synthesis have been proposed. One model is that calcium could have a direct stimulatory effect on protein synthesis (Kaplan & Richman, 1973; Reboud et al, 1972; Balasubramian & Dhalla, 1972). Kaplan and Richman found no stimulation of leucine transport in heart ventricles by calcium during a 4 hour incubation period, but protein synthesis was increased and if calcium was absent protein synthesis reached an optimum after 2 to 3 hours. Calcium appears to be required for continued protein synthesis. In plants, calcium deficiency has an inhibitory effect on protein synthesis (Fialova & Pichl, 1973), but this may be due either to reduced nitrate uptake (Burstrom, 1952), or energy metabolic disturbances that destroy the mitochondria by loss of plasmalemma integrity (Florell, 1956). Another model is that calcium enhances protein synthesis indirectly by increasing amino acid uptake (Kypson & Hait, 1971; Guroff, 1961) in accordance with a coupling of protein synthesis and amino acid transport (Hendler, 1962; Kostya, 1964; Hider et al, 1971). Indeed, both processes could be working at the same time. Calcium could increase amino acid uptake and increase protein synthesis by two separate processes.

Fungi and mammalian cell solute transport across the plasma membrane can be affected by the presence or absence of calcium. For mammalian cells only a few recent results will be described because of the preliminary nature of the investigations in this area. Calcium is required for transformation of mammalian cells during the early stages prior to DNA synthesis (Whitney & Sutherland, 1972). Phyto-

hemagglutinin (PHA), a plant lectin, can induce transformation in mammalian lymphocytes with concomitant increase in uptake of calcium and amino acids and activation of adenyl cyclase. The PHA stimulation of amino acid uptake is inhibited by EDTA, a specific chelator of calcium ions (see Whitney & Sutherland, 1972). Calcium may be involved with amino acid uptake or it may be just a result of now allowing PHA action to take place due to the calcium requirement of PHA action.

Isolated amphibian oocytes rapidly sequester proteins. The oocyte is surrounded by a layer of follicle cells which can be removed by proteolytic enzymes, EDTA, or Ca^{++} free medium. After removal of the follicle cell layer the amphibian oocytes are no longer able to sequester protein, but if calcium is added protein uptake resumes (Wallace et al, 1973). The requirement for calcium is specific and Mg^{++} is unable to substitute for calcium. Wallace and co-workers (1973) suggested that calcium is important for maintaining integrity of oocyte membrane so as to allow a functional transport of proteins through the membrane.

The liver plasma membrane is the primary site for binding and transport of asialoglycoproteins. The specific binding of serum glycoprotein not only requires the absence of sialic acid on the glycoprotein but its presence on the plasma membrane of the liver (Pricer & Ashwell, 1971). Calcium and sialic acid are required for asialoglycoprotein binding by the plasma membrane. The specific calcium requirement could not be substituted for by Mg^{++} or Mn^{++},

and ATP produced no stimulation. The binding is also pH dependent with maximum binding pH range of pH 7.5 to pH 7.8 and no glycoprotein binding occurs at pH 5.0. If calcium is absent and pH is dropped to pH 6.0, the asialoglycoprotein is released from the plasma membrane of the liver, which can be completely reversed if the pH is raised to pH 7.8 and calcium is added. Neither pH nor calcium have any effect alone. Pricer and Ashwell (1971) suggested that conditions in the body may regulate the release of bound protein into the cytoplasm prior to catabolism by the lyosomes. Recently a hepatic protein has been isolated from the rabbit liver membrane that is similar to asialoglycoprotein binding and transport protein which requires both calcium and sialic acid integrity before binding of asialoglycoprotein could take place (Hudgin et al, 1974).

Only a few recent reports which concern the effect of calcium on the transport of organic solutes in fungi have been published in the literature. When Sr^{++} replaced Ca^{++} in Neurospora crassa growth was reduced by one third and this was accompanied by an increase of amino acids in the surrounding medium. Castel (1956) concluded that calcium was important in amino acid transport, but this conclusion was critized by Bollard and Butler (1966), who suggested that the loss of amino acid is due to the reduced growth that occurs in the presence of Sr^{++}.

Recent studies using the inhibitor of protein synthesis, cycloheximide, in association with calcium have demonstrated that amino acid transport and protein synthesis are actually two separate

processes. Evans (1971) working with Euglena showed that cycloheximide inhibited both amino acid and sugar transport, as well as protein synthesis, but that the cycloheximide responses were different. This indicated that inhibition of transport by cycloheximide was not a consequence of inhibition of protein synthesis. In carbon-starved cells, glucose and amino acid membrane transport were reduced 5 to 15% by high concentrations of cycloheximide, while in rapidly growing log phase cells a low concentration inhibited membrane transport by 90%, and in both cases, protein synthesis was stopped.

In Penicillium chrysogenum cycloheximide also inhibited both protein synthesis and amino acid transport (Hunter et al, 1973), but the effects could be differentiated by calcium. Cycloheximide inhibition of L-leucine uptake in nitrogen- and carbon- starved cells was reversed by calcium, but calcium did not reverse the inhibition of protein synthesis (Hunter et al, 1973). In addition, cycloheximide promoted the continued uptake of calcium, possibly by labilizing membrane bound calcium. Hunter and Segel (1973 a, b) further suggested that calcium enhanced amino acid uptake in Achlya (Cameron & LéJohn, 1972) was due to its influence on a proton gradient across the plasma membrane. Furthermore, this may be responsible for organic solute transport in Penicillium chrysogenum.

CALCIUM

Although it cannot be proven as fact, it appears likely that the first metalloproteins to evolve on the earth were iron and magnesium proteins, followed by copper proteins (Österberg, 1974). Magnesium (enzyme catalyst) was likely important for prebiotic condensation reactions. This indirectly supported by the fact that in present day biological systems magnesium is basic to all fundamental systems, such as, protein synthesis, anaerobic energy production, and photosynthesis. Calcium's role in biological systems came later in evolution. This again is supported indirectly since calcium has little function in procaryotic systems but is very important in higher forms of life. All complex systems in higher forms of life (eucaryotes) utilize calcium in some manner: e.g. in mammalian cells; nerve transmission, hormone secretation and action, muscle contraction, bone formation and intestinal absorption; and in higher plants; hormone action, differentiation, metabolite transport, and growth. In the lower eucaryotes, the responses are similar to those mentioned for higher plants.

Growth and Differentiation

Growth Requirement

In 1946, Steinberg showed that the extent of fungal growth in the absence of calcium varies from no effect to only 14% of the original growth. Since that time, calcium has been shown to be essential for growth of the following fungi: Achlya (Reischer, 1951; Barksdale, 1962), Phycomyces sp. (Ødegard, 1952), Allomyces spp. (Machlis, 1953; Ingraham & Emerson, 1954), Neurospora sp. (Castel & Bertrand, 1954), Coprinus sp. (Fries, 1962), and Phytophthora spp. (Davies, 1959; Erwin & Katznelson, 1961). It is generally accepted that calcium is an indispensible nutrient element as rigidly defined by Arnon in 1950. For growth to occur in almost any organism at least a trace amount of calcium is required.

Sodium ions inhibit fungal mycelium growth in the marine fungi, Dendryphiella salina (Jones & Jennings, 1965) and the inhibition can be reversed by Ca^{++} in the presence of K^{+}. Calcium ions alone show no increase in the dry weight of the mycelial mat, but if Na^{+} is also present at a ratio of 20:1 (Na^{+}:Ca^{++}), there is an increase in dry weight compared to only Na^{+} present. These authors may have unconsciously encountered the problem of endogenous calcium thereby not producing any additional enhancement of growth with added calcium in the absence of sodium. Calcium appears not to be directly required for growth but prevents the inhibition of growth by sodium. Harold

(1958) showed that the requirement for calcium in Penicillium notatum during sporulation is diminished as the sodium nitrate concentration decreased.

In Achlya, the role of calcium is more direct than just reversing the effect of sodium inhibition on growth. Griffin (1966) showed that Achlya sp. has an absolute calcium requirement for the differentiation of sporangia and gemmae, and neither Mg^{++}, K^{+}, nor Na^{+} could replace Ca^{++}. Calcium is also required for oogonia production. If the concentration of Ca^{++} is reduced to less than required for maximum growth in Achlya (0.005 g/1), the number of oogonia produced decreases sharply (Barksdale, 1962). This is also true for Mg^{++} and K^{+}, but the requirement for Ca^{++} cannot be replaced by these mono- and divalent cations.

Calcium is required for maximum growth in green algae, blue green algae and diatoms (see Healey, 1973), and the requirement for calcium can be correlated with cell wall constituents (see Bollard and Butler, 1966). For example, the green algae can be divided into three groups depending on the calcium requirement. High requirement for calcium, there was a large amount of pectic material produced. Small requirement for calcium, pectic material was not essential for growth. Micro-quantity of calcium, no pectic material was found in the cell wall (O'Kelley & Herndon, 1961). This ability to classify the cell wall constituents on the basis of calcium requirement in algae cannot be extended to fungi. Fungal calcium requirements are varied and do not correlate with cell wall constituents. As indicated for fungi, calcium is also required for zoospore cell wall formation and release by the algae;

Protsiphon botryoides, and Chlorococcum (O'Kelley & Herndon, 1961).

Euglena gracilis has a calcium requirement for growth. The exact function of calcium is unknown and cannot be correlated with the cell wall pectic material (Hatner & Provasoli, 1951).

The calcium requirement by higher plants is usually very small and is generally classified as a micro-nutrient element (Burstrom, 1968), however plants do differ in their cation nutrition, and the inter-relationship between cations varies from plant to plant Collander, 1941; Falade, 1973). In 1902 Kearny and Cameron first demonstrated that calcium would neutralize the inhibitory effect of sodium ions in most plants. Wallace and co-workers (1966) suggested that beyond ameliorating toxicity of other ions, the role of calcium is that of a micro-nutrient if other required nutrients are present in the appropriate ratios. But this seems unlikely to be the only function of calcium in plants, since calcium is an integral part of the plasmalemma and cell wall. LaHaye and Epstein (1969) showed that a deficiency in calcium causes a membrane structure collapse in such a manner that normal plant growth is impossible.

Of particular interest is the calcium requirement for tip growth in higher plants. Calcium is believed to be a specific natural attractant (chemotropic agent) and presumed stimulant for pollen tube growth in all flowering plants (Mascarenhas & Machlis, 1962), since the level of calcium recorded in the ovule is found to be high in relationship to other plant sections. Calcium also acts to enhance the growth of pollen tubes as observed by Kwack and Brewbaker (1961).

The enhancement is maximum in the presence of Mg^{++} or boron, and a sugar source (Nygaard, 1970). The calcium requirement is specific in regard to tube growth with Mg^{++} replacing Ca^{++} to some extent, while Sr^{++}, Ba^{++} and Mn^{++} completely inhibit growth in the absence of Ca^{++}. In addition to calcium involvement in pollen germination and pollen tube growth, calcium ions are also antagonistic to pollen injury induced by the following chemical and physical agents: Mn^{++}, Ba^{++}, Zn^{++}, Na^{+}, 2,4-DNP, gibberellic acid and pectinase (Kwack & Kim, 1967).

Calcium apparently binds to the cell wall (Kwack, 1967). Using enzymatic and chemical inhibitors of pectin synthesis, Kwack (1967) showed that the protective effect of calcium against antagonists is due to its binding in the pectic regions of the pollen cell wall, not to protein, cellulose, or hemi-cellulose. Calcium increases the rigidity of the pollen cell wall and may arrange the cell wall material so as to promote cell elongation (Kwack & Kim, 1967), and in a similar manner affects the permeability of the wall, consequently regulating solute uptake. Calcium is more important for elongation of the pollen tube, as compared to germination of the pollen. Metabolic energy is required for germination while in cell elongation, where calcium is more active, is non-metabolic. Calcium is believed to be involved in organization of cell wall material after the initial synthesis, and again not actively involved in metabolic processes.

The growth of root hairs is similar to growth of pollen tubes; that is, growth by elongation of the apex. At low concentrations,

calcium affects initiation and growth of root hairs (Street, 1966), and calcium is incorporated in the cell wall (Ca^{++}-pectin) during root hair growth (Cormack et al, 1963).

Differentiation

Calcium and magnesium are required for the maintenance and promotion of intercellular adhesion (Curtis, 1967; Weiss, 1967b). Removal of calcium by chelators results in dissociation of early amphibian and chick embryo cells (Curtis, 1962; Zwilling, 1954), while addition of calcium to dissociated cells promotes aggregation (Armstrong, 1966), as well as adhesion to certain substrates (Takeichi & Okada, 1972). In HeLa cells, calcium increased the adhesion rate but not if cells were pre-treated with either neuraminidase or trypsin. Glycoproteins containing terminal sialic acid residues on the surface of normal cellular membrane are essential for intercellular adhesion. Calcium's apparent action is through direct binding to sialic acid which has been demonstrated by Long and Mouat (1971) and Shlatz and Marinetti (1972), attenuating the adverse repulsion effect of sialic acid.

During differentiation, the surface charge of animals can vary depending on the required tightness of associated cells. Harris and Zalik (1974) showed that early chick blastoderm cells have a very high affinity for calcium as compared to differentiating cells and adult cells. The calcium binding is believed to

be associated with charged ionic groups on the cell surface. As cells become committed to various differentiation patterns, calcium binding sites may be redistributed so that they are no longer available on the cell surface (Forstner & Manery, 1971), or they may be shed as normal surface turnover (Kapeller et al, 1973; Deman et al, 1974). After treatment of early chick embryo blastoderms with trypsin, calcium binding still remains high which suggests that some trypsin susceptible calcium binding sites are removed, but that the majority are not. In chick embryo cells, calcium binding sites are usually clustered in associated cells, and homogeneously spread over the surface of dissociated cells. The clusters of calcium binding sites may give the characteristic high affinity calcium property to early chick embryo blastoderms (Harris & Zalik, 1974). Another property of chick embryo blastoderms is that the cells are constantly dissociating and reassociating. Since calcium is involved in both adhesion and detachment of cells to substrate (Takeichi & Okada, 1972), the discrete localization of calcium binding sites on the cell surface of chick embryo blastoderm cells may play an important role in association and dissociation of cells.

In the slime mold, Dictyostelium discoideum, cyclic AMP initiates aggregation of motile single cells to form a 'slug' which differentiates into a fruiting body (Konijn et al, 1967). Calcium is required for this aggregation process (Rasmussen, 1970), and the calcium content of Dictyostelium discoideum varies throughout differentiation (see Maeda & Maeda, 1973). The calcium content

of free moving amoebae decreases as growth stops and aggregation starts. During this stage calcium is extruded. Why this occurs is unknown but cell adhesion effect of calcium may be important (Weiss, 1967a; Curtis, 1967). After the formation of papilla-like masses, cell calcium content increases to slug stage and continues to culmination stage. The slug consists of a pre-stalk cells and pre-spore cells. Calcium present in large amounts in pre-stalk cells is probably involved in cellulose synthesis that is formed for formation and aggregation of the tightly packed stalk cells. The pre-spore cells contain less calcium and are loosely packed.

Calcium and Cyclic AMP

Communication between animal cells takes place by either direct communication of the nerve system or indirect communication through chemical messengers. Calcium is important in both types of cellular communication, (see Table V). At the basis of the hormonal control mechanisms is calcium, cyclic AMP, protein kinases and other enzymes involved in synthesis and breakdown of cyclic AMP.

Cyclic AMP in animal cells acts as a second messenger of communication systems throughout the body, such as, exocrine and endocrine hormonal systems, and nerve transmission (Sutherland & Rall, 1958; Breckenridge et al, 1967; Robinson et al, 1968; Goldberg & Singer, 1969). The second messenger concept developed

TABLE V. TABLE OF CALCIUM ACTION

GENERAL FUNCTION	SPECIFIC FUNCTION*	LITERATURE REFERENCE
Cell proliferation	regulation of mitosis	Perris, A.D. (1971) in Cellular Mechanisms for Calcium Transfer and Homeostasis (G. Nichols Jr. & R.H. Wasserman, eds). Academic Press, New York, pp. 101-131.
	regulation of fertilization in sea urchin	Castaneda, M.A. & A. Tyler (1968) Biochem. Biophys. Res. Commun. 33 783-787.
	regulation of mitosis and required for cell division in higher and lower plants	Sorokin, H. & A.L. Sommer (1940) Amer. J. Bot. 27 308-318 and Hewitt, E.J. (1963) in Plant Physiology (F.C. Steward, ed.), Academic Press, New York
	transformation of small lymophocytes	Whitney, R.B. & R.M. Sutherland (1972) J. Cell. Physiol. 80 329-338.
Hormone action	release of insulin	Grodsky, G.M. (1972) Diabetics 21 (Suppl. 2) 584-
	release of thyroid stimulating hormone	Vale, W., R. Burgus & R. Guillemin (1967) Experientia 23 853-855.
	release of lutenizing hormone	Samli, M.H. & I. Geschwind (1968) Endocrinology 82 225-231.
	release of vasopressen	Douglas, W.W. & A.M. Poisner (1964) J. Physiol. 172 19-30.
	melanocyte-stimulating hormone	Dikstein, S., C.P. Weller & F.G. Sulman (1963) Nature 200 1106).

* located in mammalian tissue unless otherwise stated.

Table V. Continued.

GENERAL FUNCTION	SPECIFIC FUNCTION	LITERATURE REFERENCE
	release of growth hormone	Schofield, J.G. (1967) Nature 215 1385-1386.
	release of oxytocin	Dicker, S.E. (1966) J. Physiol. 185 429-444.
	release of thyroxine	Zor, V., I.P. Lowe, G. Bloom & J.B. Field (1968) Biochem. Biophys. Res. Commun. 33 649-658.
Enzymes	amylase secretion of the prancrease salivary gland is calcium dependent	Douglas, W. & A.M. Poisner (1962) (Nature) 196 379-381.
	stabilization of deoxyribonuclease against proteolytic attack	Price, P.A., T.-Y. Liu, W.H. Stein & S. Moore (1969) J. Biol. Chem. 244 917-923 and Melgar, F. & D.A. Goldthwait (1968) J. Biol. Chem. 243 4409-4416.
	pancrease phospholipase	Jungalwala, F.B., N. Freinkel & R.M.C. Dawson (1971) Biochem. J. 123 19-33.
	variety of proteolytic enzymes	Gorinin, L. & C. Fromageot (1949) Compt. Rend. 229 559-561.
	stability of α-amylase of B. subtilis and human saliva	Hsiu, J., E.H. Fischer & E.A. Stein (1964) Biochemistry 3 61-66.
	stimulates phosphodiesterase activity	Kakiuchi, S.R. Yamazaki, R., Y. Teshima, Y. & K. Uenishi (1973) Proc. Natl. Acad. Sci. U.S. 70 3526-3530.
	calcium binding protein activates phosphodiesterase activity	Wolff, D.J. & C.O. Brostrom (1974) Arch. Biochem. Biophys. 163 349-358.

Table V. Continued.

GENERAL FUNCTION	SPECIAL FUNCTION	LITERATURE REFERENCE
Nerve transmission	stimulates release of neurotransmitter, norepinephrine	Douglas, W.W. & R.P. Rubin (1961) J. Physiol. 159 40-57.
	stimulates release of neurotransmitter, acetylcholine	Katz, B. & R. Miledi (1965) Proc. Roy. Soc. B. 161 496-503.
	generation of action potential via 'calcium current' in neurons	Bulbring, E. & T. Tomita (1970) in Calcium and Cellular Function (Cuthbert, ed.), St. Martin's A.W. Press, London, pp. 249-
	binding of transmitters to post-synaptic receptors	Takagi, K., I. Takayanagi & C.S. Liao (1972) Eur. J. Pharmacol. 19 330-342
Miscellaneous	assembly of microtubules	Weisenberg, R. (1972) Science 177 1104-1105.
	maximum cell fusion in muscle cells	Bosch van der, J., Chr. Schudt & D. Pette (1973) FEBS Lettr. 32 296-298.
	blood clotting, conversion of prothrombin to thrombin	Esnouf, M.P. & R.G. Macfarlane (1968) Adv. Enzymol. 30 255-315.
	glycogenesis	Gevers, W. & H.A. Krebs (1966) Biochem. J. 98 720-735.
	gluconeogenesis	Kimmich, G.A. & H. Rasmussen (1969) J. Biol. Chem. 244 190-199.

by Sutherland and colleagues involves a chemical stimuli as the primary messenger that interacts with a specific target tissue causing activation of the membrane bound adenyl cyclase which in turn leads to an increased level of cyclic AMP in the cytoplasm. Cyclic AMP acts as a second messenger causing activation of a series of protein kinases involved in activating one or more processes or enzymes.

Protein kinases are common throughout the cell cytosol compartments and can also be membrane associated. Protein kinases are found in a variety of mammalian tissues, such as, liver, brain, and muscle, and resemble each other in kinetic and catalytic properties. The kinases have a variety of regulatory units and apparently, common catalytic units (Yamamura et al, 1973). Membrane bound protein kinases can now be added to this list since these enzymes resemble other soluble protein kinases and carry out the same phosphorylation reactions (Shimomura et al, 1974). Membrane associated cyclic AMP-dependent protein kinases have been found attached to brain microsomal membrane (Weller & Rodnight, 1971), brain synaptosomal membrane (Johnson et al, 1971), liver plasma membrane (Shlatz & Marinetti, 1972), mammary gland membrane (Majumder & Turkington, 1972), pituitary gland membrane (Lemaire et al, 1971), and human erythrocyte membrane (Duffy & Schwartz, 1972; Rubin et al, 1972; Shimomura et al, 1974). These intrinsic cyclic AMP dependent protein kinases transfer ATP terminal phosphate to seryl groups in membrane structure. In erythrocyte ghost membrane, cyclic AMP-stimulated phosphorylation of membrane proteins has been demonstrated by Guthrow et al (1972), Rubin and

Rosen (1973), Roses and Appel (1973), and Shimomura et al (1974). Shimomura and colleagues showed that protein substrate molecules are of multiple species, one intrinsic (65,000 daltons), possibly the major glycoprotein of the red blood cell, and numerous salt and/or EDTA extractable proteins ranging in molecular weight from 12,000 to 30,000 daltons. The cyclic AMP-stimulated protein kinase isolated by salt and/or EDTA extraction of the membrane cannot be stimulated by cyclic AMP in vitro, but this may be due to a requirement for an absent regulatory subunit. The substrate proteins are relatively stable to heat whereas the protein kinases are easily inactivated by heat treatment.

After cell excitation by an external stimulus, the rise in intracellular cyclic AMP is accompanied by a rise of intracellular calcium (see Rasmussen, 1970). The external stimulus produces a rise in cyclic AMP whether calcium is present or not, but the physiological response occurs only in the presence of calcium. The source of calcium may be extracellular or from an intracellular storage source, such as, the mitochondria or the sarcoplasmic reticulum. A model involving coordination of calcium and cyclic AMP as mediators of cell response has been suggested by Rasmussen (1970, 1971). After the extracellular stimulus produce a rise in intracellular level of cyclic AMP and enhances calcium uptake, cyclic AMP causes activation of protein kinases and redistribution of intracellular calcium from the storage centres into the cytosol. Calcium in the cytosol now can activate enzymes (Ozawa & Ebashi, 1967), produce a permeability change in the cell mem-

brane to monovalent cations (Romero & Whittam, 1971), and inhibit the membrane bound adenyl cyclase (Rasmussen & Nagata, 1970). In agreement with this model, Prince and co-workers (1972) showed that both cyclic AMP and calcium are interconnected second messengers of 5-hydroxytryptophan (5-HT) in the fly salivary gland secretion system.

Nerve Transmission

Neurotransmitter release from nerve ending in response to electrical stimulation and neurohormones requires a transient increase in calcium ion level and cyclic AMP within the nerve cell (Shimizu et al, 1970). Calcium is involved in intracellular mediation of neurohormone response probably via the cyclic AMP system (Greengard et al, 1972). One way that calcium can be linked with the cyclic AMP system is through its role in reducing the level of intracellular cyclic AMP by activating cyclic nucleotide phosphodiesterase activity. Ca^{++}-dependent cyclic nucleotide phosphodiesterases have been isolated from pig brain (Wolff & Brostrom, 1974), bovine heart (Teo, Wang & Wang, 1973; Teo & Wang, 1973), and rat cerebral cortex (Kakiuchi et al, 1973). Approximately two-thirds of the brain phosphodiesterase activity is dependent on calcium (Kakiuchi et al, 1973).

A heat stable calcium binding protein isolated from brain tissue by Teo and Wang (1973) and Wolff and Brostrom (1974) activates phosphodiesterase hydrolysis of cyclic AMP. The calcium binding protein has been previously characterized by Wolff and Siegel (1972) and may

be identical to the calcium binding acidic phosphoprotein (see Table II). The heat stable calcium binding protein apparently mediates the calcium dependent phosphodiesterase activity in the brain with a half maximal stimulation of 4×10^{-6} M calcium.

Cell Proliferation

The calcium level in extracellular fluid directly determines the extent of cell proliferation in bone marrow and thymus gland. When parathyroid hormone (PTH), which is the major hormonal regulator of the calcium level in the extracellular fluid of animals, is removed from rats, the cell proliferation in bone marrow and thymus gland is reduced (Perris, 1971). Another example of calcium involvement in initiation of cell division is erythropoiesis. After loss of erythrocytes by hemorrhaging, the hormone erythropoietin stimulates PTH synthesis, which in turn raises the level of extracellular calcium and as a result erythrocytes divide rapidly to restore lost blood cells (Perris et al, 1971). Also, in both higher and lower plants, calcium is required for mitosis and cell division (Sorokin & Sommer, 1940; Hewitt, 1963; Moss, Thomas & O'Kelley, 1971).

At the molecular level, calcium requirement for cell proliferation of normal cells is connected with the level of cyclic AMP in the cell (Whitney & Sutherland, 1973). Calcium acts by regulating the initiation of DNA synthesis, but is not required for continued DNA synthesis. Since the extracellular calcium level in a normal animal

is maintained at a constant level by hormonal control, proliferating cells are more permeable to calcium which allows initiation of DNA synthesis (Balk, 1971). Cyclic AMP is probably the calcium mediator since cyclic AMP stimulates lymphoblast cell proliferation even in the absence of calcium (Whitfield et al, 1971). Magnesium also promotes lymphocyte proliferation, but this action appears to be due to enhancement of cyclic AMP-activated protein kinases involved in DNA synthesis (Morgan & Perris, 1974). How calcium influences the level of cyclic AMP is unknown but may act by inhibiting the phosphodiesterase breakdown of cyclic AMP (Borle, 1972a; Rasmussen, 1971). In agreement, Morgan and Perris (1974) recently demonstrated by the use of the sex hormone, oestradiol, that the mode of calcium and cyclic AMP enhancement of rat thymic lymphocyte proliferation can be separated. Oestradiol inhibits parathyroid hormone and calcium promoted cell proliferation while it has no effect on cyclic AMP or mitogens that stimulate adenyl cyclase activity and promote cell proliferation.

Calcitonin, the natural antagonist of PTH and cell division, inhibits the calcium induced lymphocyte proliferation, although ultimately it exerts its stimulatory action on the cyclic AMP system. Calcitonin may act by preventing access of calcium to cyclic AMP phosphodiesterase. Whitfield and co-workers (1973) suggested a similar model where calcium acts by lowering the membrane bound phosphodiesterase activity thereby raising the membrane cyclic AMP activity which acts at a membrane site where the ultimate mediator of DNA synthesis is located. This may lead to cell proliferation.

Calcium has a second mode of action in cell proliferation. Hormonal stimulation of cyclic AMP production at optimum levels of calcium produces cell proliferation, but if the concentration of calcium is raised, the cyclic AMP level still increases, however the cells do not divide. Consequently, depending on the amount of calcium present, it can influence cyclic AMP to become either an activator or an inhibitor of cell proliferation. Calcium appears to be required for the production of a mitogenic initiator and the high levels of cyclic AMP produced from excess calcium may block cell proliferation by releasing calcium from a mitogenic active site.

In liver cells, 2.5 hours after the start of cell proliferation, cyclic AMP level peaks, then falls to normal level at 8 hours, but by 12 hours cyclic AMP again peaks which is just prior to DNA synthesis. Apparently two peaks of cyclic AMP level are required for the initiation of DNA synthesis in liver cells, kidney cells, and parotide gland (Short et al, 1972). What function calcium has in cell proliferation of these cells is unknown. Calcium and cyclic AMP are also implicated in cell proliferation of normal adrenal cortical cells initiated by adrenocorticotrophic hormone (Gill, 1972).

Calcium is specifically required for transformation of small lymphocytes by phytohemagglutinin (PHA) (Whitney & Sutherland, 1972; 1973). PHA, a mucoprotein of bean Phaseolus vulgaris, is a common mitogenic agent for lymphocyte transformation. Normally small lymphocytes of the thymus do not divide, but if PHA is supplied large immature cells that may divide are produced. Transformed lymphocytes

now go through the cell cycle G_1, S (DNA synthesis), G_2, and M (mitosis) repeatedly. G_1 is the stage prior to DNA synthesis which involves synthesis of RNA and proteins needed for DNA synthesis, and G_2 is the stage between DNA synthesis and mitosis which involves synthesis of metabolites required for mitosis. PHA acts as an external stimulant which increases the cytoplasmic level of cyclic AMP by enhancing adenyl cyclase activity (Rasmussen, 1970), and by increasing the uptake of calcium in lymphocytes (Allwood et al, 1971). Calcium is required during the early inductive phase of transformation and recently Kay (1971) showed that calcium is associated with PHA binding to the membrane since the inhibition of PHA binding by EDTA can be reversed by calcium. As PHA-induced early RNA synthesis is also inhibited by EDTA, Kay (1971) suggested that the inhibition was a direct result of inhibiting PHA binding. However, calcium appears to be involved in other subsequent roles. EGTA (a specific chelator of calcium) could inhibit transformation when added up to 12 hours after the initiation of PHA induced transformation (Whitney & Sutherland, 1972), and only calcium can antagonize this inhibition. Whitney and Sutherland (1972) found that calcium is involved in initiation of low molecular weight RNA synthesis, but they do not rule out the possibility that some earlier step is involved. The synthesis of the low molecular weight RNA may be connected with the postulated ultimate mediator of DNA synthesis, since this is the step or one of the steps calcium is required during cell proliferation.

A fundamental property of transformed cells is that they multiply autonomously with the control mechanisms of normal cells being no longer

effective. Adult chick fibroblasts transformed by Rous sarcoma virus can multiply rapidly in low calcium concentrations, and calcium ions can no longer promote proliferation as can be shown for normal fibroblasts (Balk, 1971; Whitney & Sutherland, 1973).

Of particular interest is the induction of rice cells to form callus. Callus induction in rice in synthetic medium can be mediated by the auxins indole-3-acetic acid, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid (Yamada et al, 1967). During callus induction phosphate uptake is inhibited while calcium uptake is enhanced (Nishi, 1970). Calcium may be required for auxin mediated callus induction.

AUXIN

To understand calcium involvement in growth and differentiation, the function of auxin in growth and the mechanism of growth itself should be reviewed first. The relationship between auxin and plant growth has recently been reviewed by Burstrom (1968), Davies (1973), Street (1966), Ray, 1974, Scott (1972), Evans (1974), and Shantz (1966). Before growth or cell wall extension can occur the plant tissue must be under applied stress or turgor. The reason why stress is required for growth is unknown, but Cleland and Rayle (1972) suggested that in the presence of auxin, loose points in the cell wall break and reform, while only in the presence of turgor will reforming bonds produce extension growth. Cell growth can be separated into four phases. First, extensibility

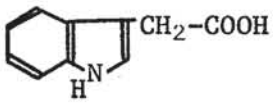
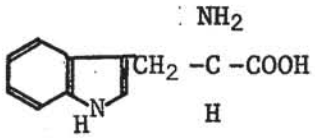
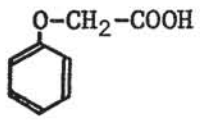
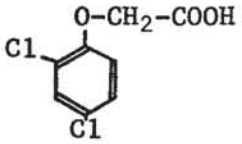
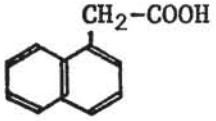
of the cell wall is increased. Second, osmotic uptake of water caused by decrease in cell water potential in the cell as a result of cell extension. Third, stretching of the cell wall. Fourth, the increased cell volume leads to cell wall synthesis.

Chemistry

The auxins are a group of compounds that are involved in cell wall synthesis and elongation of plants. Auxin can be defined as any substance by reason of its chemical characteristics and/or biological activity that resembles indole-3-acetic acid (IAA) (Overbeek et al, 1954). The structure required for an active molecule must obey the following criteria: (i) ring form or easily cyclizable; (ii) at least one double bond in the ring; (iii) a side chain on the ring adjacent to th double bond; (iv) a carboxyl group or potential carboxyl group separated from the double bond by a minimal length; (v) generally one to two carbons on the side chain; and (vi) the correct isomer geometry (see Clement, 1970). Table VI shows the chemical structure of auxin and also lists other auxin-like compounds that have growth promoting ability.

Trytophan is the precursor for IAA synthesis in plant tissue (Erdmann & Schiewer, 1971), and there are four possible pathways for conversion of tryptophan to indole-3-acetic acid, indole pyruvic acid (IPyA), tryptamine (TNH_2), indoleacetaloxine (IAOx), and trytophol (TOL) pathways (Schneider & Wightman, 1974). The indolepyruvic acid pathway is the most widely used pathway in plants. The first step in

TABLE VI. TABLE OF AUXIN COMPOUNDS

NAME	ABBREVIATION	STRUCTURE
indole-3-acetic acid	IAA	
tryptophan		
phenylacetic acid	PAA	
2,4-dichlorophenoxy-acetic acid	2,4-D	
naphthaleneacetic acid	NAA	

the indolepyruvic acid pathway is transamination of tryptophan to produce IPyA. The enzyme involved is nonspecific using tryptophan, tyrosine, phenylalanine, aspartic acid, and glutamic acid as amino-group donors. IPyA is next decarboxylated by IPyA decarboxylase to IAAld followed by conversion to IAA by either a mutase, a dehydrogenase, or an oxidase. The tryptamine pathway is common in some species, but yet unidentified in many common plants such as pea, bean, squash, and cabbage. The formation of tryptamine from tryptophan is catalyzed by tryptophan decarboxylase, and then amino oxidase catalyzes the conversion of tryptamine to IAAld. The final step is the same as for the indolepyruvic acid pathway. IAOx and TOL pathways occur only in a few plant species. Why the utilization of one pathway over another occurs is unknown.

In plants, auxins are usually produced in the apex of the stem and move in a polar direction to the roots. Auxin is isolated by ether or alcoholic extraction of tissues followed by either ether, ethyl acetate, or acid chromatography. After extraction, auxin activity is often associated with the water soluble faction, that is, free unbound auxin (Woodruffe et al, 1970). Indoleacetic acid can be inactivated by being broken down by peroxidase and phenolase, or forming conjugates with macromolecules, inositol, aspartic acid, ammonium, and glucose (see Clement, 1970).

Within the plant, IAA is frequently ester linked but very little is known of the physiological role of IAA esters. The bound

auxins may be either storage forms of IAA, detoxification forms, transport forms, or physiologically active hormonal compounds. Whatever the role of IAA esters, Bandurski and Piskornik (1973) have recently shown that 50% of IAA in mature corn kernels, Zea mays is ester linked. The bound auxin is a high molecular weight lipophilic cellulosic glucan. The only carbohydrate present is glucose which represents 50% of the molecule. IAA represents from 1% to 7% of the glucan, probably one molecule of IAA bound per molecule of glucan. The remainder of the molecule is yet unidentified, but traces of the amino acids aspartic acid, glutamic acid, serine, proline, glycine, alanine, and leucine are present. However, the total nitrogen content cannot be accounted for by these amino acids. Bandurski and Piskornik suggested that polyhydric amines are linked to the glucan by a glycosidic bond. Davies and Galston (1971) also demonstrated IAA-polysaccharide complexes in pea stem segments after incubation in radioactively labelled IAA.

Mechanism of Auxin Biological Activity

A survey of auxin effects is presented in Table VII (Evans, 1974). The majority of auxin effects recorded in Table VII are immediate effects having a latent period less than 15 min where there is no increase in growth rate of plant coleoptiles or stem segments. After the latent period, there is a 3-15 min period when the growth rate increases rapidly to a higher steady state level or in some cases falls to a new steady state level. Since the mediated effects re-

TABLE VII. TABLE OF PRIMARY AUXIN EFFECTS

GENERAL EFFECT	SYSTEM	SPECIFIC EFFECT	LITERATURE REFERENCE
Metabolism	isolated mitochondria	stimulation of respiration	Sarkissian, I.V. (1966) Physiol. Plant. 19 328-334.
	Avena coleoptile	stimulation of CO_2 uptake	Yamaki, T. (1954) Sci. Papers Coll. Gen. Educ. Univ. Tokyo 4 127-154.
	pea stem	decreased ATP/ADP ratio	Trewavas, A.J., I.R. Johnston & E.M. Crook (1967) Biochim. Biophys. Acta 136 301-311.
	pea stem	increased specific activity of nuclear protein	Datta, A. & S.P. Sen (1965) Biochim. Biophys. Acta 107 352-357.
	pea stem	stimulation of glycerophosphate synthesis	Neumann, P.M. (1971) Planta 99 56-62.
	isolated chloroplasts	stimulation of photophosphorylation	Tamas, I.A., B.D. Aktins, S.M. Ware & R.G.S. Bidwell (1972) Can. J. Bot. 50 1523-1527.
Increased enzyme activity level	Avena coleoptile	β-1,3-glucanase	Masuda, Y. & R. Yamamoto (1970) Develop. Growth & Differentiation 11 287-296.
	pea stem	glucan synthetase	Ray, P.M. (1973) Plant Physiol. 51 601-608.
	pea stem	benzoylaspartate synthetase	Venis, M.A. (1964) Nature 202 900-901.
	purified enzyme from caster bean	citrate synthetase	Sarkissian, I.V. (1966) Physiol. Plant. 19 328-334.
Electrophysiology	bean root	change in membrane potential	Jenkinson, I.S. (1962) Australian J. Biol. Sci. 15 101-114.

Table VII. Continued

GENERAL EFFECT	SYSTEM	SPECIFIC EFFECT	LITERATURE REFERENCE
Electro-physiology	mung bean root tip	increased negative surface charge	Tanada, T. (1973) Plant Physiol. 51 150-153.
	barley root tip	decreased negative surface charge	Tanada, T. (1973) Plant Physiol. 51 154-157.
Permea-bility	pea stem	increased permeability to water	Kang, B.G. & S.P. Burg (1971) Proc. Natl. Acad. Sci. U.S. 68 1730-1733.
	Avena coleoptile	increased chloride uptake	Rubinstein, B. & E. N. Light (1973) Planta 110 43-56.
	pea stem	release of xyloglucan from wall	Labavitch, J. & P.M. Ray (1973) Plant Physiol. Suppl. 59
	Helianthus annus hypocotyl	stimulated uptake of glutamic acid	Reinhold, L. & R.G. Powell (1958) J. Exptl. Bot. 9 82-96.
		stimulated uptake of Rb^+ and K^+	Higinbotham, N., M. J. Pratt & R.J. Foster (1962) Plant Physiol. 37 203-214.
Growth and wall extensibility	Avena coleoptile	stimulation of growth	Evans, M.L. & P.M. Ray (1969) J. Gen. Physiol. 53 1-20.
	Avena coleoptile	stimulation of growth at elevated temperature	Nissl, D. & M.S. Zenk (1969) Planta 89 323-341.
	Avena coleoptile	increased wall extensibility	Cleland, R. (1967) Planta 74 197-209.

Table VII. Continued

GENERAL EFFECT	SYSTEM	SPECIFIC EFFECT	LITERATURE REFERENCE
Miscellaneous	tobacco leaf	bursting of isolated protoplasts	Power, J.B. & E.C. Cocking (1970) J. Exptl. Bot. 21 64-70.
	bean leaf pulvinus	increased water uptake	Brauner, L. & N. Arslan (1951) Rev. Fac. Sci. Univ. Istanbul B 16 257-300.

corded are immediate, a correlation between mediated effect and auxin action in growth response may be drawn. Characterization of the latent period of auxin action resulted in controversy as to what was actually occurring. Rayle et al (1970) showed that the latent period of auxin action is concentration independent, while Nissel and Zenk (1969) demonstrated that auxin action during the latent period is IAA concentration dependent. Recently, Zenk was able to reconfirm his results using the material and methods of Rayle's group and showed that indeed auxin effect is concentration dependent. Part of the dependency is apparently due to the time required for IAA uptake (Durand & Zenk, 1972). Within this latent time period of 15 min auxin action in growth response may be involved in decreased ATP/ADP ratio (Trewavas et al, 1967); stimulation of RNA synthesis (Masuda & Kamiska, 1969); nuclear protein synthesis (Datta, 1965); glycerophosphate synthesis (Neumann, 1971); glucose synthetase activity (Ray, 1973); increased permeability to water (Kang & Burg, 1971); wall extensibility (Burstrom et al, 1970); stimulation of protoplasmic streaming (Timann & Sweeney, 1937), and hydrogen ion efflux (Cleland, 1973a, b; Rayle, 1973).

Non-hormonal Auxin-like Factors

To aid in the elucidation of the mechanism of auxin action, nonhormonal factors are very useful. By comparing nonhormonal factors with auxin on growth response during the latent period (primary auxin

effect) in plant tissue a possible mode of action of auxin action can be envisaged. Nonhormonal factors that have a similar growth response to auxin are acid pH, CO_2, victorin, pathotoxins, and heavy water. In all cases, nonhormonal factors have a much reduced latent period as compared to auxin (IAA). Recent reviews and reports concerning non-hormonal factors as producing auxin-like responses in plant tissues can be found by Evans (1973, 1974), Cleland (1973a, b), Rayle and Cleland (1970), Davies (1973), Edwards and Scott (1973), Ilan (1973), Barkley and Leopold (1973), and Marre et al (1974).

In 1934, Bonner first demonstrated that hydrogen ions promoted the growth of Avena coleoptile segment. Hydrogen ion efflux into the cell wall is the most probable mode of auxin action. Acid conditions initiate elongation after a lag of one min, and the acid promoted elongation continues for one hour then gradually declines. The acid and auxin responses are similar in terms of Q_{10}, growth rate effects on wall extensibility, and normal tension for the wall (Rayle & Cleland, 1972). The acid response differs from the auxin response in the following respects: (a) metabolic inhibitors inhibit auxin response but not the acid response; (b) in frozen-thawed segments only acid will induce increased cell wall extensibility; and (c) in vivo acid response is shortlived (Rayle & Cleland, 1972).

In 1965, Harrison first showed that CO_2 induced cell elongation. Elongation of coleoptile segments is strongly promoted by CO_2 with a latent period of 1 min or less (Evans et al, 1971). No CO_2 response can be obtained at neutral pH, but at pH 3 to 4 growth is promoted in

1 min, indicating that a major part of the action of CO_2 depends on its ability to reduce the pH. Rayle and Cleland (1972) observed that CO_2 at pH 3.8 produces an additive effect on elongation of Avena coleoptile in relation to acidic conditions (pH 3.8). When the pH is raised to pH 5.9, CO_2 produces a growth effect but none can be shown in the absence of CO_2 at this pH. After terminating the response to pH by buffering the growth medium (pH 7.0) only a second response to CO_2 could be obtained, not to acid pH. Similarly Barkley and Leopold (1973), using green pea stem tissue, suggested that the action of CO_2 can be separated from acid pH. If the epidermis is removed to allow penetration of hydrogen ions no growth response could by recorded for acid pH, while CO_2 did produce a growth response. Evans (1974) has questioned the validity of Rayle and Cleland (1972) and Barkley and Leopold's (1973) experimental protocal, since Avena coleoptile are normally quite impermeable to hydrogen ions which would allow CO_2 to penetrate the cuticle and epidermis more readily than H^+. A second growth response to acid conditions can be shown if the end of the tissue segment is cut, and green tissue is usually acid-insensitive with a small CO_2 response as compared to acid-sensitive tissue. Consequently, the CO_2 growth response is largely due to its acidifying effect as earlier found by Evans et al (1971), but there may be a smaller direct effect of CO_2 on growth depending on plant tissue.

Other compounds that have auxin-like action are fusicoccin and victorin. Fusicoccin is a fungal toxin produced by Fusicoccum amygdali Del. that can stimulate water uptake and cell elongation in etiolated pea stem segments (Lado et al, 1973). Growth promotion by fusicoccin is accompanied by a decrease in pH in the surrounding medium from pH 6.4 to pH 4.9 during a four hour period, while auxin only produces a

pH drop of one pH unit. The fusicoccin mode of action is similar, but more extensive than auxin producing a greater increase in elongation and hydrogen ion extrusion. The pathotoxin, victorin, stimulates elongation to sensitive coleoptile segments of Avena (Evans, 1973). The growth response is rapid occurring in 3 min but it is short lived, and the magnitude of growth stimulation is only one half that produced by auxin. Victorin may act by increasing membrane permeability (Samaddar & Scheffer, 1971), but Saftner and Evans were unable to show that victorin produces any significant leakage of hydrogen ions from the sensitive peeled segments of Avena coleoptile (see Evans, 1974).

If coleoptile segments are transferred from water to heavy water (D_2O) for a short period of time, then transferred back to water, an increase in elongation rate occurs that is comparable to the increase produced by auxin (Evans et al, 1971). The mode of action of heavy water enhancement of elongation is unknown, but heavy water may act on the xyloglucan-cellulose fibril H-bond linkages by tightening the binding. When the coleoptiles are returned to water, wall loosening occurs allowing cell elongation (Evans, 1974).

Auxin stimulated H^+-pump

Since the similarities between auxin and acid growth response are considerable, Hager et al (1971) suggested that a primary mode of auxin action involves a membrane bound ATPase hydrogen ion pump. They suggest that auxin functions as an effector of the hydrogen pump, promoting the extrusion of hydrogen ions from the cell cytoplasm into

the cell wall. The hydrogen ions in the cell wall would act as secondary messengers causing a break in the H-bonds joining the xyloglucan polymers to the cellulose microfibrils. When the cell wall is under tension the xyloglucan can now 'creep' along the microfibrils allowing extension of the cell wall. In the absence of auxin the membrane bound ATPase would be inactive.

Hertel et al (1972) found auxin-specific receptor sites in the particulate fraction of corn coleoptiles. Auxin binding reaches an equilibrium condition in 15 min and the apparent K_m value correlates to physiologically effective concentrations of auxin. Hertel's group suggested that the auxin specific receptor may be analogous to the membrane bound ATPase hydrogen ion pump earlier described by Hager et al (1971). The ATPase activity found in plant cell membranes by Leonard et al (1973) and the possible stimulation of this membrane bound ATPase by cations (Leonard & Hanson, 1972; Fisher et al, 1970) may be part of the auxin stimulated H^+ extrusion pump.

Experimental data generally complies with Hager's model. Auxin stimulation of H^+ efflux is specific (Rayle & Johnson, 1973), and the stimulation is inhibited by antagonists of auxin growth, such as abscisic acid, KCN, and cycloheximide (Cleland, 1973a, b; Rayle & Johnson, 1973). Using pea internode segments and isolated squash, Marre's group supported Hager's model by demonstrating that the extrusion of protons is accompanied by a significant increase in the negative transmembrane potential (Marre et al, 1974).

However, in some cell types substantial H^+ efflux can be observed in the absence of auxin (Evans, 1973). It appears that the

induction and the maintenance of auxin induced hydrogen ion leakage is itself dependent on pH (Cleland, 1973b). A feedback mechanism may be operating whenever the pH drops. It is known that in peeled Avena coleoptile segments, when the pH drops to 4.6, auxin-stimulated hydrogen ion efflux ceases.

In some cases the auxin induced proton extrusion does not correlate with auxin induced growth response. H^+ extrusion in Helianthus hypocotyl segments requires K^+ and takes 2 hours to detect, whereas auxin growth response occurs in 10 min (Uhrstrom, 1969) and shows no dependence on K^+. Also, Cleland (see Evans, 1974) was unable to detect auxin induced hydrogen ion efflux in either sunflower hypocotyl or pea epicotyl segments. Green pea stems respond normally to auxin, but show no acid response (Barkley & Leopold, 1973). Since there are a number of differences between green tissue and etiolated tissue, another mode of auxin action may occur in green tissue. In green tissue which readily responds to CO_2, it is likely that auxin and CO_2 are linked (Pope & Black, 1972) with acid pH playing a secondary role (Barkley & Leopold, 1973).

Ilan (1973) argued that lowering of the pH was not involved in auxin primary action since the pH of the surrounding medium of sunflower hypocotyl segments remains constant at pH 6.8, while auxin produces a substantial increase in dry weight. Also Durand and Zenk (1972) had demonstrated that during auxin primary action the surrounding medium for the coleoptiles is constant at pH 6.3. Although the pH in the surrounding medium was recorded not to have changed, this does not

indicate that the pH of the cell wall milieu has not changed. Until precise measurements of the pH of the cell wall can be made, pH drop in the cell wall without a pH drop in the surrounding medium is still feasible.

If auxin is pH-mediated, auxin should have no effect on stored growth at acid pH. Stored growth is growth caused by transferring cells first to a growth inhibiting solution of mannitol, followed by a transfer to normal growth conditions (absence of mannitol). Cleland and Rayle (1972) did not observe any effect of auxin on stored growth at acid pH as was expected if auxin action is pH-mediated. However, other research workers have demonstrated that stored growth is affected by auxin (Green & Cummins, 1971; Kobayashi et al, 1956).

It is unlikely that protein synthesis is involved in the initial response to auxin (Durand & Zenk, 1972; Addink & Meijer, 1972; Evans & Ray, 1969), but it is generally accepted that the sustained growth response to auxin is dependent on protein synthesis (Davies, 1973). Since the latent period can be reduced essentially to zero, the involvement of protein synthesis in such a short process is unrealistic. Nissel and Zenk (1969) reduced the latent period to zero by raising the temperature from 21° to 40° C for Avena coleoptile segments responding to 5 mM IAA, and Barkley and Leopold (1973) reduced the latent period to 1 min in green pea stem segments by reducing the pH of the growth medium from pH 6.3 to pH 3.0 at the same time that auxin was being added.

The question whether protein synthesis is directly involved in auxin action under normal physiological conditions has not yet been completely settled. Cleland (1970; 1973a) suggested that protein synthesis is required for growth in the presence of auxin, and that the specific protein required for hydrogen ion extrusion is unstable and must be continually resynthesized, since KCN, CCCP, or cycloheximide can block immediate auxin induced hydrogen ion excretion. The rapidly turning over protein may be the unidentified growth limiting protein which was earlier postulated by Cleland (1970). In agreement Marre and co-workers (1974) suggested that the auxin activated H^+ pump proposed by Hager is itself rapidly turning over, and protein synthesis is required to sustain it because CCCP (phosphorylation uncoupler) and two completely different protein inhibitors, cycloheximide and puromycin, inhibit auxin-induced growth that occurs within a few minutes. A recent report by Penny et al (1972) also came to the same conclusion as Marre's group, because when lupen hypocotyls were preincubated in cycloheximide, auxin action was reduced.

RNA synthesis may also be involved in auxin growth response (Key, 1969; Key & Ingle, 1968), even though initial auxin action is apparently at the cell surface. Hardin and co-workers (1972) suggested that these apparently opposing views are not necessarily mutually exclusive. Auxin may have a common base interaction with the cell membrane. Auxin stimulated H^+ pump could promote transcription of messenger RNA in some unknown manner by enhancing the RNA polymerase activity. The authors presented no direct evidence to support this hypothesis, but it still stands as a possible means of dispelling conflicting evidence.

In summary, evidence does support the hypothesis that auxin stimulates elongation and growth in some plant tissues by a mechanism involving hydrogen ion influx into the cell wall. Although an auxin-activated, possibly cation-dependent ATPase that is membrane-bound, hydrogen ion extrusion pump that may be rapidly turning over has been postulated, little is known what protein molecules are involved, how they are associated with each other, and in what fashion possible allosteric effectors interact with the postulated ATPase?

Cell Wall Growth

Structure: Before the interaction of plant hormones and other metabolites on cell growth can be investigated a description of the cell wall chemistry, metabolism, and growth should be discussed. Recent reviews on this subject can be found by Lamport (1970), Northcote (1972), and Cleland (1971). The basic composition of the primary cell wall of higher plants closely resembles the cell wall structure of Phycomycetes. The cell wall of suspension cultured sycamore is one of the best examples where the cell wall structure has been described in detail (Keegstra et al, 1973; Lamport, 1970). Albersheim's group isolated different fractions of the sycamore cell wall, and chemically and physically characterized the different components. After conducting a thorough study and by fitting the isolated fractions back together in the most probable way, a tentative model of cell wall structure was postulated by Albersheim. Xyloglucan which has been shown to bind tightly to purified cellulose as well as to cell wall

(Aspinall et al, 1969; Bauer et al, 1973) would be in juxtaposition with the cellulose elementary fibril. The reducing ends of the xyloglucans are proposed to be attached to the 4-linked galactan side chains of the rhamnogalacturonan. The rhamnogalacturon polymer is the main chain of the pectic polysaccharide. The pectic polysaccharides are linked in some manner to the hydroxyproline-rich protein while the proposed connection between 3, 6-linked arabinogalactan and the serine residue of the protein had yet to be shown conclusively by Albersheim's group. Recently Lamport (1973) showed that the major polysaccharide (arabinogalactan) is attached to hydroxyproline-rich protein via the serine hydroxyl group. The components of the wall are connected by covalent bonds except xyloglucan and cellulose which are connected by numerous H-bonds that make the connection as strong as covalent bonding. Albersheim proposed that the protein-glycan backbone of plant cell wall is analogous to the peptide-glycan network found in bacterial cell walls (Keegstra et al, 1973). In summing up, the primary structure of sycamore cell wall, the components are tightly interconnected with the protein-glycan backbone covered by cellulose elementary fibril network.

Lamport's model of the primary cell wall structure closely resembles that of Albersheim's group (Lamport, 1970; 1973). The involvement of the protein called extensin in the cell wall structure in Lamport's model has received considerable scrutiny. The structure of extensin is ---ser(gal)-(hyp)$_4$-ser(gal)-lys---- with hydroxyproline being O-substituted by a tetraarabinose. The tetraarabinose sidechains

possibly act as stabilizers for the hydroxyproline backbone. Disulfide bonds are important between cross linkages of wall proteins (Lamport, 1965). Lamport also suggested that the extensin complex is analogous with the peptidoglycan network of bacteria (Strominger et al, 1967). As an additional note of interest, Ginzburg (1961) showed that calcium is involved in cross-linkages in cell wall protein glycan.

The structure and possible mode of synthesis of the cell wall of the unicellular algae, Polyphysa (A.) cliftonii has been recently investigated by Göke and co-workers (1974). The amino acid composition of cell polypeptide is rich in glycine, valine, glutamic acid, and especially alanine and aspartic acid, other minor amino acids are also present; arginine, phenylalanine, tyrosine, leucine, and isoleucine, and traces of methionine, proline and hydroxyproline. Göke suggested that this polypeptide is similar to 'extensin' as proposed by Lamport (1970). As only a small amount of hydroxyproline could be demonstrated, it is possible that the backbone of the extensin-like cell wall polypeptide of Acetabularia may have a different amino acid composition. The amino acid composition of the cell wall varies quantitatively in stalk and cap, which indicates a possible role in cell wall synthesis during differentiation. Also, cell wall polypeptide can alter in amino acid composition during growth in other plants (Robinson & Preston, 1972; Nickerson, 1963).

The primary cell wall has its own complement of enzymes and possibly ribonucleic acids (Phethean et al, 1968). The enzymes are involved in transfer, synthesis and hydrolysis of cell wall macro-

molecules, modification of extracellular metabolites to facilitate their transport across the cell membrane, and defence mechanisms. The majority of cell wall enzymes are glycosidic hydrolases, but in intact corn roots there is also, cellobiose, pyrophosphatase, ATPase, RNase, and DNase activity, but no protease activity is present at the cell surface (Chang & Bandurski, 1964). An interesting cell surface enzyme is peroxidase which is located in the cell wall of Japanese radish. It is rich in hydroxyproline (Shannon et al, 1966), and upon alkaline hydrolysis releases hydroxyproline-O-arabinosides similar to those found in extensin.

Although the basic structure of the fungal cell wall resembles the primary cell wall structure of higher plants, the composition varies as is characteristic of all species whether of higher or lower plant origin. Fungal cell wall structure and morphology have recently been reviewed by Bartnicki-Garcia (1968), Taylor and Cameron (1973), Aronson (1965), and LéJohn (1974). The cell is composed of 80 to 90% polysaccharide, and the remainder is protein, lipid, and possibly nucleic acids.

Oömycete cell walls are comprised of β-D-glucan with cellulose present but the main polymer is a non-cellulose β-(1→ 3)-glucan with branches at the C-6 of the glucose unit (Bartnicki-Garcia & Lippman, 1967). Also the main storage carbohydrate in the vegetative mycelium of all Oömycetes is β-(1→ 3)-glucan with C-6 branches (Zevenhuizen & Bartnicki-Garcia, 1970; Faro, 1972). This neutral storage carbohydrate is used for cell wall synthesis during starvation and differentiation

(encystment). The neutral carbohydrate is present in sporangia, zoospore, and cysts of Phytophora palmivora but the majority of carbohydrates are phosphoglucans (Wang & Bartnicki-Garcia, 1973). The glucan portion of the phosphoglucan is identical to the neutral glucan and there are five possible phosphoglucans (PI through to PV) varying in degree with stage of vegetation cycle. PI and PII predominate in sporangia but PIII and PIV are the major phosphoglucans during zoospore stage. During encystment, both neutral and phosphoglucans are used for cell wall synthesis. Phosphoglucans must be completely utilized since none occur in the vegetative mycelium. Only traces of mannose, glucosamine, galactosamine, and ribose are found in the cell wall (Bartnicki-Garcia, 1966). It is a general characteristic of the Oömycete cell wall that it contains little or no chitin, but recently Lin & Aronson (1940) have shown by X-ray analysis that Apodachlya does have chitin as a major consituent of its cell wall, and Dietrich (1973) showed that chitin may be present in four species: Achlya, Dictyuchus, Saprolegnia, and Pythium.

Bartnicki-Garcia and Nickerson (1962) established that protein (3-5%) is an integral part of the fungal cell wall. In Phytophthora cinnamomi, the cell wall contains high amounts of glutamic acid, aspartic acid, serine, threonine, and alanine which represents 55% of the amino acid composition; moderate amounts of proline, hydroxyproline, valine, lysine, arginine, and leucine; and trace amounts of tyrosine, phenylalanine, isoleucine, histidine, cysteine, and methionine (Bartnicki-Garcia, 1966). Part of the cell wall protein

is enzymatic. Achlya cell wall contains the enzyme cellulase which is involved in morphogenesis (Thomas & Mullins, 1967). Cellulase appears to be located in the cell wall because two hours after induction to branch by antheridiol, a sterol hormone, the enzyme is released into the medium. In the growing mycelium of Pythium, Mitchell and Sabar (1966) found a correlation between growth and autolytic activities of glucanase and protease, since the growth antagonist, coumarin was found to inhibit growth by a direct interference with the hydrolytic enzymes (cellulases). Dietrich and Valio (1973) suggested that cellulases are predominant at the growing tip of the mycelial cell wall and are involved in growth. The presence of hydroxyproline is a distinguishing feature of Oömycete cell walls (Crook & Jonston, 1962) since it is not found in the chitinous cell walls of fungi. Although, Chataway et al (1968) showed that the fungi, Candida utilis and Trememlla mesenterica also have hydroxyproline in their cell walls.

The lipid fraction is mainly composed of saturated fatty acids which may confer structural stiffness and hydrophobic properties to the fungi. Nucleotides and nucleo-bases, can be detected in significant quantities in some fungi, for example in the filamentous cell walls of Mucor rouxii (Bartnicki-Garcia & Nickerson, 1962). The function of the nucleotides or the nucleo-bases in the cell wall is unknown, but they may be involved in cell wall synthesis.

Synthesis: The majority of enzymes responsible for cell wall polysaccharide synthesis are found in the cytoplasm. Nucleotide sugars are the precursors of cell wall polysaccharides (Hassid, 1967). In general, biosynthesis of plant cell wall resembles

bacterial cell wall synthesis (Strominger et al, 1967) and occurs by polymerization of repeating units. All reactions for bacterial cell wall synthesis occur inside the cell except transpeptidation, which joins the repeating units of peptidoglycan. Lipid intermediates that are recovered from the plasma membrane may represent a transport mechanism for the transport of prefabricated units of the cell wall across the membrane to the cell wall. The location of synthesis and incorporation of the hydroxyproline-rich glycopeptides into the plant cell wall was characterized by Chrispeel's group. The first steps in the synthesis of the hydroxyproline-rich glycopeptide occur in the cytoplasm including protein synthesis and enzyme modification steps (Sadava & Chrispeels, 1973). The hydroxyl group of the hydroxyproline-rich glycopeptide is hydroxylated by a soluble peptidyl proline hydroxylase. The carrot root phloem parenchyma cells synthesize in the cytoplasm and secrete to the cell wall via the smooth endoplasmic reticulum and the Golgi apparatus, the hydroxyproline-rich glycoproteins (Chrispeels, 1970).

In plants, oligopeptide units in connection with membrane bound lipids are already under study as possible intermediates in cell wall synthesis (Villemez, 1970; Kauss, 1969; Van der Woude, 1973). Kauss isolated a mannosyl lipid and suggested that the lipophilic carrier of mannose is isoprenoid and by analogy with bacterial systems is straight chain rather than a sterol. Van der Woude (1973) using the cytochemical stain chromic phosphotungstic acid to stain plant plasma membrane, suggested that this reagent stains glycolipids which serve as intermediates in the synthesis of cell wall polysaccharides.

Auxin: As was previously discussed under auxin stimulated H^+-pump section, auxin action during growth may occur at the xyloglucan-cellulose H-bond linkage. Further evidence was presented by Labavitch and Ray (1973), who showed that IAA promotes wall turnover only on pectinase-extractable xylose and glucose, and the auxin stimulated xyloglucan turnover is as rapid as auxin stimulated cell extension.

However, other connections may be points of breakage during extension growth since the cell wall contains several polymer connections. It is possible that the connection between pectic polysaccharide and the hydroxyproline-rich glycoprotein may be hydrolyzed by cell wall degradative enzymes, producing cell wall loosening, see Table VII. Again auxin action involving extrusion of hydrogen ions into the cell wall could be important in hydrolysis mediated by acidic pH dependent hydrolases or activation of hydrolases by hydrogen ions. Most plant polysaccharides have an acid pH optimum. For example Johnson et al (1973) have shown that β-galactosidase in Avena coleoptile cell wall has an optimum of pH 4 to pH 5 and has a 3-10 fold auxin-stimulated increase in activity at pH 7, but that it takes 30 to 60 min for auxin to stimulate the enzyme, and the extent of stimulation of enzyme does not correlate with auxin growth responses. Another enzyme stimulated by auxin is β-1, 3-glucanase (Masuda & Yamimoto, 1970). But doubt has been cast on the role β-1,3-glucanase might play in cell growth since it is a stable enzyme and a short-lived auxin product would seem more appropriate. If auxin is removed growth rate decreases immediately, consequently a stable enzyme such as β-1,3-glucanase would seem an

unlikely candidate for auxin regulation in growth. As added support, Dela Fuente and Leopold (1970) showed that auxin has no direct enhancement on the purified β-1,3-glucanase in vitro. A number of other reports argue against the involvement of polysaccharidases in auxin action. Cleland (1968) showed that auxin-induced wall loosening is readily reversible in the presence of metabolic inhibitors while polysaccharidase activities are essentially irreversible in the presence of metabolic inhibitors (Hassid, 1967), and a number of enzyme inhibitors have little effect on acid-induced growth (Rayle & Cleland, 1972). Sulfhydryl inhibitors that interfere with enzyme activity have little effect on acid-induced growth (see Evans, 1974).

Another possible breakage point could be the galactosyl-serine linkage of the glycoprotein (Lamport, 1973). Heath and Northcote (1971) had shown that hydrazinolysis does not degrade cell wall protein, but Lamport (1973) found that cell walls treated with hydrazine lost significant amounts of serine. Usually serine in non-glycosylated proteins, in the presence or absence of carbohydrate is stable to hydrazinolysis. Lamport suggested that serine in extensin represents a special case where serine can be broken down to permit cell wall growth. The hydrazine-stable serine decreases with plant culture age, that is, increased glycosylated serine. The effect of auxin in this assay has not yet been tested. Also, in Lamport's model of cell wall structure, disulfide bonds between wall proteins are important in the assembly of components and may be the growing points. Auxin could act by increasing the level of NADPH (reduced state of the membrane) and

consequently stimulate the reduction of sulfhydryl bonds of the cell wall protein to permit cell wall elongation. In addition, IAA would increase the level of ATP (Lamport, 1965). The exact nature of the actual growing point in the cell wall is still unknown, although several models have been postulated. But, in general, the requirement of a raised hydrogen ion concentration in the cell wall induced by auxin or analogous non-hormonal factor must occur before there can be cell wall extension.

Calcium: Classically, the effects of auxin and calcium on cell wall growth are opposite. Calcium ions at high concentrations inhibit the effect of auxin on elongation and cell wall extensibility. In 1965, Bennet-Clark proposed the Ca^{++}-bridge theory. The rigidity of the cell wall depends upon cross linked pectin molecules with calcium binding the pectin molecules together through the carboxyl groups. Auxin would remove the calcium thereby loosening the cell wall. Wall pressure would subsequently decrease due to decreased water potential and the cell would elongate. This passive stretching would lead to irreversible growth. Recent experimental data do not support this hypothesis. Calcium appears to have little effect on initial growth responses to auxin, and the converse is also true. Auxin has little effect on cell calcium content (Uhrstrom, 1969). Complexing of calcium with auxin is not chemically acceptable (Masuda, 1959; Cohen et al, 1958). Masuda (1959) suggested that IAA might be bound to RNA forming a IAA-RNA-Ca^{++} complex, and thereby remove calcium from the cell wall. But no evidence for such a complex has been found. Since auxin appears to have a direct effect on

the induction of cell wall elongation, Masuda's proposal seems unlikely as calcium would have to play the major role in cell wall extension with auxin only playing a passive role.

Calcium's role in cell wall synthesis is likely secondary to the action of auxin. Uhrstrom (1969) suggested that calcium present in the cell wall gives the cell rigidity. If cells are deficient in calcium, they cannot elongate because the microfibrils in the cell wall no longer have the support required for extension growth. When calcium and auxin action on extension growth are viewed together, auxin action involves a dissolution or sliding of some structural components in the cell wall which is followed by a subsequent synthesis of new cell structure with calcium involved in organization of the new cell wall.

Calcium may also be involved in cell wall growth at a level other than organization of synthesized wall material. The regulation of growth by calcium could be by the maintenance of the correct structural conformation of the proposed H^{+} pump of Hager et al (1971) (refer to previous section of Historical) or like the persumptive auxin effect proposed by Albersheim and co-workers (1973), by being an allosteric effector of the hydrogen ion pump.

Calcium is also required for the optimum uptake of auxin, but this may be due to general membrane structural function of calcium, since in the absence of calcium, intensity of auxin transport is lowered while velocity remains unchanged (Epstein, 1972). In addition, EDTA inhibited auxin transport in the sunflower stem can be reversed by calcium ions, and also Mg^{++} and La^{+++} to a lesser extent (Dela Fuente & Leopold, 1973).

Auxin in the Fungi

Since the identification and characterization of auxin action in higher plants, auxins have been investigated in relationship to fungi, although only a few reviews can be found by Gruen (1959), Janke (1939), and Went and Thimann, (1937). Nielsen (1928) first reported the presence of a substance excreted from Rhizopus suinus and Absidia ramosa that promotes the growth of Avena coleoptiles. Studies prior to 1959, demonstrated that auxin was produced in 90% of the fungal species tested (Gruen, 1959). The presence of tryptophan in the medium is generally required to obtain appreciable levels of auxin production by fungi, but in some cases tryptophan is not required, and in other fungal species tyrosine can readily substitute for tryptophan. Table VIII lists the Phycomycetes that have been examined for auxin production. The Avena curvation test was used to detect the presence of auxin for all the fungi listed in Table VIII although the test is not specific for indole-3-acetic acid.

Negative findings on auxin production must be viewed with caution if based on a single test because it has been shown that auxin production is dependent on the stage of the growth cycle of the fungal species (Hirata, 1957). Also, the auxin released in the medium often varies with time, aeration and temperature (see Gruen, 1959). In general, acid pH produces the highest yields of auxin in the medium (Gruen, 1959). Production of auxin cannot be correlated with growth and varies depending on the species.

TABLE VIII. AUXIN PRODUCTION BY PHYCOMYCETES

ORGANISM	AUXIN	LITERATURE REFERENCE
Blastocladiales		
Allomyces arbuscula	+	Machlis, L. & E. Ossia (1953) Amer. J. Bot. 40 465-468.
Peronosporales		
Pythium mamillatum	+	Ronsdorf, L. (1935) Arch. Mikrobiol. 6 309-325.
P. intermedium	+	
P. splendens	--	
P. debaryanum	--	
Mucorales		
Absidia ramosa	+	Nielson, N. (1928) Planta 6 376-378.
Phycomyces nitens	+(IAA)*	Heyn, A.N.J. (1935) Koninkl. Ned. Akad. Wetenschap. Proc. 38 1074-1081.
P. blakesleeanus	+(IAA)	Kögl, F. & B. Verkaaik (1944) Z. Physiol. Chem., Hoppe-Seyler's 280 162-166.
Rhizopus suinus	+	Nielson, N. (1928) Planta 6 376-378.
R. suinus	+(IAA)	Thimann, K.V. (1935) J. Biol. Chem. 109 279-291.
R. delemar, R. reflexus R. tritici	+	Kögl, F. & A.J. Haagen-Smit (1931) Koninkl. Ned. Akad. Wetenschap. Proc. 34 1411-1416. Kögl, F., A.J. Haagen-Smit, & H. Erxleben (1933) Z. Physiol. Chem., Hoppe-Seyler's 214 241-261.
R. nigricans	+(IAA)	Nielson, N. (1928) Planta 6 376-378.

* auxin extracted, purified, and crystallized to indole-3-acetic acid

In phytopathogenic fungi, it is of interest to investigate auxin relation to pathological symptoms in higher plants, but only a beginning has been made in elucidating the causes of hyperauxiny. Nothing is known regarding the role of auxin in fungal diseases. Although in crown gall a lot is known of auxin involvement (Braun, 1954), nevertheless, other hormonal factors play vital roles as well.

Auxin action on Phycomycete growth is negligible or has no effect. NAA either has no effect or inhibits growth at high concentrations in Achlya sexualis and Saprolegnia ferax, and similar results were found for the following with IAA: Achlya dubia (Murdia, 1939), Achlya sp., Isoachlya anisopora var. indica, Saprolegnia monica, and Brevilegnia gracilis (Bhargava, 1946). Although in the following fungi: Pyronema (Kerl, 1937), Psalliota (Fraser, 1953), and Neurospora (Nysterakis, 1954), IAA promotes growth if the fungi are grown in a basal medium.

Although auxin production among fungi is widespread, no role for growth regulation by auxin has been shown. However, hormones do play an important role in growth (branching) and sexual reproduction of fungi (Barksdale, 1969). If a possible correlation between action of auxin on fungi and their hormonal regulation could be drawn, it would be possible to make a comparison with auxin regulation in plants. Fungal cell walls are similar in structure to higher plant primary cell walls, and unlike animal cells are able to differentiate and dedifferentiate quickly.

In the yeast, Saccharomyces cervisiae, a link between auxin involvement in higher plants and auxin relationship to hormone action in yeast has been suggested by Yanagishima and Shimoda (1973). Yeast protoplasts are insensitive to auxin, while in the intact yeast cell auxin induces expansion (Yanagishima & Shimoda, 1968). Similar to higher plants, RNA and protein metabolism is involved in yeast sustained auxin response. Shimoda and Yanagishima (1969) showed that both β-1, 3-glucanase and special RNAs are important in the auxin response. Mutant yeast cells that do not respond to auxin also do not respond to β-1,3-glucanase induction of growth.

Sex specific receptors are located on the yeast cell surface (Yanagishima & Shimoda, 1973; Crandall & Brock, 1968). Auxin and sex hormones are closely related both genetically and physiologically, since sex hormones in fungi can increase cell wall hydrolase activity in much the same way as auxin does in yeast. Also animal sex hormones and auxin have a similar action on yeast cells and pea plants. Consequently, there may be an evolutionary relationship between animal, plant, and fungi hormones in promoting growth.

In Achlya ambisexualis, a fungal hormone increases the cellulase activity upon induction of the antheridial branch (Thomas & Mullins, 1967). Recently, Mullins showed that the steroid hormone (antheridiol) induction of antheridial hyphae in male strains of Achlya required the concomitant synthesis of RNA (Kane et al, 1973). The increase in cellulase activity is completely inhibited by pre-incubation in cycloheximide (1 μg/ml) for 1 hour. These authors concluded that both trans-

lation and transcription are required for cellulase hormonal induction. Also vegetative branching with a simultaneous rise in cellulase activity can be produced by casein hydrolysate and an amino acid mixture which again suggests that protein synthesis may be involved in promoting branching in rich growth medium.

CYTOKININS

In plants, not only calcium and auxin are required for normal cell growth and differentiation, but an additional substance was found to be required which promotes cell division (Jablonski & Skoog, 1964). The substance is present in liquid endospern of coconut, germinating seedlings, and young fruit, such as apple or pear containing cells that are dividing vigorously, and autoclaved DNA. Kinetin was the first of such a growth substance to be isolated and characterized. A number of these compounds, cytokinins (cytokinesis) have been isolated from biological tissue or chemically synthesized in plant tissue. A detailed discussion of the chemistry and function of cytokinins can be found in Miller (1961), Srivastava (1967), Letham (1967, 1969), Helgeson (1968), Fox (1969), Skoog and Armstrong (1970), Kende (1971), Hall (1973), and Stevenson (1974).

Chemistry

N^6-substituted adenines are the most effective compounds for producing cytokinin biological activity (Skoog & Leonard, 1967; Skoog & Armstrong, 1970). The chemical structures of a variety of cytokinins, natural and synthetic are listed in Table IX. Any changes in the adenine

TABLE IX. CYTOKININ COMPOUNDS

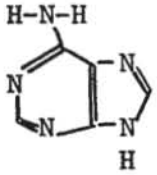
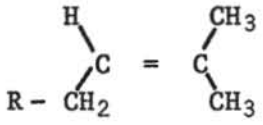
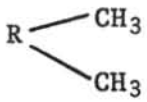
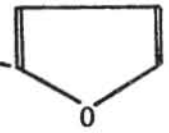
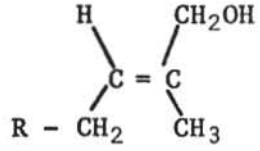
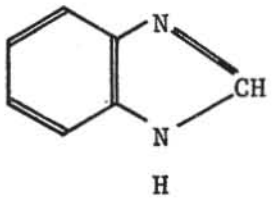
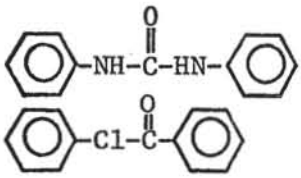
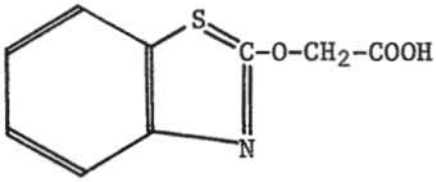
NAME	ABBREV.	STRUCTURE
adenine		
6-amino-3-dimethylallyl purine (triacanthine)		(^{3}N-isomer of isopentenyladenine)
6-benzylaminopurine (benzyladenine)	BAP	R - CH_2 - 
6-benzylmercaptopurine	BMP	(S-atom replaces 6-amino group)
6-butoxypurine	BxP	R - O-$CH_2CH_2CH_2CH_3$
6-butylaminopurine		R - $CH_2CH_2CH_2CH_3$
6-(γ,γ-dimethylallyl)aminopurine or N^6-(Δ^2-isopentenyl)adenine	i^6ade, IPA	
6-(γ,γ-dimethylallyl)aminopurine, or N^6-(Δ^2-isopentenyl)adenine	i^6Ado, IPAdo	
6,6-dimethylaminopurine	DMAP	
6-heptylmercaptopurine	HpMP	
6-hexylaminopurine	HAP	R - $(CH_2)_5$ -CH_3
6-hexylmercaptopurine	HMP	
6-histaminopurine	HiP, HisAP	R - $C_3H_3N_2CH_2CH_2NH_2$
6-furfurylaminopurine (kinetin)	Kn, FAde	R - CH_2 
6-furfurylaminopurine riboside (kinetin riboside)		
6-methylaminopurine	MAP, MeAP	R - CH_3

Table IX. Continued

6-(trans-4-hydroxy-3-methyl-but-2-enyl)amino purine, (zeatin)	Zea	
6-propylaminopurine		R - $CH_2CH_2CH_3$
6-propylmercaptopurine	PMP	R - S-$CH_2CH_2CH_3$
Benzimidazole		
N'N'-diphenylurea		
N-3-chlorophenol-N'-phenyl urea		
2-benzthiazolyl oxyacetic acid		

ring, especially any sutstitutions at 1-position results in reduced activity. Also the 2- and 3-positions should be free as biological activity is reduced if they are blocked but not to the same degree as the 1-position changes. At the 9-position certain alterations can be made without reducing biological activity (see Fox, 1969). However, a variety of N^6-substitutions on the adenine ring can occur without reducing activity when compared to the kinetin furan group as standard. Furthermore, some compounds have increased activity.

Increased activity can be produced by the following alterations at N^6-position of the adenine ring: (i) lengthening the hydrocarbon chain up to 5-carbon units, (ii) a double bond at any position on side chain, and (iii) presence of polar groups. In summary, any substitutions in the N^6-side chain that makes the molecule more planar increases the biological activity (Skoog & Armstrong, 1970). A number of non-purine compounds have cytokinin activity. Examples of these are the urea derivatives, N-3-chlorophenol-N'-phenyl urea and N', N'-diphenyl urea.

Bioassay

Concomitant with isolation and synthesis of cytokinin compounds, numerous bioassay systems have evolved to determine cytokinin effects on a variety of cell tissues and cellular metabolism. The first bioassay for cytokinins was developed by Murashige and Skoog (1962) and Miller (1963). This was based on measurement of the increase in dry weight

of soybean or tobacco callus grown with cytokinin. This assay procedure required a waiting period of two to four weeks before results could be obtained, but recently a cytokinin bioassay system was developed by LéJohn (1975) that is completed within 10 min and still meets all the defined criteria of a cytokinin bioassay system.

A number of criteria for the assay must be met to show the validity of measuring cytokinin activity. The assay should be linear over a wide cytokinin range and specific for natural occurring cytokinins at physiological levels even in the presence of possible contaminating biological antagonists. Also the bioassay should be easily performed in a short time. Table X lists bioassay procedures, and a viewpoint is expressed as to whether the criteria of the cytokinin bioassay is being discerned (after Stevenson, 1974).

Mechanism of Cytokinin Biological Activity

Since the discovery of cell division promoting ability of cytokinin, a number of other biological roles may be cytokinin regulated, cell growth, mitosis and cell division, differentiation, metabolic synthesis, seed dormance and germination senescence and degradation, respiration and transformation, transport membrane, enzyme activity, etc. (see Stevenson, 1974). When studying cytokinin action numerous difficulties are encountered. The effect of cytokinin depends on other plant hormones, physiological condition, and type of plant tissue. The mechanism of cytokinin action is impossible to state as one simple theory due to the variety of processes involved and the question of what actually is the

TABLE X. COMPARISON OF DIFFERENT CYTOKININ BIOASSAYS

TEST SYSTEM & REFERENCES	PARAMETER OBSERVED	BIOASSAY TIME	SENSITIVITY & SPECIFICITY	ADVANTAGES & DISADVANTAGES
Cell Division Tests				
(a) Tobacco pith (1)	Fresh and dry weight of callus increase linearly, within certain limits, with log increase in cytokinin concentration in culture medium.	2-4 wks	For some cytokinins, linear relation between callus plug weight and 0.1 nM to 1 μM cytokinin. Auxin required for effect.	Extreme sensitivity makes it ideal for pure cytokinins. Growth period very long, and must be aseptic. Problem with explant uniformity.
(b) Soybean callus (2)	as above	as above, but some indication in 48 hr	as above, but with a broader "window" of sensitivity, up to 100 μM.	Sensitivity and more consistent linear response make it useful for both pure and impure preparations. Disadvantages as above.
(c) Carrot phloem (3,4,5)	Weight and number of cells in phloem explants grown with cytokinins increase with log cytokinin concentration.	2-3 wk. indication in 7-10 days	As sensitive as above, but less specific than earlier callus assays.	Material readily available and easily stored. Can be made sterile. Thiamine, glycine, nicotinic acid inhibit. Adenine has effect.

Table X. Continued

Senescence Tests				
Richmond-Lang Effect on chlorophyll degradation (6,7)	Chlorophyll is extracted from test material incubated with cytokinin in the dark. Chlorophyll measured spectrally. (leaves used include Xanthium, corn, tobacco, Chinese cabbage, and radish)	48 hr	Linear relation between concentration of cytokinin and chlorophyll remaining. Detects as low as 0.1 μM, and as high as 5-50 μM non-quantitatively. Lacks specificity.	Rapid, therefore sterility not a problem. Leaves must be prepared in dim light prior to use. Seasonal changes can affect leaf responses. Simple test to perform. Interference from purines and high sugar concentrations.
same (8)	Same test using detached oat or wheat seedling blades.	as above	as above	Secondary advantage is that only a small drop of cytokinin is required.
Cell Enlargement Tests				
(a) Leaf disc expansion with radish leaves (9)	Cell enlargement of immature leaves aged in the dark for 24 hr. Discs are cut out and floated in test solution for 18 hr in the light. Discs then blotted dry and weighed.	18 hr	Cell weight increases with increase in cytokinin concentration. Sensitivity similar to callus assays but limit is 1 nM.	Rapid, therefore need not be sterile. Seasonal changes can affect leaves. Special leaf preparation required.
(b) Leaf discs of etiolated bean seedlings (2,10,11)	Same as above, but using discs from 7-9 days old dark-grown bean leaves with a main lateral vein. Discs floated in the dark. Increases in diameter or weight measured.	48 hr	Less sensitive than the radish system. Compares with chlorophyll retention assay in sensitivity.	Gibberellins, red light, and cobalt may interfere. May be useful to investigate interrelationships of effects.

(c) Radish cotyledon enlargement (12, 13)	Radish cotyledons excised immediately after germination and soaked in test solution in the light. Cotyledon weight used as measure of size increase.	3 days	Weight increase is essentially a linear function of log cytokinin concentration. Sensitivity as for callus assays.	Seeds easily germinated, and can be surface sterilized. Relatively rapid and specific. Cotyledons require preparation.
(d) Pea stem section enlargement (14)	4 mm stem sections of 6 day old seedling stems put in test solution. Length and diameters of sections are compared before and after.	24 hr	Increase in size is sensitive over a wide range	Uses effects on cell division. Rapid. Sucrose, IAA, gibberellins, cobalt have effects. Response varies with sections used. Time-consuming measurements.
Germination Tests				
Lettuce seeds (15, 16, 2)	Seeds soaked in test solution in the dark for 8 hr then transferred to filter paper wet with the solution for 24-60 hr. Germination percentage scored.	32-68 hr	Germination in abscence or presence of red light measured. Sensitivity poor, with permeability problems. Detects 1 nM cytokinin in some cases	Requires high concentration and large amounts of cytokinin. Red light and gibberellins will affect. May have sterility problem.
Lettuce seed embryos (17)	Excised embryos in cytokinin plus abscisic acid solution are tested for cytokinin overcoming inhibition by abscisic acid	46 hr	Relatively sensitive with linear response.	Depends on double effect of two hormones. Must consider penetration and endogenous levels in embryos.

Frond Expansion Test					
(a)	Water lily (*Lemna minor*) (3, 18)	Single plant in basal medium, incubated in the light for test period. Plant weighed, and frond number and area determined.	12 days	Frond number or weight increase relate to log cytokinin concentration. Not sensitive; lowest detection at about 0.25 μM.	Long test period. Low sensitivity and poor selectivity. Extraneous factors influence system.
(b)	*Spirodela* (3)	Cytokinins satisfy the non-photosynthetic red light requirement for growth. Grown in dark; other conditions as above.	14 days	as above	as above
Differentiation Tests					
(a)	Moss buds in *Tortella caespitosa* (19, 20), *Pohlia nutans* (21), & *Funaria hygrometrica* (23, 23)	Cytokinin induces bud formation in moss proteonemata. Buds are counted before and after exposure to test solution.	48 hr	Number of buds increases logarithmically with cytokinin concentration. Broad specificity and fairly sensitive. Detects at 10 nM levels; linear correlation to 100 μM.	All-or-nothing response easy to measure. Rapid and selective for purine compounds. Somewhat tedious, and some non-purine active compounds not effective.
(b)	Tobacco pith buds (2)	Cytokinins promote adventitious budding from tobacco stem slabs. Incubation without auxin leads to bud formation in 12 days; with auxin and cytokinin buds appear much later (5 wks).	less than 12 days	Not very sensitive; trace amounts of auxin inhibit buds. Adenine has some effect. Linear correlation with cytokinin concentration when system works.	Auxin antagonism. Certain cytokinin concentrations inhibit, therefore not quantitative. Sterility problems.

(c) Apical dominance release (24)	Application of substance to lateral bud at second node of 6-day old pea plants with apical bud intact releases apical dominance effect. Bud cut off and weighed after 3 days; compared to control bud.	3 days	Reasonably specific for adenine cytokinins, no effect with some other compounds. Only moderately sensitive.	Fairly rapid. Auxin may affect results.
Metabolic Synthesis Tests				
(a) Nitrate reductase induction (25)	Increased induction of nitrate reductase activity in Agrostemma githago embryos by nitrate when cytokinins present.	30-60 min	Activity of enzyme increases with cytokinin between 10 nm and 100 μM.	Very rapid response, but not assessed for suitability as an assay.
(b) Betacyanin (pigment)	Cytokinins induce betacyanin production in Agrostemma seedling cotyledons and hypocotyls when incubated in the dark with tyrosine. Pigment extracted and measured spectrally.	18-72 hr	Broad specificity. Extracted pigment increases in optical density with the log of cytokinin concentration from 1 nM to 10 μM.	Can measure small amounts of pigment pure or in extracts. Rapid. Disadvantages are in manipulation of seeds, and processing of extracts leading to error.
(c) Deoxyisoflavone synthesis (29)	Induction of deoxyisoflavone correlates with soybean growth in culture. Callus is chopped finely and incubated in liquid medium, then ethanol or acid extracted. Optical density read.	45 hr	Reasonably sensitive to cytokinins. Correlates with growth of soybean.	Quantitative measure of effect. Auxin may affect synthesis. May be measuring a secondary hormone effect. Deoxyisoflavone is alkali-sensitive.

Metabolite Transport and Binding Tests

(a)	Inhibition of active uptake by Achlya (30)	Cytokinins inhibit the active transport of amino acids, sugars, and nucleosides in germinated spores. Membrane filter assay used to measure effect on uptake.	2-10 min	Decrease in uptake with increased cytokinin levels between 0.1 μM to 1 mM. Selective for cytokinin bases; ribosides not active.	Rapid, specific. Requires radioactive substrates and equipment.
(b)	Enhanced uptake and binding of auxin and tryptophan to Achlya germlings (30)	Cytokinins enhance auxin and tryptophan binding to a cell wall glycopeptide. Can measure with filter assay as above.	as above	Specificity as above. Increases in binding with concentrations from 1 nM to 1mM.	As above. Interaction of auxin and cytokinin can be assessed.

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active cytokinin molecule. Cytokinins are found free in cell tissue but are also present in tRNA (see Hall, 1971), and as various free soluble derivatives (Dyson et al, 1972; Fox et al, 1972).

Cytokinins are found in significant numbers in tRNA (Hall, 1971). The number of cytokinin molecules per tRNA molecule appears to increase with evolutionary complexity which reflects more complex regulatory roles of tRNA. Modified nucleosides in tRNA determine the characteristic secondary structure, loops and base pairing of tRNA molecules (Hall, 1971), and modified nucleosides adjacent to the 3' end of the anticodon may be involved in binding of the ribosome to the messenger RNA. If the modified purine at the 3' anticodon is changed or modified, this reduced or eliminated the ability of tRNA to function in protein synthesis (Fittler & Hall, 1966; Gefter & Russell, 1969; Faulkner & Uziel, 1971). In a model proposed by Fuller and Hodgson (1967) for the role of 3' anticodon modified purine in tRNA, the modified purines give specificity and conformational flexibility to the reading of the anticodon triplet.

Although cytokinins present in tRNA molecules are important in protein synthesis, it seems unlikely that biological functions attributed to cytokinins are regulated via tRNA cytokinin. This concept would imply that cytokinin molecules would be directly incorporated into tRNA molecules (Fox, 1966; Burrows et al, 1971). The weight of evidence is to the contrary. It appears that specific adenosine residues are modified during synthesis of tRNA molecules (Peterofsky, 1968; Fittler et al, 1968; Kline et al, 1969; Chen & Hall, 1969). As

a number of non-purine compounds also have cytokinin activity and exogenous cytokinins produce biological effect, there is now little doubt that the majority of the biological activity of cytokinin is not mediated via tRNA.

It is possible that degraded tRNA may serve as the cytoplasmic source of active cytokinin (Hall, 1970; Dyson & Hall, 1972; Hall et al, 1972). Therefore it was deemed important to investigate enzymes involved in cytokinin catabolism. In some animal bone marrow tissue such as chicken, rabbits, and humans, the enzyme adenosine amino-hydrolase converts isopentenyladenosine to inosine (Hall et al, 1971; Hall & Mintsiolis, 1973). An analogous enzyme exists in cultured tobacco cells which convert isopentenyladenosine to adenosine instead of inosine (Pacés et al, 1971). After conversion of isopentenyladeno-sine to isopentenyladenine in mammalian cells or N^6-(3-hydroxy-3-methyl-butyl)-adenine in pea seedling (McLennan et al, 1968), these products are converted to 8-hydroxy- and 2,8-dihydroxy- derivative by xanthine oxidase (McLennan & Pater, 1973a). It is likely that different cell tissue and organisms break down isopentenyladenosine to a variety of products. For example, 5'monophosphate ester of isopentenyladenosine may be the active cytokinin in chicken liver, kidney, and some tissue culture (McLennan & Pater, 1973b). However, sources of cytokinin other than tRNA catabolism may provide the active form of the cytokinin molecule.

In tobacco and soybean tissue, 75% of exogenously added N^6-benzyladenine is rapidly broken down by cleavage of the side chain (Fox & Chen, 1967; 1968). The remaining 25% forms stable derivatives benzyladenosine monophosphate and benzyladenosine (Dyson et al, 1972; Fox et al, 1972). After a period, these derivatives are converted to 6-benzylamino-7-glucofuranosylpurine (Deleuze et al, 1972; Fox et al, 1972). This stable compound has also been isolated from tobacco tissue, potato tubers, and lupin seedlings (Fox et al, 1973) and found to be a metabolically active as cytokinin. The role of this stable compound as the actual active cytokinin in plant tissue has been questioned by Stevenson (1974) as to the known ability of cytokinins to rapidly respond to changing conditions. Some cytokinin effects occur too rapidly to allow the formation of glucoside derivatives. The glucoside derivatives may be involved in only one specific function in cytokinin regulation of metabolism.

Cytokinins affect the nitrogen metabolism in plants. Cytokinins, but not auxin or gibberellic acid enhance nitrate reductase activity in the embryo of Agrostemma githage (Borriss, 1967). Nitrate reductase activity of plants is induced by nitrate in the surrounding medium only in the presence of light (Beevers & Hagerman, 1969). Lips and Roth-Bejerano (1969), using tobacco leaves, suggested that cytokinin can replace the light requirement of nitrate reductase synthesis, and recently the light requirement has been related to general induction of protein synthesis (see Knypl, 1973). Kende and colleagues (1971) showed that the effects of benzyladenine and nitrate on Agrostemma

githago were additive and distinct from each other, but it is probable that de novo synthesis of nitrate reductase is induced by both nitrate, for tobacco tissue (Zielke & Filmer, 1971), and cytokinin, for Agrostemma embryo (Hirchberg et al, 1972). Knypl (1973) suggested that in the Agrostemma system, cytokinin acts specifically at the genome level, but this cannot be stated as a general mechanism of action of cytokinins. For instance, Sahulka (1972) demonstrated that kinetin reduced the activity of nitrate reductase in excised pea roots. IAA promoted an increase in glutamate dehydrogenase activity, while kinetin had an antagonistic effect on IAA and no effect when added alone. Sahulka (1972) suggested that cytokinin inhibition of root growth may be due to inhibition of nitrogen metabolism, but cautioned that results vary with time of incubation of hormone with plant tissue, combination of hormones used, and enzymes of nitrogen metabolism. Yet, in another plant tissue, carrot callus, Werner and Goglin (1970) showed that kinetin at physiological concentrations enhanced glutamate dehydrogenase activity. Stevenson (1974) was able to demonstrate that IPA significantly increased glutamate dehydrogenase specific activity in Achlya when grown in the presence of glucose, but when grown under induction conditions (glutamate in place of glucose) IPA had no effect on the enzyme level. Stevenson (1974) suggested that IPA enhanced glutamate dehydrogenase activity could be by several, yet to be distinguished, possibilities: (i) enzyme synthesis or stability, (ii) decreased degradation of messenger RNA for an enzyme, or (iii) activation of a pre-existing enzyme molecule. The effect of cytokinin on enhancement of enzyme level

could not be correlated to a general effect on either RNA or protein synthesis. In Achlya, IPA inhibits general RNA and protein synthesis either by antagonistic effect on solute transport, synthesis itself or RNase activity.

Dorée, Terrine, and Guern (1972b) correlated the uptake of cytokinin with the metabolic activity in the cell. Using cultured cells of sycamore, Acer pseudoplatanus, these authors proposed that peripheral binding sites exist for kinetin and other cytokinins which are distinct from actual uptake sites. These sycamore cells have two rates of cytokinin uptake, an initial rapid rate, and a slower steady rate up to one hour then levelling off. The majority of kinetin taken up was immediately metabolized to a variety of nucleotides (Terrine et al, 1972). Another suggestion by Sadorge and co-workers (1970) was that kinetin is catalyzed by the highly active enzyme purine phosphoribosyl transferase during membrane transport in sycamore. A similar model for cytokinin action applies to the moss cells. Cytokinins are known to be present in moss cells (Bauer, 1966; Klein, 1967; Beutelmann, 1973) and they enhance bud formation (Gorton et al, 1957; Bopp, 1968). Brandes and Kende (1968) proposed that the hormone (cytokinin) receptor sites were present on the surface of target cells of Fumaria hygrometrica protonemata. Removal of surface binding sites at different stages during bud formation results in dedifferentiation. However, the intracellular interactions after cytokinin binding are unknown.

The rate of degradation of kinetin nucleoside may be important in regulating kinetin uptake (Dorée et al, 1972a). The wide specificity

enzyme, adenosine hydrolase converts kinetin nucleosides to adenosine, guanosine, and inosine in several plant tissues (Terrine et al, 1969). However, in further studies, Dorée (1973) showed that N^6-substituted nucleotide synthesis is directly catalyzed by the transfer of a 5-phosphoribosyl pyrophosphate group instead of the two step reaction involving nucleoside phosphorylase and adenosine kinase. The N^6-side group is likely removed from N^6-substituted monophosphate by AMP deaminase (Terrine et al, 1972; Hall et al, 1971). It is suggested that AMP deaminase regulates the uptake of exogenous cytokinin (Dorée & Guern, 1973). It cannot be determined if degradation of nucleosides directly controls rate of cytokinin uptake, but some correlation between degradation rate and uptake of cytokinin does exist since intracellular pools appear linked to cytokinin uptake.

Another mechanism for cytokinin action has been proposed by Wood and Braun (1967). They suggested that cytokinin molecules activate a chemically distinct system that is involved in cell division, called cell division promoting factor (CDF). In crown gall tumor cells, Vinca rosea, 3, 7-alkyl-2-alkylthio-7-purinone with attached glucose molecule was found to be a cell division promoting factor (Wood, 1970). In tumor cells, the growth factor is produced continually, but in normal cells, exogeneous application of cytokinin is required before the growth factor is produced. Also CDF will replace the cytokinin requirement of normal tissue cell cultures (Wood & Braun, 1967). Braun and colleagues proposed a mechanism of cytokinin action linked to cAMP metabolism (Wood et al, 1972; Lundeen et al, 1973). CDF can replace either

cytokinesin I or 8-Br-cAMP (stable cAMP analogue) action in cell differentiation (Wood & Braun, 1973; Basile et al, 1973). As a note of caution, Wood and co-workers showed that CDF has no cytokinin activity in moss-bud production assay although non-purine cytokinins also show no cytokinin activity in moss-bud production assay (Valadon & Mummery, 1971).

Some reports describe the hormone effect as mediated by a reactive protein that binds to chromatin in vitro (Matthysse, 1970; Matthysse & Phillips, 1969; Mondal et al, 1972; Johri & Varner, 1968). Matthysse and Abrams (1970) proposed that a protein factor isolated from chromatin or purified DNA forms a complex with kinetin or zeatin, and interacts in some manner with DNA to enhance RNA synthesis. The basic mechanism of how such a model would work is entirely speculative. Mondal and co-worker (1972) have recently proposed a model that agrees with cytokinin effect mediated by a protein factor.

It is unlikely that one single mechanism of action can explain cytokinin activity in the wide variety of plant tissues that exist. In a recent report, Menon and Lal (1974) suggested that kinetin does not mediate bud formation in Physomitrium pyriforme alone, but involves interaction with other regulators such as abscisic acid. This plant system like that of animal systems follows the general rule: differentiation is affected by a variety of interacting components as described by Maltzahan (1959), Szweykowska (1962), Johri and Desai (1973), and Sood and Chopra (1973).

Cytokinins in Transport

Cytokinin interaction at the surface of the membrane may affect transport of other metabolites. Feng (1973) proposed that kinetin affected a protein component of (Allium cepa) onion cell membrane thereby increasing membrane permeability to polar metabolites such as, urea and thiourea. Cytokinin in a similar manner to calcium may be involved in maintenance of the cell membrane (Shibaoka & Thimann, 1970).

Both cytokinin and abscisic acid (ABA) are important in plant ion transport. In isolated maize roots both kinetin and ABA affect K^+, Ca^{++}, and Cl^- movement (Collins & Kerrigan, 1972). Kinetin and ABA increase the net flux into root, while kinetin reduces ion and water transport from the root. ABA exerts its effect rapidly while kinetin is slow to react. Calcium fluxes were the least affected by the hormones, but this tissue transports calcium poorly. Transport of monobasic cations K^+, Rb^+, and Li^+ in sunflower leaf discs and Na^+ in bean primary leaves is enhanced respectively by kinetin and benzylaminopurine (Ilan, 1971; Ilan et al, 1971; Jacoby & Dagan, 1970).

The active transport of nucleosides, amino acids and sugars in Achlya is inhibited by cytokinin (Stevenson, 1974; Goh, 1975), but facilitated transport processes are unaffected. Kinetic characterization of the inhibitory effect of cytokinin shows that cytokinin effect is concentration dependent, immediate, non-competitive, and pH dependent. The uptake of all the amino acids is inhibited except tryptophan with the

relative effectiveness of major cytokinins established as follows: isopentenyladenine > benzylaminopurine ≈ (kinetin ≈ zeatin) > hexylaminopurine > propylaminopurine. The apparent activation of tryptophan transport is due to increased binding to a site on the membrane distinct from the actual tryptophan transport system.

Plant Pathogenesis

Disease in plants is most likely a response by the host to readjust hormonal imbalances produced by pathogens (see Stevenson, 1974). In an investigation of pathogenic attack, two criteria must be observed. (i) Increased level of cytokinin produced either by the pathogen or the host in response to pathogenic attack. (ii) An overall effect produced by cytokinin interaction with other hormones.

Cytokinin has been shown to be related to disease symptoms in a number of plants. Corynebacterium fascians infection or kinetin application produces 'fasciation disease' (Thimann & Sachs, 1966). Isopentenyladenine was reported to be produced by Corynebacterium fascians (Helgeson & Leonard, 1966; Klambt et al, 1966), but experimental isolation procedure has been criticized in a recent report by Rathbone and Hall (1972). Isopentenyladenine may also be present in Agrobacterium tumefaciens (see Kende, 1971), a bacterium which is capable of initiating crown gall tumor growth.

Fungi or insect larvae upon infection of leaves are able to attract nutrients to the point of infection (Shaw & Samborski, 1956;

Engelbrecht, 1971). This could be one of the reasons why infecting pathogens developed the ability to produce cytokinin (Thimann & Sachs, 1966). Cytokinins are also produced by mycorrhizal fungi, Amanital rubescens, Rhizopogon roseolus, R. japonicum, and R. leguminosarum (Miller, 1967; Phillips & Torrey, 1972), and may be associated with root diseases produced by these organisms.

Senescence

Richmond and Lang (1957) suggested that kinetin delays senescence in detached leaves of Xanthium. Since that time, numerous reports have been published attempting to elucidate the mode of cytokinin action in delaying aging. Cytokinin was postulated to act as a mobilizing agent which causes plant metabolites and auxin to come to the point of cytokinin application and, at the same time, prevent movement of metabolites out of the treated area (Mothes & Engelbrecht, 1961). Also, presumably, it increases macromolecular synthesis (Richmond & Lang, 1957; Mothes & Engelbrecht, 1961; Osborne, 1962; 1965). However cytokinin action due to increased synthesis has been critized by Tavares and Kende (1970), Kende (1971), and Trewavas (1972), because the enhancement may in fact be secondary to the pool sizes and the degradation rate. Reduced degradation rates do occur in the presence of cytokinin (Tavares & Kende, 1970; Kuraishi, 1968). In addition, kinetin suppresses increased activity of DNase, RNase, and peptidase in senescing barley leaves (Balz, 1966; Atkin & Strivastava, 1969), and this ability was recently

substantiated by Dove (1972) and Shibaoka and Thimann (1973).

Another mode of cytokinin action in delaying senescence was suggested by Thimann and colleagues. The amino acid L-serine counteracted the delaying effect of senescence produced by cytokinin, auxin, and adenine (Shibaoka & Thimann, 1970). L-Serine also enhanced proteolytic enzyme activity in detached leaves. Shibaoka and Thimann (1970) suggested that L-serine may be present at the active site of proteolytic enzymes. In a follow up, Thimann, Shibaoka and Martin (1972) proposed that cytokinins may promote the synthesis of a protein component that inhibits proteinase formation. In agreement with this hypothesis, Martin and Thimann (1972a, b) showed that serine promoted proteinase activity in senescing tissue and cytokinin and cycloheximide antagonized serine effect, thereby linking action to protein synthesis. Other amino acids, such as, arginine, cysteine, alanine, and glycine were also involved in counteracting cytokinin effects on leaf senescence but regulation was extemely complex. Martin and Thimann (1972b) suggested that the interaction between amino acid and cytokinin is important in leaf senescence.

The surrounding medium is also important in the mechanism of cytokinin action in Lemna minor (fern) leaf senescence (Trewavas, 1972). When cytokinin was added in the presence of water only the degradation rates were altered, but if cytokinin was added in the presence of sucrose-mineral salts, synthetic rates were also increased. In this fern, the greatest antagonistic effect produced by cytokinin occurred in the absence of calcium. Calcium, in addition to nitrate and

sulfate, reduced the synthetic rate and increased the degradative rate. However, Poovaiah and Leopold (1973) showed that calcium deferred senescence in corn, (Rumex) leaves and ripening fruit. In this instance, calcium appears to influence the maintenance of the membrane integrity and overcome increased membrane permeability, depressing the respiratory rise and other phenomena associated with senescence.

Plant and Hormone Interaction

In addition to auxin and cytokinin, other plant hormones and effectors gibberellins, ethylene, ascorbic acid and abscisic acid regulate plant growth and differentiation (Clement, 1970; Hill, 1973). The overall action of hormones on cell growth and metabolism cannot be separated but must be viewed as a result of numerous interactions between the growth factors. For example, tobacco pith cells in culture require the presence of both cytokinin and auxin to grow (Skoog & Miller, 1967). The concentration ratio of cytokinin to auxin is important for determining what type of growth occurs. If the kinetin to auxin ratio is high, bud formation occurs; equal, callus formation; and low, root growth takes place. Another example of auxin-cytokinin relationship is apical dominance (Clement, 1970). Auxin produced in the apex moves downward preventing lateral bud formation, while kinetin moving upward from the roots tend to suppress longitudinal growth and promote transverse growth and lateral bud formation. An example of too much cytokinin being present is 'witches broom'. This disease is caused by the invading pathogenic fungi in willow trees.

Ascorbic acid is believed to function in a similar manner to cytokinin. Apical dominance due to auxin inhibition of lateral bud formation in Pisum sativum L. is antagonized by application of ascorbic acid (Johnnykutty & Khudairi, 1972). Table XI lists a variety of effects produced by ascorbic acid in plant tissue.

TABLE XI. TABLE OF ASCORBIC ACID EFFECTS

EFFECT	LITERATURE REFERENCE
induction of nodule formation in *Cicer arietinum*	Swaraj, K. & O. P. Garg (1970) Physiol. Plant. 23 889-897.
cell elongation in *Avena* coleoptiles	Merkys, A. & O. Rupainiene (1965) Lietuvos T. S. R. Mosklu Akad. Carbasen. C. 1 97-107 (English Abstr.)
promotes bud and seed germination in presence of red light	Khudairi, A. K. (1968) Phytologia 17 441-444 and Khudairi, A. K. (1968) Plant Physiol. 43 (suppl) 48
release of apical dominance produced by auxin	Johnnykutty, A. T. & A. K. Khudairi (1972) Physiol. Plant. 26 285-288.

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM AND GROWTH

A. Classification

The fungal strain employed throughout most of this thesis study was obtained from Dr. J. S. Lovett, Purdue University, U.S.A., and has been previously described and designated Achlya sp. (1969) by LéJohn and Stevenson (1970). Achlya sp. (1969) was choosen because it is easy to grow in its asexual life cycle. Occasionally, studies on Pythium heterothallicum (female strain) CBS* 202.68 and Blastocladiella emersonii were carried out to allow comparison of results between different water moulds. Blastocladiella emersonii was also obtained from Dr. J. S. Lovett, Purdue University, U.S.A.

* Centralbureau voor schimmelcultures, Baarn, Netherland.

Achlya: A systematic account of Achlya sp. (1969) is as follows: class, oomycetes; order, saprolegniales; and family, saprolegniaceae (Alexopoulos, 1962; Dick, 1973). The eucarpic thallus construction is filamentous, mycelial, nonseptate, and has unlimited capacity for growth. Members of the saprolegniales have the stoutest hyphae of any fungus ranging from 40 μm to 100 μm in diameter with a diameter of 20 μm at the

growing tip. Most of the family are saprophytic on plant and animal debris, and inhabit fresh water and moist soils.

In asexual reproduction which is diploid, sporangia development is initiated by the streaming of cytoplasm into the tip area of the hypha and the arrest of hyphal growth, followed by formation of basal septum separating the dense cytoplasm in the tip from the rest of the hypha. Sporangiospores are formed from individual nuclei and are released by way of a papilla. The family saprolegniaceae is diplanetic in nature having both a primary and secondary spore in the life cycle. The primary spores may or may not have two apical flagella, are short lived, and are feeble swimmers. The flagella are retracted on encystment. In the species Achlya, the first swimming stage is largely suppressed. The secondary zoospores (kidney shaped) have two flagella; one whiplash type, and the other tinsel type, both inserted in a deep lateral groove. These easy swimming zoospores can be motile for several hours. The flagella are presumed cast off upon encystment. Germination is by a thin germ tube which widens to form a hypha.

The vegetative reproductive cycle of Achlya sp. is easily discernable but sexual reproduction characteristic of the saprolegniaceae is not demonstrable. Somatic hypha bear oogonia (female sex organ) which are thick-walled spheres containing free oospheres, and antheridia (male sex organ) which are smaller and multinucleated. Fusion between oogonium and antheridium occurs producing diploid zygotic nuclei. A thick wall develops around each oosphere forming oospores, and after a rest period the oospores are released from the disintegrated wall of the oogonium which, upon germination, form hypha or hyphal stalks bearing zoosporangia.

Pythium: Pythium heterothallicum, as Achlya sp. (1969), belongs to the class, oomycetes, although it belongs to a different order, peronosporales. This coenocytic water mould has a vegetative cycle similar to Achlya, but under normal laboratory conditions it is almost impossible to obtain asexual spores. Pythium sexual reproduction cycle resembles Achlya having well differentiated gametangia (Alexopoulos, 1962).

Blastocladiella: Blastocladiella emersonii belongs to the class chytridiomycetes, and order blastocladiales. The thallus of this microscopic water mould has a rhizoidal appendage in the substratum which is non-living while above the stratum it has a bulb-shaped thallus that is the living part of the cell. In asexual reproduction, the entire thallus is converted into a sporangium which releases haploid zoospores, each containing a posterior flagellum. Blastocladiella is believed to have a sexual life cycle, although it has not been demonstrated in the past 50 years, where one surface thallus donates its nuclei to another forming a diploid gametangia which undergoes meiosis, and releases haploid zoospores.

B. Growth Media

Throughout the course of this study, the following media were most frequently used.

GY Medium:

yeast extract (Difco)	0.5 g
glucose	5.0 g
tap distilled H_2O	to 1 liter

GTY Medium:

yeast extract (Difco)	0.5 g
glutamate (monosodium)	5.0 g
tap distilled H_2O	to 1 litre

PYG Medium:

yeast extract (Difco)	1.0 g
peptone	1.0 g
glucose	3.0 g
tap distilled H_2O	to 1 litre

Czapek Dox Medium (modified): (commerical medium from Oxoid Ltd., London, England)

$NaNO_3$	2.0 g
KCl	0.5 g
magnesium glycerophosphate	0.5 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
sucrose	30.0 g
distilled H_2O	to 1 litre

Defined Medium: (Barksdale, 1963)

per litre de-ionized H_2O:

monosodium glutamate	0.5 g
glucose	2.8 g
*L-cystine	0.02 g
KH_2PO_4	1.5 mM
KCl	2.0 mM
$MgSO_4 \cdot 7H_2O$	0.5 mM
$CaCl_2$	0.5 mM
Na_4EDTA	0.01 g
Tris	1.2 g
Metal Mix # 4 solution (2 mg/ml)	10.0 ml

* Dissolve in 2 N HCl.

The pH was adjusted to 6.8 with 1 N NaOH. Metal Mix # 4 was prepared by grinding together 28.9 g $Fe(NH_4)(SO_4)_2$-$6H_2O$, 8.8 g $ZnSO_4$-$7H_2O$, and 3.1 g $MnSO_4$-H_2O. The final solution was prepared by mixing 200 mg Metal Mix # 4 and 100 mg sulfosalicylic in 100 ml de-ionized H_2O. The final concentration of trace metals is 20 mg/ml Fe^{+++}, 10 mg/ml Zn^{++}, and 5 mg/ml Mn^{++}.

C. Growth of Organism

The procedures for growth all pertain to growth of Achlya sp. (1969) follow below. For glycopeptide study, Blastocladiella emersonii was grown at 18° for 16 h in Cantino's PYG liquid medium as described by LéJohn and Jackson (1968). Pythium heterothallicum (female strain) was grown as stationary cultures in modified Czapek-Dow liquid medium for 2 days at 24°.

Preparation of spore inoculum: Stock cultures of Achlya and other fungal species used in experimental studies were maintained on Cantino's PYG agar slants (Cantino & Lovett, 1960). Sterile technique was ultilized for handling of all fungal species. Cultures were started by removal of several mycelial threads from a PYG agar slant and inoculated into 20 ml of GY medium at pH 6.5 in standard plastic tissue culture Petri dishes. The mycelial mat was allowed to grow to maturation, eventually sporulating at 22°. After this initial growth, a working stock of spores was maintained by inoculating 2 ml of spore suspension into 20 ml of GY medium. Sporulation occurs after 24 h at 22°. After 7 days, mycelial mats were stiff enough that they could be easily picked up with an inoculating needle and suspended in tap distilled water. A constant

proportion of four mycelial mats was transferred to 100 ml sterile tap distilled water. Spores were released from the mat by vigorously shaking for 30 s. The mats were removed from the spores by filtration through cheese cloth and used as inoculum. For some studies a further purification of the inoculum was required. The spore suspension was filtered through cotton gauze to trap all mycelial threads and fungal germlings except spores. This procedure was repeated weekly to maintain a constant stock supply of standardised inoculum for experiments. Periodically re-isolation of Achlya sp. from a large stock of PYG agar slants was carried out to ensure that the original fungal characteristics of the strain were maintained.

Preparation of spores: Roux flasks containing 80 ml GY medium were inoculated with 10 ml Achlya spore inoculum prepared as described above without cotton filtration. After incubation at 22° for 40 h, the spent medium was removed by filtering through four layers of cheesecloth. The cheesecloth was secured around the mycelial mats and put in the requisite resuspending medium, vigorously shaken, and then the mycelial mats were removed. The suspension containing spores was next filtered through cotton gauze. The final spore density was in the range of 2.5×10^4 to 5.0×10^4 spores/ml. The spores were frequently used in studies of metabolic macromolecular synthesis and metabolite transport.

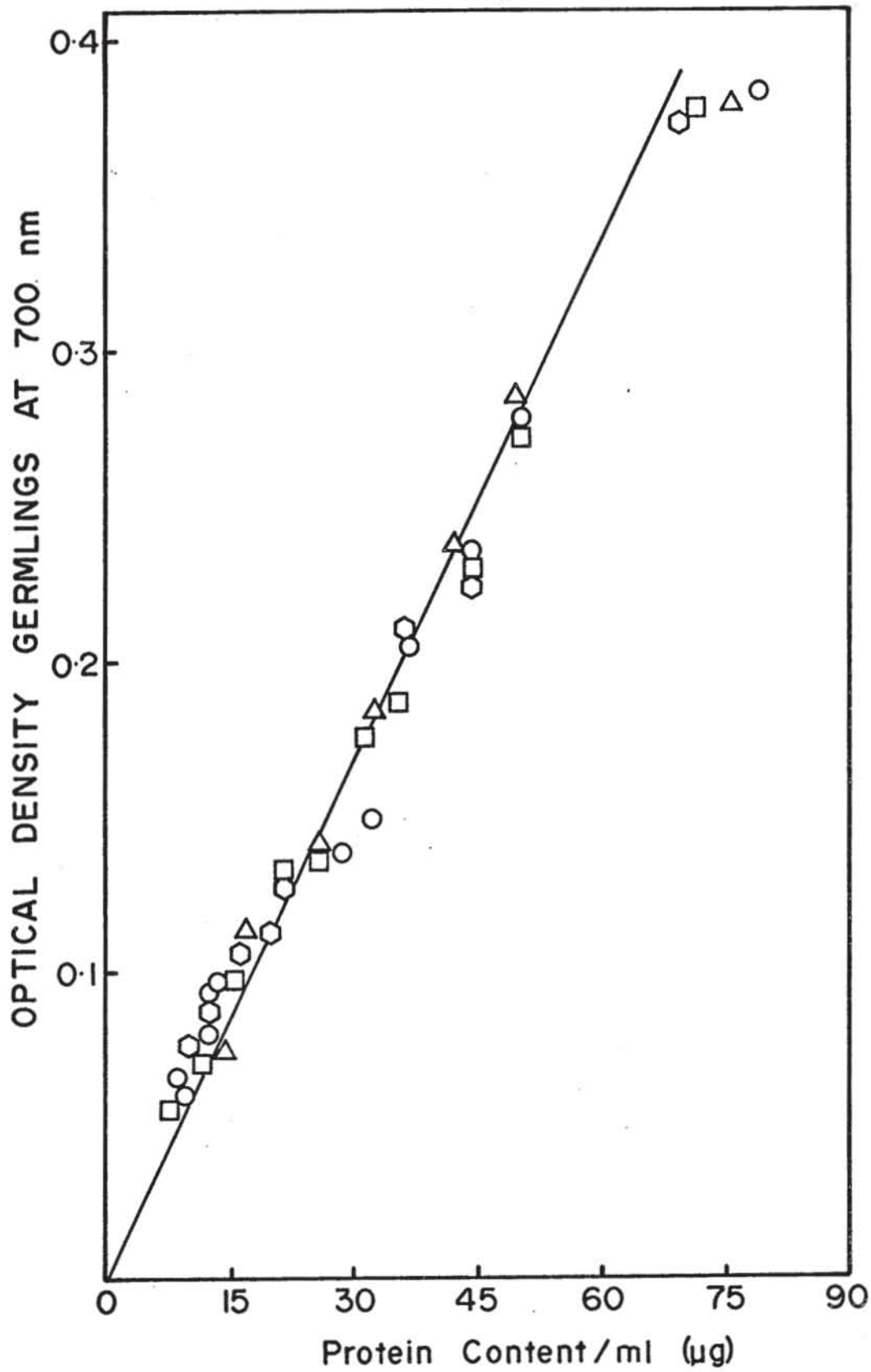
Preparation of germinated spores (germlings): Roux bottles containing 80 ml GY medium were inoculated with 10 ml Achlya spore inoculum. After incubation at 22° for 40 h to allow sporulation, the contents of roux bottles were filtered through four layers of cheesecloth into a wide mouth fernbach flask. The spore suspension (in spent medium) was

incubated at 11.5° for 18 h to germinate the spores to sizes of 120 μm ± 20 μm with a diameter of 5 μm. These germlings were collected on 61 μm nylon mesh cloth obtained from Henry Simon Ltd., Stockport, England, washed with resuspending buffer, and resuspended in the appropriate buffer or growth medium. After stirring for a short period, the suspension was again filtered through one layer of cheesecloth to remove any clumps that had formed during handling, and the cell density was adjusted to that required for a particular experiment.

For large scale production of germinated spores, the spores were collected as described for preparation of spores with a constant ratio of 20, 100-ml roux flasks culture mats per litre suspension medium, which was generally GY medium (density of 10^5 spores per ml). Germination of spores was carried out in Bellco spinner flasks at 27° for 2 h at which time there was 70% germination and the germlings were 30 to 50 μm long with a diameter of 5 μm.

Germinated spore density: The cell density was determined from a predetermined protein-optical density correlation. For each point on the standard curve, the soluble protein content (Lowry) and optical density at 700 nm, using a Gilford Model 2400 recording spectrophotometer were determined (Fig. 1). The soluble protein content was determined as follows. After collecting the cells on 0.45 μm Millipore HAWP filters, they were frozen in liquid N_2, the soluble protein extracted by suspending the frozen cells in 1 ml of 10 mM Tris-acetate buffer, pH 7, then sonicated until they were completely disintegrated (Insonator Model 1000, Ultrasonic System Inc., N. Y.), and finally centrifuged to remove the cell debris. Optical density of cell suspension for transport studies was maintained as constant as possible. Germinated spores were at 0.19

Figure 1: Correlation between soluble protein and optical density at 700 nm of germinated sporangiospores of Achlya. Each symbol represents an average of three experiments determined over a period of two weeks.


OPTICAL DENSITY GERMLINGS AT 700 nm
0·4
0·3
0·2
0·1
0
15
30
45
60
75
90
Protein Content / ml (µg)

$\pm$ 0.01 $A_{700\ nm}$, and osmotically shocked germinated spores at 0.32 $\pm$ 0.2 $A_{700\ nm}$.

Preparation of large-scale mycelium: When fungal cell extract was required for enzyme assay or RNA extraction, cells were grown as vigorously aerated cultures in either 8 or 15 litre cultures. For slow growth GY medium was used and for rapid growth PYG medium. After 18 h at 22°, mycelia were collected on Whatman No. 1 filters via suction filtration, washed with water and sucked dry and stored at -76° or used immediately.

METHODS

A. General Procedures

Liquid scintillation counting: For all the studies reported here that involved the use of radioactive material, the resulting data was computed through liquid scintillation counting techniques. Generally, radioactive cells were first collected by filtration on HAWP Millipore filters, and either cells plus filter or cells alone were put in scintillation vials. Frequently liquid samples ($\leq$ 1 ml) were put directly into the scintillation vials. The scintillation fluid used was Bray's (Bray, 1960) solution. Bray's solution contains the following components: 60 g naphthalene (scintillation grade), 4 g PPO, 0.2 g POPOP, 20 ml ethylene glycol, 100 ml methanol, and ρ-dioxane to 1 litre. Radioactivity was determined using either a Packard Tri-Carb or Beckman Model LS-230 liquid scintillation spectrometer standardized to disintegration per minute routinely.

B. Macromolecular Studies

Protein, RNA and DNA synthesis: For studies of macromolecular synthesis cells were grown either in GY medium or defined medium of Barksdale (1963) in Bellco spinner flasks at 27°. At time zero, radioactive material was added. Effector(s) were usually added at this time or de-

pending on experimental protocol at a specified time interval before or after zero time. After removing 1 ml samples at designated time intervals, the samples were immediately mixed with 1 ml ice-cold 10% trichloroacetic acid containing unlabelled carrier and stored on ice for at least 30 min. Subsequently the samples were filtered on HAWP Millipore filters, washed 3 times with 5 ml aliquots of 5% trichloroacetic acid and the radioactivity of the sample on the filter determined by liquid scintillation counting after immersing in Bray's fluid.

Enzyme activity: Enzyme activity was frequently assayed concomitantly with RNA and protein synthesis. To obtain enough material for assay at designated time intervals, 10 ml aliquots of labelled cell material was mixed with 90 ml samples of an identical large culture (unlabelled), and immediately filtered onto HAWP Millipore filters. After washing the filter with 50 ml of the appropriate medium, the sample was quickly frozen with dry ice. The frozen samples were removed from the filter, resuspended in 1 ml buffer of the composition, 50 mM Tris-acetate - 10 mM potassium phosphate buffer, 1 mM EDTA, at pH 7.3, and completely disrupted within 10 min by sonication at 10° in a water-cooled Raytheon 10 kc sonic oscillator. Cell breakage was monitored by microscopic examination. The cell debris was removed by centrifugation at 5,000 x g for 5 min and the resultant supernatant was used to assay protein content, enzyme activity and radioactivity after percipitating a sample with an equal volume of 10% trichloroacetic acid containing 0.1% vitamin-free casamino acids.

Enzyme activity was assayed using a Gilford Model 2400 recording spectrophotometer at 24° with a 3 ml silica cuvette of 1 cm light path. Activities were calculated from optical density changes during reduction

of NAD^+ and oxidation of NADH. One standard International Enzyme Unit represents the rate unit change of 0.001 O.D. per min multiplied by 4.8×10^{-4}. Protein was determined by the method of Lowry et al (1951) and the specific activity is expressed as total counts per sample per mg protein.

The enzyme activity assay mixtures in a total volume of 3 ml are as follows:

Glutamate dehydrogenase:

10 mM ∝-ketoglutarate, pH 8
0.083 mM NADH
0.167 mM $NADP^+$
0.5 M $(NH_4)_2SO_4$
33.3 mM Tris-acetate, pH 8

Isocitrate dehydrogenase, NADP-specific:

3.33 mM isocitrate
0.167 mM $NADP^+$
1.67 mM $MnCl_2$
67 mM Tris-acetate, pH 8

RNA extraction: The RNA profile was determined by sedimentation analysis in linear sucrose gradients (5% to 20%) using extracted RNA from radioactive cells labelled with either ^{14}C- or ^{3}H- uracil. At specified time intervals, 50 ml aliquots of labelled cells were removed from the incubation culture, filtered, washed with 3, 50 ml aliquots of buffer of composition: 0.01 M Tris-HCl, pH 7.4, 0.01 M Mg-acetate, and 0.05 M KCl, and immediately frozen on dry ice. Cells were disrupted by grinding in liquid nitrogen environment. The broken cell extract was taken up in buffer containing 0.2% diethylpyrocarbonate and 0.1% acid-washed bentonite. The RNA was extracted with buffer-saturated phenol (1:1 ratio of

phenol to RNA solution) continuously until an $A_{254:280}$ ratio of 2 in the aqueous phase or better was obtained, and precipitating the RNA with a 2 fold volume of absolute ethanol containing 0.3% 1 M Na acetate, pH 5, two times. After centrifugation at 3,000 x g for 10 min, the RNA pellet was resuspended in 10 mM Na acetate, pH 5.

RNA species were separated by sucrose density centrifugation for 2 h at 50,000 rpm in a SW 50L rotor in a Spinco ultracentrifuge. The 5% to 20% sucrose gradient (in 5 ml) was prepared in buffer containing 5 mM Na acetate, pH 5 and 1 mM $MgCl_2$, over a 0.3 ml cushion of 20% sucrose buffer. Samples of the fractions (0.14 ml) collected were directly assayed for radioactivity. Fractions were also monitored at $A_{260\ nm}$.

C. Uptake Kinetic Studies

Uptake assay buffer: The following buffers were used for uptake kinetics.

Buffer A:

Tris-acetate, pH 7	5 mM
NaCl	1 mM
KCl	1 mM
glucose	5 mM

Buffer B:

Tris-acetate, pH 7	10 mM
NaCl	2 mM
KCl	2 mM
glucose	10 mM

When the effect of IAA, cytokinin, or cytokinin-like compounds on transport was being studied, potassium phosphate buffer, pH 6.5, was substituted for Tris-acetate. The Tris-acetate concentration was

reduced to 1 mM for pH and ionic strength studies.

Uptake with time assay: To determine whether a compound is taken up and to initially characterize transport of this compound uptake studies with time are useful. Overnight germinated spores or osmotically shocked cells were resuspended in Buffer A or GY medium to the required optical density. The spore suspension was incubated with stirring throughout the duration of the experiment. Effector was generally added with the label although sometines it preceded or followed the label. Samples were removed at specified intervals, filtered through HAWP Millipore filters, washed tree times with Buffer A containing 1 mM unlabelled carrier and the radioactivity determined.

Initial uptake assay: Germinated spores prepared by overnight germination were resuspended in buffer B at a final concentration of 0.19 ± 0.01 A_{700}. Germlings were pre-incubated with stirring for 15 min. Uptake was initiated by transferring 1 ml germling suspension to 1 ml assay reaction system, the mixture incubated for 5 min at 22^{o} and rapidly filtered through HAWP Millipore filters using a 30-chamber Millipore manifold under suction generated by vacuum pumps, and washed three times with 5 ml Buffer A within 12 s. Both cells and filter were put into scintillation vials, but if label used readily bound to the filter only the cells were put into the vials. Such cells were gently removed from the filter by a thin blade spatula. The radioactive material taken up by the cells was determined by liquid scintillation counting.

Spores germinated by growing in GY medium at 27^{o} for 2 h were used to study the initial transport rate of metabolites in osmotically

shocked cells. After growth, the germlings were either immediately cold osmotically shocked or maintained in ice at 4^{o} (control). Such germlings were suspended in Buffer B at 0.32 ± 0.2 $A_{700\ nm}$ and equilibrated with stirring for 15 min prior to start of assay. The remainder of the initial rate transport assay for osmotically shocked cells is identical to initial uptake in whole cells.

Ca^{++} efflux assay: Spore inoculum and radioactive material were homogeneously dispersed in either GY medium or defined medium containing 1 mM $^{45}Ca^{++}$, and a 20 ml aliquot of the mixture was added per Petri dish. After incubation for 40 h in either defined medium or GY medium (precautions were taken to prevent sporulation either by slow growth or addition of 10 mM NaCl), efflux assay was carried out using one mycelial mat per assay tube. This method was devised because of cell clumping that results from mycelial fibres. Efflux assay tubes were prepared containing 10 ml of 1 mM Tris-acetate, pH 7, with or without effector(s). Mycelial mats from each Petri dish were collected by filtration through SMWP Millipore 5 μm pore size (47 mm) membrane filters, immediately suspended in efflux assay buffer, incubated for 10 min and filtered through HAWP Millipore filters. One ml aliquots were removed from the filtrate of each sample and the radioactivity determined. After washing twice with 5 ml of 1 mM Tris-acetate, pH 7, mycelial mats were removed from membrane filters by thin metal spatulas and radioactivity determined.

D. Isolation of Glycopeptides

Cold osmotic shock treatment: After large scale production of germinated spores, they were collected by suction filtration using 47 mm SMWP Millipore filters and immediately used for cold osmotic shock procedure. Using a slight modification of the Nossal and Heppel (1966) procedure of cold osmotic shock treatment for bacterial cells, fungal cells were readily osmotically shocked. One litre of germinated spores of Achlya (density of 10^5 spores/ml) were collected by suction filtration and gently resuspended in 25 ml hypertonic solution consisting of 0.5 M sucrose, 0.05 M Tris-acetate, pH 8 and 0.1 M EDTA. After incubating the hypertonic mixture at 30° for 15 min the cells were collected by filtering through 47 mm SMWP Millipore filters, the filtrate discarded, and the cells rapidly resuspended in a test tube in 12.5 ml ice cold 0.5 mM $MgCl_2$. This mixture was kept on ice for 10 min with frequent swirling. For large scale experiments, the ratio of 1.25×10^7 Achlya cells to 1.25 ml shock buffer to 1 ml ice cold 0.5 mM $MgCl_2$ was maintained. By filtering again, the bulk of the cells was removed, and the filtrate containing the osmotic shockable material collected. The filtrate was centrifuged at 17,000 x g for 15 min at 0° and the supernatant retained was the crude preparation of the glycopeptide.

Osmotic shock treatment of Pythium and Blastocladiella was identical to that described for Achlya except an overnight growth of mycelial mat was used in place of 2 h germinated spores. Occasionally, similar large Achlya hyphal fibers were osmotically shocked.

Purification of Achlya glycopeptide: All steps of the purification procedure were carried out at 4^{o}. The first step in purification was the concentration of the crude osmotic shock fluid by micro-ultrafiltration under N_2 pressure of 70 psi using a Diaflo UM-10 Amicon membrane system, Model 8MC. The UM-10 Amicon membrane retained molecules with a molecular weight greater than 10,000 daltons while allowing smaller molecules to pass through. The glycopeptide passed through the membrane. Thereafter, UM-2 Amicon membranes which retained all molecules greater than 1,000 daltons were used.

The concentrate retained by the Diaflo UM-2 Amicon membrane filter (3 to 6 mg protein/ml) was applied to either a CM-Sephadex or CM-cellulose (1.5 x 30 cm) column that had been equilibrated with 10 mM Na acetate, pH 7. The protein profile was continuously monitored at A_{280} using an UA-4 ISCO ultraviolet analyzer. The first protein void peak was collected, pooled, and again concentrated by using the UM-2 Amicon membrane filtration system under a nitrogen pressure of 70 psi. At this stage the calcium binding glycopeptide was relatively pure containing only a few small contaminants. This concentrate was put on a Sephadex G-50 (1.5 x 30 cm) column pre-equilibrated with 1 mM Tris-acetate, pH 7, buffer, which was also the running buffer. One ml fractions were collected at a flow rate of 0.56 ml/min using an Instrumentation Specialities Co. fraction collector. The protein peak eluting at fraction 52 was the purified calcium binding glycopeptide. This was again concentrated by the Diaflo UM-2 Amicon membrane ultrafiltration system to give a final concentration of 0.5 to 1.0 mg protein/ ml as determined by the method of Lowry et al (1951).

E. Glycopeptide Binding Assays

Equilibrium dialysis: The capacity of the glycopeptide to bind calcium and other effectors was determined by equilibrium dialysis. Corex centrifuge tubes of 30 ml volume contained 25 ml buffer, usually 1 mM Tris-acetate, pH 7 or 1 mM potassium phosphate, pH 6.8, and the radioactive material with or without effector(s). For rate-concentration binding, 0.1 ml aliquots were removed from each tube and put in scintillation vials. Aliquots of 0.25 ml calcium binding glycopeptide were dispensed in visking dialysis sacs tied with waxed dental floss. Dialysis tubing (Canlab, No. D161-1T, 8/32 inch) had been previously cleaned by boiling with 1 mM EDTA for 30 min, and exhaustively washed with de-ionized H_2O. The type of dialysis tubing is of particular importance since other dialysis tubing that was tested permitted the small calcium binding glycopeptide to escape into the dialysis buffer. Each dialysis sac containing a sample was immersed in the dialyzing mixture in the corex centrifuge tube (25 ml), plugged with a rubber stopper, and stirred for 3 h at 4°. After this, 0.1 ml samples were removed from outside and inside the dialysis sac, and put into scintillation vials and radioactivity determined. Dissociation constants for glycopeptide binding were determined from Scatchard plots (Scatchard, 1949).

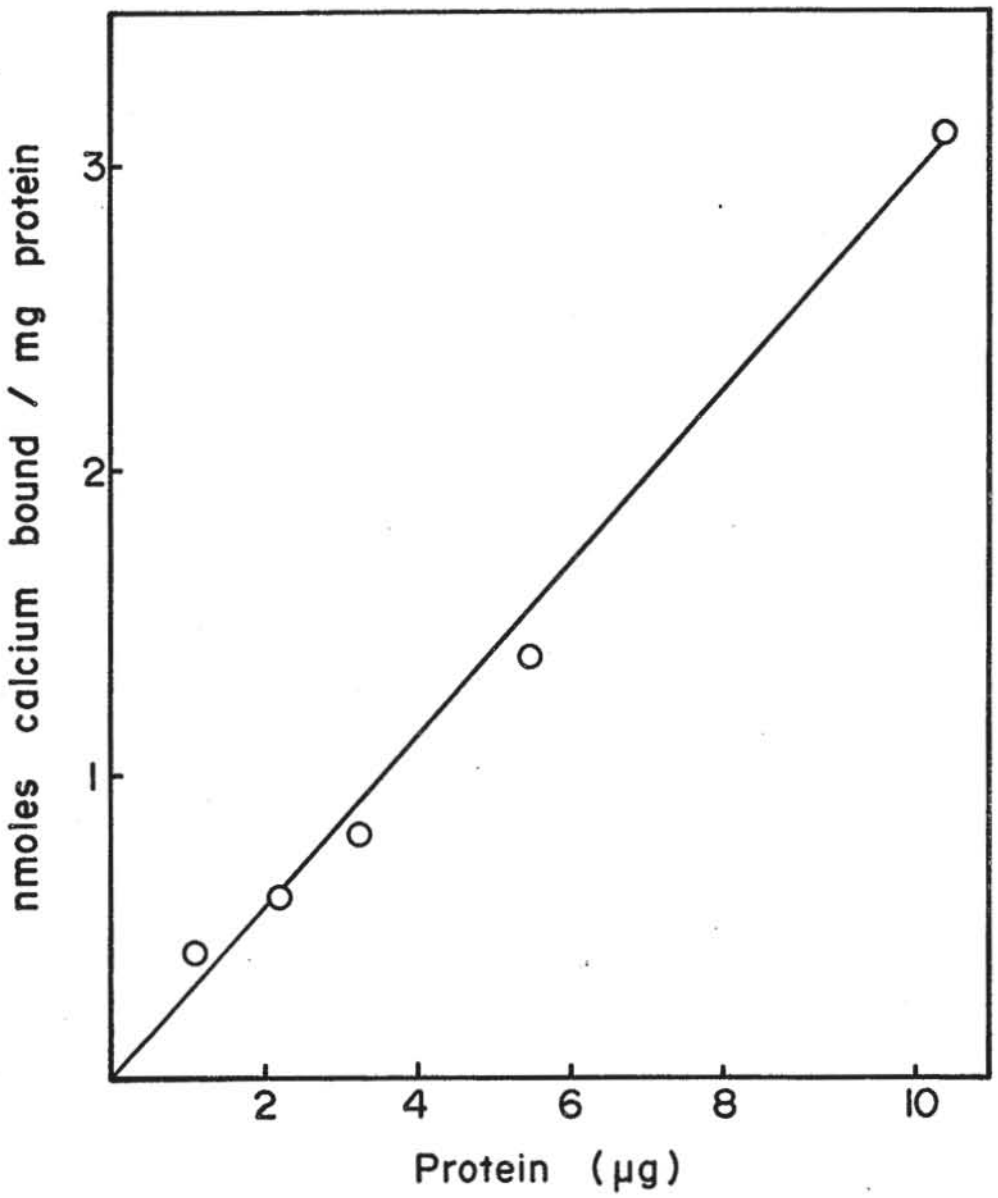
Equilibrium binding: Binding of labelled compounds to calcium binding glycopeptide was also determined by equilibrium binding on a Sephadex G-50 column (1.5 x 30 cm). Although this method required more time than equilibrium dialysis a greater amount of versatility was attained, since the determination of auxin and cytokinin binding by equilibrium dialysis was difficult due to interference by the visking dialysis

tubing. A Sephadex G-50 (1.5 x 30 cm) column was pre-equilibrated at 4^{o} with either 1 mM Tris-acetate, pH 7, or 1 mM potassium phosphate, pH 6.8 buffer containing radioactive material with or without effector(s), which was also the running buffer. After the glycopeptide sample (0.5 to 1.0 mg protein) was applied to the column, 1 ml fractions were collected at an efflux rate of 0.56 ml/min. Usually 100 µl samples were removed from each fraction and dispensed in scintillation vials. The radioactivity bound was determined by liquid scintillation counting. The binding of labelled compound by the glycopeptide was characterized by a peak of radioactivity followed by a trough due to the displacement of label from equilibrium by the glycopeptide as it moves down the column.

In addition to $^{45}Ca^{++}$ equilibrium binding on a Sephadex column, a disc electrophoresis system was also devised. The Tris-barbital, pH 8 gel electrophoretic system was equilibrated with 0.01 mM $^{45}Ca^{++}$ by adding it to both the Tris-barbital, pH 8 buffer and to the acrylamide gel solution before dispensing in gel tubes. Tris-barbital pH 8 gel electrophoretic system is described under physical characterization of the calcium binding glycopeptide. Samples of glycopeptide were applied and electrophoresis was carried out for 2 h at a current of 3 ma per gel. The electrophoresed polyacrylamide gels were then sliced and the radioactivity of each slice determined by liquid scintillation counting. During electrophoresis the $^{45}Ca^{++}$ had a tendency to move toward the gel top (cationic) while the anionic glycopeptide would move towards the gel bottom (anionic). The $^{45}Ca^{++}$ binding peak was characterized by a peak with a slight trailing trough towards the gel top and a build up of label towards the gel bottom preceding the $^{45}Ca^{++}$ binding peak.

Anionic exchange resin Dowex 2-X8 binding: This assay method is based on the ability of the anionic exchange resin Dowex 2-X8 (50-100 mesh) to bind calcium. The amount of calcium on the Dowex 2-X8 column bound by the glycopeptide is directly proportional to the protein concentration of the glycopeptide put on the column (Fig. 2). A mixture of glycopeptide, $^{45}Ca^{++}$, and effector(s) in a final volume of 0.1 ml 1 mM Tris-acetate, pH 7 was incubated on ice for 30 min. The sample was poured onto a small Dowex 2-X8 (50-100 mesh) conical shaped column that had been equilibrated with 0.5 mM $MgCl_2$. When the column was prepared, glass wool was used to seal the lower end and 3 cm of 0.5 mM $MgCl_2$ equilibrated Dowex 2-X8 resin was put in the column. Excess liquid was allowed to run down to the top of the Dowex resin before the top of the column was capped. After application of the sample, the reaction tube was washed with 0.25 ml 0.5 mM $MgCl_2$ and the wash was also put onto the column. When the sample plus wash had run into the column, the column was stoppered for an incubation period of 5 min to allow calcium to bind to the resin. The column was then washed with two 10 ml volumes of 0.5 mM $MgCl_2$, dried by vacuum suction for 1 min, and the resin put into a scintillation vial. Radioactivity bound by the glycopeptide on the resin was determined. This method of assaying radioactive binding by the glycopeptide was useful due to the rapidity of the test, but the degree of sensitivity was low compared to equilibrium dialysis.

Figure 2: Linear relationship of calcium binding by Achlya calcium binding glycopeptide and glycopeptide protein concentration on Dowex 2-X8 (50 - 100 mesh).


nmoles calcium bound / mg protein
3
2
1
2
4
6
8
10
Protein (µg)

F. Glycopeptide Chemical Characterization

Protein: Determination of protein concentration by the method of Lowry et al (1951) was used as a standard measurement of calcium binding glycopeptide, and occasionally the protein concentration of the glycopeptide was determined using the Gilford recording spectrophotometer at $A_{280\ nm}$.

Sugar: Carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al (1956) using both glucose and mannose for standard curves. Total hexose was determined by the Anthrone reaction (Spiro, 1968). Both of these chemical tests are readily applied to neutral sugar analysis of the glycopeptide since glycopeptides do not have to be hydrolyzed prior to assay.

Amino acid hydrolysate: A known dry weight of lyophilized glycopeptide was hydrolyzed in 1 ml of 6 N HCl (redistilled) for 24 h at 105° in a sealed bomb tube evacuated with nitrogen gas. The bomb tube was rotated several times during the hydrolysis period. The HCl was removed by vacuum evaporating at 37° and resuspended in 10 ml de-ionized H_2O. This procedure was repeated twice, vacuum evaporated again and resuspended in a final volume of 1 ml de-ionized H_2O. After the amino acid hydrolysate was evaporated over a saturated NaOH solution in a desiccator for 20 h, the final precipitatewas resuspended in 1 mM Tris-acetate, pH 7, and stored at 4°. Storage in a freezer at either -20° or -76° caused precipitation of the amino acid hydrolysate.

The glycopeptide amino acid hydrolysate was analyzed using a

Beckman Model 120 automatic amino acid analyzer. In addition, amino acids were separated and characterized by paper chromatography. Two large chromatography (22 cm x 40 cm) chambers were saturated with running buffer; one, n-butanol:acetic acid; H_2O (60:20:20, v/v/v) and the other, phenol:H_2O (75:25, w/v), and the descending trough was filled with appropriate running buffer. No. 1 Whatman chromatography paper was spotted with standard samples (5 µl of 10 mM solution) and unknown sample, dried under a flow of warm air, and the folded V-shaped top dipped in the trough. Descending paper chromatography was carried out at 22° for varying time periods (9 h to 48 h) depending on ease of separation of samples, after which the chromatograph paper was removed and air dried in a fume hood for several hours. When doing two-dimensional paper chromatography the Whatman No. 1 paper from the first descending chromatography (n-butanol: acetic acid:H_2O was dried for 15 h in a fume hood, and subsequently put in the second chamber (phenol:H_2O) trough at right angles to the original running direction. Paper was removed, dried for several hours, and sprayed with the appropriate spray to detect the components. Amino acids were determined by comparing to known standards running concurrent with the glycopeptide sample after amino acid spots were detected using Sigma Chemical Co. commercial ninhydrin spray (air dried at 105° for 10 min). Spots were the usual characteristic colors: purple, aspartic acid; blue, most amino acids; and yellow, hydroxyproline and proline.

To determine the amino acid optical rotation, glycopeptide amino acid hydrolysate was first treated with either D- or L- amino acid oxidase for 3 h at 37° before the two dimensional paper chromatography system for amino acid separation was run concurrent with untreated sample of hydrolysate. Prior to treatment of hydrolysate with amino acid oxidase,

amino acid oxidase was desalted as described in Separation News (September, 1972) Parmacia Fine Chemicals for "A rapid procedure for desalting (enzymes) and buffer exchange". A small plastic, conical shaped column (1 cm x 5 cm) was filled with 0.75 ml of Sephadex suspension which was prepared by swelling 0.5 g of Sephadex G-50 (medium) in 7.5 ml de-ionized H_2O for 10 min. After allowing 0.75 ml of de-ionized water to just run through the column, 0.25 ml of D- or L- amino acid oxidase (5 mg/ml) was applied to the column and the column was sealed after the enzyme had run into the Sephadex. The effluent was discarded. One ml de-ionized H_2O was added and the entire 1 ml eluate was collected as desalted amino acid oxidase diluted 4 fold.

Carbohydrate hydrolysate: Analysis of the calcium binding glycopeptide for the presence of sialic acid, neutral sugar, amino sugar, and uronic acid was based on the method of Simkin et al (1964) as modified by Jamieson et al (1972). In an attempt to determine the presence of sialic acid, purified glycopeptide (2 mg dry weight) in 2 ml 0.05 N H_2SO_4 was heated in a sealed bomb tube at 90° for 60 min, and the entire hydrolysate was applied to a Dowex 2-formate form (1 cm x 7 cm) column. Both the tube and the column were washed with 7 ml H_2O and the eluate discarded. The sialic acid fraction was collected by washing the Dowex column with 15 ml 0.3 N formic acid. The formic acid was removed by vacuum evaporation at 35° to one third the original volume. This step was repeated twice after bringing up the volume to 15 ml with de-ionized H_2O and finally evaporated to dryness. The residue that should contain sialic acid was assayed by the thiobarbituric acid method for sialic acid as described by Warren (1959) using N-acetylneuraminic acid as the standard.

For analysis of neutral sugars, amino sugars, and uronic acid, a lyophilized sample of glycopeptide (2 mg dry weight) was dissolved in 0.25 ml H_2O and mixed with 0.5 ml of Dowex 50 - 0.46 N HCl. The mixture was put in a bomb tube, evacuated with N_2, sealed and heated in a 100° oven for 30 h. The bomb tube placed at a 45° angle was frequently rotated throughout the heating period. To fractionate the sample, the entire hydrolysate mixture was poured onto a column of Dowex 50 (H^+ form) (1 x 7 cm) superimposed over a Dowex 2 (formate form) (1 x 7 cm) column, and the sample allowed to pass through both columns. To collect the neutral fraction, 15 ml of de-ionized H_2O was poured in 1 to 2 ml aliquots and allowed to pass through both columns. The eluate collected from the Dowex 2 (formate form) column was the neutral fraction. The amino sugar fraction was collected by first shutting off the Dowex 50 column, after all the fluid from the neutral fraction had eluted then adding 1 ml of de-ionized H_2O and washing the hydrolyzate-resin mixture with 0.25 ml 2 N HCl, twice using 0.25 ml de-ionized H_2O, followed by 0.25 ml 2 N HCl. After this washing procedure, 3 ml of 2 N HCl was used to eluate the amino sugar fraction from the Dowex 50 column. After allowing the neutral fraction to pass through the Dowex 2 (formate form) column, 8 ml of 1.2 N formic acid was used to eluate the uronic acid fraction. All fractions were vacuum evaporated.

Descending paper chromatography (22 x 40 cm chamber) with Whatman No. 1 chromatograph paper was used to qualitatively analyze the sugar fractions. A wide range of sugar standards for each fraction was run coincident and concurrent with the fraction sample. Neutral and amino sugars were detected by spraying with acetylacetone reagent followed by *p*-dimethylaminobenzaldehyde reagent as described by Partridge and

Westall (1948), and uronic acid was detected by spraying with benzidine-trichloroacetic acid-HCl (Chan & Cain, 1961). The presence of glucose in the neutral fraction was verified by the glucostat test (Worthington Biochemical Corporation, Freehold, U.S.A.).

G. Glycopeptide Physical Characterization

Ultrafiltration: Using the Amicon ultrafiltration system under a N_2 pressure of 70 psi, a rough calculation of the glycopeptide molecular weight was made by varying the pores size of the Diaflo-membrane filters. The Diaflo UM-2 membrane retains all molecules with a molecular weight greater than 1,000 daltons, while the UM-10 Diaflo membrane allows passage of all molecules with a molecular weight smaller than 10,000 daltons.

Sephadex gel filtration: A Sephadex G-50 (1.5 x 30 cm) column equilibrated with 1 mM Tris-acetate, pH 7, was used to determine the molecular weight of the glycopeptide. The protein peak was determined by a Gilford recording spectrophotometer or an UA-4 ISCO ultraviolet analyzer at A_{280}. Calcium binding glycopeptide peak was also determined by $^{45}Ca^{++}$ equilibrium binding assay at a concentration of 0.01 mM on a Sephadex G-50 (1.5 x 30 cm) column. A Sephadex G-50 calibration curve was prepared by methods described by Parmacia using proteins as standards. Table XII lists the standard protein K_a values and molecular weights. Blue dextran was used to determine the void volume, V_o. The K_a value

Table XII

Standard proteins used for molecular weight determination by Sephadex gel filtration and SDS gel electrophoresis. For Sephadex gel filtration each protein standard was run separately through the column to determine V_e value. The protein peak was determined at optical density A_{280} using a Gilford spectrophotometer. For SDS gel electrophoresis each standard protein and test sample was run separately, concurrent with a protein and test sample mixture. Protein was determined by staining with Coomassie blue.

Protein	Molecular Weight	Sephadex G-50 K_a Value	SDS Electrophoretic Mobility
Ribonuclease A	13,700	0.45	
Chymotrypsinogen	25,000	0.35	0.48
Ovalbumin	45,000	0.064	0.39
Cytochrome C	12,384	0.59	0.57
*Insulin	5,700	0.92	

* In the presence of 10 μM NEM.

for each protein standard was calculated as follows:

$$K_a = \frac{V_e - V_o}{V_t - V_o}$$

V_e is the elution volume of the standard protein and V_t is the total volume of the column occupied by the Sephadex gel.

Polyacrylamide disc gel electrophoresis: The basic procedure for pH 8 polyacrylamide disc gel electrophoresis (Tris-barbital) used in this study was described by Davis in 1964. Stock solutions for pH 8 gel electrophoresis were prepared as follows:

Solution A:

1 N HCl	48 ml
Tris	6.85 g
TEMED	0.46 ml
de-ionized H_2O	to 100 ml

Solution C:

Acrylamide	30 g
BIS	0.8 g
de-ionized H_2O	to 100 ml

A 7.5% acrylamide gel was prepared by first mixing 1 part A, 2 parts C, and 1 part H_2O. This solution was mixed with an equal portion (v/v) of freshly prepared potassium persulfate (0.14 g/100 ml H_2O) and immediately dispensed in gel tubes. A 10% polyacrylamide gel was prepared by mixing 1 part A with 2 parts C, then adding an equal amount of potassium persulfate. Individual gel samples were prepared by mixing with an equal portion of glycerol to increase density. The tracking dye, bromophenol blue (0.05%), was also mixed with the sample before appling to the gel. Tris-barbital buffer, pH 8, containing 5.25 g

barbital diethylbarbituric acid, 1 g Tris, and de-ionized H_2O to 1 litre was the buffer system used for both anionic and cationic chambers during electrophoresis. A current of 3 ma was applied to each gel and the running time was generally 2.5 h or until the tracking dye was ¼ cm from the lower level of the gel.

After removal of gels from tubes, they were either stained for protein or sugar, or sliced for fractionation of radioactive material. Protein was stained with 1% Coomassie brillant blue solution in 10% acetic acid and 25% methanol. After staining for a period of two hours, gels were submerged in 4 litres of destaining solution containing 75 ml glacial acetic acid, 50 ml methanol, and de-ionized H_2O to 1 litre. Destaining in a Bio-Rad electrophoresis diffusion destainer proceeded for a period of 48 h. An attempt to stain the glycopeptide was made using Periodic acid-Schiff stain for protein-bound carbohydrate by the method of Clarke (1964).

A Bio-Rad hand slicer was used to fractionate polyacrylamide gels containing radioactive labelled samples. After removal from tube, the gel was immediately frozen on a metal plate over dry ice placed in methanol. When the gel turned white, it was removed from the dry ice and allowed to partially thaw. A prechilled hand slicer containing razor edges at every 2 mm interval was used to slice the gel. Individual slices were put in separate scintillation vials to which 0.2 ml H_2O_2 was added to dissolve the polyacrylamide gel. Vials were capped and put at 37° for 20 h before radioactivity was determined by liquid scintillation counting.

The SDS gel electrophoresis system of Weber and Osborn (1969) was used as the basis for the determination of glycopeptide molecular

weight. Standard samples and glycopeptide were prepared by dialyzing against 0.01 M phosphate buffer, pH 7 containing 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol for 2 h at 37°. Polyacrylamide gels were prepared as described by Weber and Osborn (1969). After mixing gel buffer which contains 7.8 g NaH_2PO_4-H_2O, 38.6 g Na_2HPO_4-H_2O, and 2 g SDS per litre de-ionized H_2O with one half volune glycerol, bromophenol blue, and sample (10 - 50 ug), the mixture was applied to the gel and the disc gel electrophoresis chamber was filled with a one to one dilution of gel buffer. Electrophoresis was carried out for a period of 8 h at 5 ma per tube, and the gels were stained and destained as before.

The molecular weights of the standard proteins used and their corresponding electrophoretic mobilities are listed in Table XII. Electrophoretic mobility was calculated as follows:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{gel length after staining}} \times \frac{\text{gel length before staining}}{\text{distance of dye migration}}$$

MATERIALS

Chemicals

Biochemicals used in this study were obtained from Sigma Chemical Co., Calbiochem., and Nutritional Biochemicals. All amino acid ultilized were the L-optical isomers. Media and inorganic chemicals were of standard laboratory quality and purchased from commerical sources.

Enzymes

Enzymes used for the preparation of molecular weight standard curves were purchased from Pharmacia Fine Chemical Inc. as a molecular weight calbration kit with the exception of insulin which was obtained from Connaught Medical Research Laboratories, Toronto, as Zn-insulin crystals. Pronase was B grade (lot No. 901107) having an activity of 45,000 PUK units/g and lysozyme was grade I from egg white (30,000 units/mg), both obtained from Calbiochem.

Other Materials

CM-cellulose, CM-Sephadex, Sephadex gel, and equipment were obtained from Pharmacia, and disc gel electrophoresis chemicals from Eastman Chemicals. Ultrafiltration filters were obtained from Amicon and filters used for collection of labelled and unlabelled cells from Millipore Corp. and Gellman Instrument Co.

Radioisotopes

The list of radioisotopes used in these studies is given below together with their specific activities. The majority of radioisotopes were obtained from Amershan/Searle, Illinois, and exceptions are noted in the following list.

Inorganic compounds:

$^{45}Ca^{++}$ as $CaCl_2$ in aqueous solution (17.9 mCi/mg)

$^{22}Na^{+}$ as NaCl in aqueous solution (8.98 μCi/g)

$^{54}Mn^{++}$ as $MnCl_2$ in 0.5 M HCl (51.5 mCi/mg)

Amino acids:

alanine [-^{3}H(G)] (1.1 Ci/mmole)

arginine [-5-^{3}H] monohydrochloride (22 Ci/mmole)

asparagine [-^{3}H(G)] (100 mCi/mmole)

aspartic acid [-^{14}C(U)] (231 mCi/mM)

cysteine [-^{14}C(U)] hydrochloride (38.6 mCi/mmole)

glycine [1-^{14}C] (2 Ci/mmole)

glutamine [-^{14}C(U)] (48 mCi/mmole)

glutamic acid [-^{14}C(U)] (10 mCi/mmole)

histidine [-2,5-^{3}H] (58 Ci/mmole)

isoleucine [-4,5-^{3}H(n)] (17.7 Ci/mmole)

leucine [-4,5-^{3}H] (1.0 Ci/mmole)

methionine [-methyl-^{14}C] (46 mCi/mM)

3-phenylalanine [-ring-4-^{3}H] (58 Ci/mmole)

proline [-^{3}H(G)] (178 mCi/mmole)

serine [-3-^{3}H] (500 mCi/mmole)

threonine [-^{14}C(U)] (228 mCi/mmole)

tryptophan [-^{3}H(G)] (4.7 Ci/mmole) (New England Nuclear)

tyrosine [-3,5-^{3}H] (40 Ci/mmole)

valine [-2,3-^{3}H(n)] (31.6 Ci/mmole)

Nucleic acids:

adenosine [-^{3}H(G)] (12.1 Ci/mmole)

cytidine [-^{3}H(G)] (2.5 Ci/mmole)

uridine [-^{3}H(G)] (6.4 Ci/mmole)

thymidine [-methyl-^{3}H] (19.5 Ci/mmole)

guanosine [-8-^{3}H] (11 Ci/mmole)

adenine [-2-^{3}H] (20 Ci/mmole)

cytosine [-2-^{3}H] sulfate (59 Ci/mmole)

uracil [-2-^{14}C] (60 mCi/mmole)

thymine [-2-^{14}C] (60 mCi/mmole)

adenosine-3,5-cyclic monophopsphate ammonium salt (20.7 Ci/mmole)

Cytokinins* and auxins:

6-benzylaminopurine [-8-^{14}C] (24 mCi/mmole)

3-indolyl acetic acid [-2-^{14}C] (55 mCi/mmole)

N^{6}-isopentenyladenine [-^{3}H(G)] (crude preparation supplied by Amersham/Searle, purified by Stevenson (1974))

kinetin [-8-^{14}C] (7.82 mCi/mmole)

1-naphthyl acetic acid [-1-^{14}C] (44 mCi/mmole)

* All radioactive cytokinin compounds were resuspended in DMSO.

Other compounds:

citric acid [-1,5-^{14}C] (0.02 mCi/mg) (New England Nuclear)

ethylene diaminetetraacetic acid [-2-^{14}C] (7 mCi/g) (New England Nuclear)

fumaric acid [-2,3-^{14}C] (13 mCi/mM)

D-glucose [-1-^{14}C] (3.49 mCi/mM)

DL-isocitric acid [-5,6-^{14}C] (3.87 mCi/g) (New England Nuclear)

α-ketoglutaric acid [-5-^{14}C] (17.1 mC/mM) (New England Nuclear)

L-malic acid [-^{14}C(U)] (28 mCi/mM)

protein hydrolysate [-^{14}C(U)] containing 15 common amino acids (54 mCi/mAtom)

succinic acid [-2,3-^{14}C] (41 mCi/mM)

RESULTS AND DISCUSSION

PART I

TRANSPORT SYSTEM FOR CALCIUM

RESULTS

A. Characterization of Calcium Transport Processes

Kinetic values and specificity: The ability of germinated sporangiospores of Achlya to take up calcium was initially examined under growth conditions in GY medium as a function of time, as shown in Figure 3. The time course uptake of calcium indicates that calcium uptake is not a diffusion process due to the rapidity of saturation reaching half maximal equilibrium conditions within 7 min. The rate concentration plot of calcium uptake, as shown in Figure 4 shows initial saturation of the calcium transport process, which also indicates that calcium uptake is a carrier mediated process. The Lineweaver-Burk form inset shows that calcium transport is bi-phasic with a relatively high affinity site (apparent $K_m = 5 \times 10^{-6}$ M) and a low affinity site (apparent $K_m = 5 \times 10^{-5}$ M). The second apparent binding site is likely due to nonspecific surface binding and diffusion uptake since calcium uptake tends to gradually increase after the initial saturation plateau.

Figure 3: Calcium uptake in germinated sporangiospores of Achlya as a function of time.

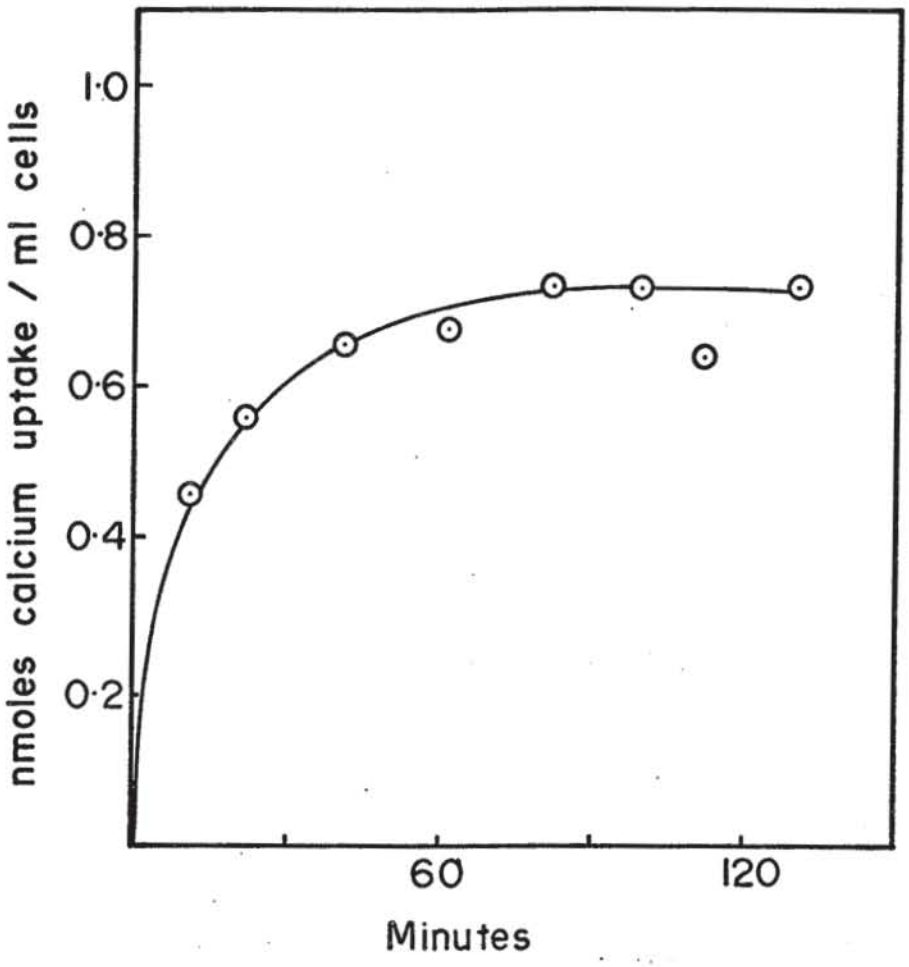
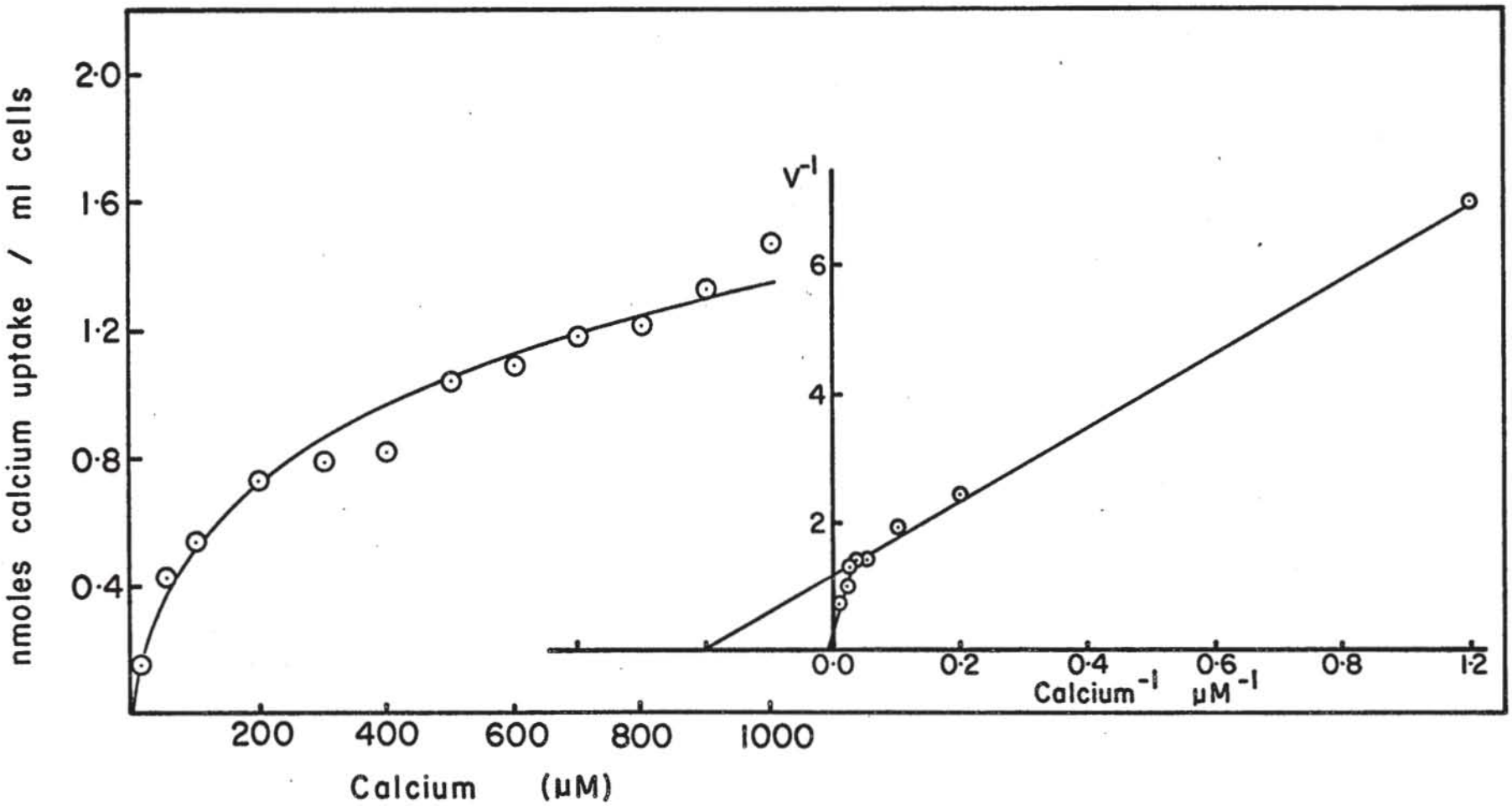

nmoles calcium uptake / ml cells
1·0
0·8
0·6
0·4
0·2
60
120
Minutes

Figure 4: Rate-concentration and Lineweaver-Burk plots (inset) of calcium uptake in germinated sporangiospores of Achlya.


nmoles calcium uptake / ml cells
2·0
1·6
1·2
0·8
0·4
200
400
600
800
1000
Calcium (μM)
V⁻¹
6
4
2
0·0
0·2
0·4
0·6
0·8
1·2
Calcium⁻¹ μM⁻¹

Support for the conclusion that calcium is a mediated process came from competition studies. Other cations inhibited calcium uptake to varying degrees, as recorded in Table XIII. The degree of inhibition depended on mono-, di-, or tri- cationic form, ratio of antagonizing cation to Ca^{++}, and specificity for the calcium uptake system. When the inhibition of calcium uptake was computed at a constant ratio of one to one for competing cation to calcium, the decreasing affinity for calcium transport was as follows: $Pb^{++} > Ba^{++} > Sr^{++} > Cd^{++}$. These divalent cations competitively inhibited calcium uptake. Figure 5 shows the competitive inhibition pattern of one such divalent cation, Sr^{++}, on calcium uptake.

Metabolic inhibitors: Calcium uptake is antagonized by metabolic energy and electron transport inhibitors, CCCP, 2,4-DNP, azide, and cyanide as shown in Table XIV, but inhibition was never greater than 55% at concentrations of antagonist that normally abolish active transport of nucleosides (Stevenson, 1974), amino acids (Singh, 1974), and sugars (Goh, 1975) in germinated sporangiospores of Achlya. The effect of metabolic inhibitors, 2,4-DNP and azide on calcium uptake is shown in Figure 6. The Lineweaver-Burk inset shows that inhibition of calcium transport is non-competitive. Calcium uptake apparently is not linked to glycolysis as an energy source since iodoacetate has no effect on transport. A further test to determine if active transport of calcium was being observed was done by an efflux experiment as described under Methods. The results show that neither azide nor 2,4-DNP caused a release of accumulated $^{45}Ca^{++}$ (Fig. 7), which indicates that a dis-

TABLE XIII

Cationic inhibition of calcium uptake in germinated sporangiospores of Achlya. Inhibition was assayed by the initial uptake assay using the method previously described.

Cation	Concentration (μM)	Calcium concentration (μM)	Percent calcium uptake
none	-	50	100%
$Sr(NO_3)_2$	5	50	85.7
	50	50	69.5
$BaCl_2$	5	50	92.9
	50	50	58.9
Pb-acetate	1	100	93.8
	100	100	43.8
Cd-acetate	1	100	86.7
	100	100	71.1
$CoCl_2$	10	100	99.6
	100	100	80.1
$La(NO_3)_3$	100	250	60.4
	250	250	50.3
	500	250	35.3
	1000	250	23.1
$FeCl_3$	10	100	100
	100	100	100
$ZnCl_2$	10	100	100
	100	100	100

Figure 5: Competitive inhibition of calcium transport by Sr^{++} supplied at the following concentrations : (□), 0 mM; (○), 0.1 mM; (△), 0.5 mM; and (●), 1.0 mM.

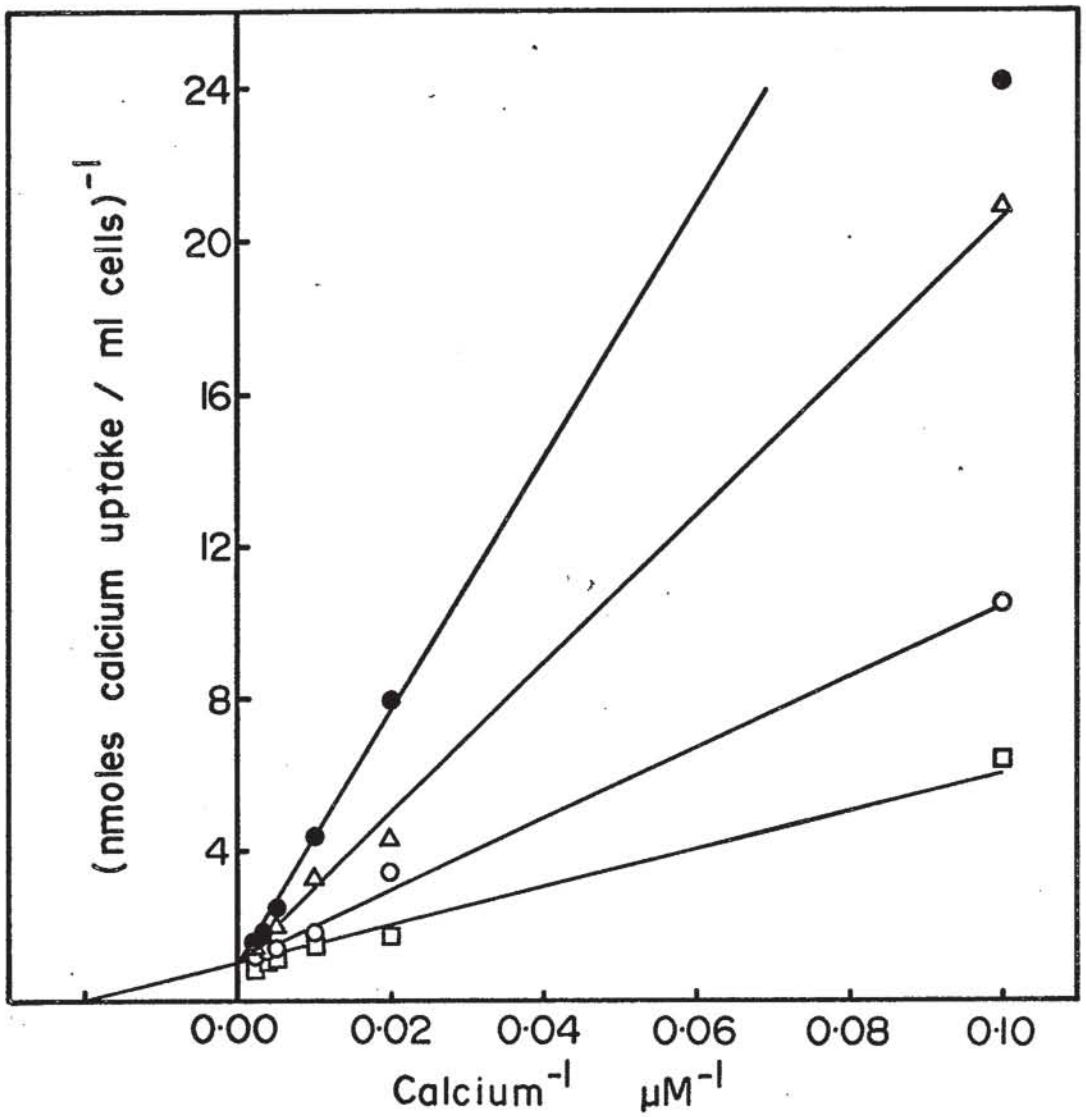

24
20
16
12
8
4
(nmoles calcium uptake / ml cells)$^{-1}$
0·00
0·02
0·04
0·06
0·08
0·10
Calcium^{-1} μM^{-1}

TABLE XIV

Effects of metabolic energy inhibitors and electron transport uncouplers on calcium transport is germinated sporangiospores of Achlya.

Inhibitor	Concentration (μM)	Percent calcium uptake (200 μM)
none	-	100%
CCCP	50	85
2,4-DNP	100	72.4
	500	68.5
	1000	61.6
azide	100	84.4
	500	71.7
	1000	71.4
cyanide	100	60.5
	500	56.4
	1000	55.1
iodoacetate	10	100
	1000	100

Figure 6: Rate-concentration plots and Lineweaver-Burk forms of calcium transport: (a) effect of 2,4-DNP (○), 0 mM; (●), 0.1 mM; (△), 0.25 mM; and (□), 0.5 mM, and (b) effect of Na azide (○), 0 mM; (●), 0.1 mM; (△), 0.25 mM; and (□), 1.0 mM on calcium uptake.

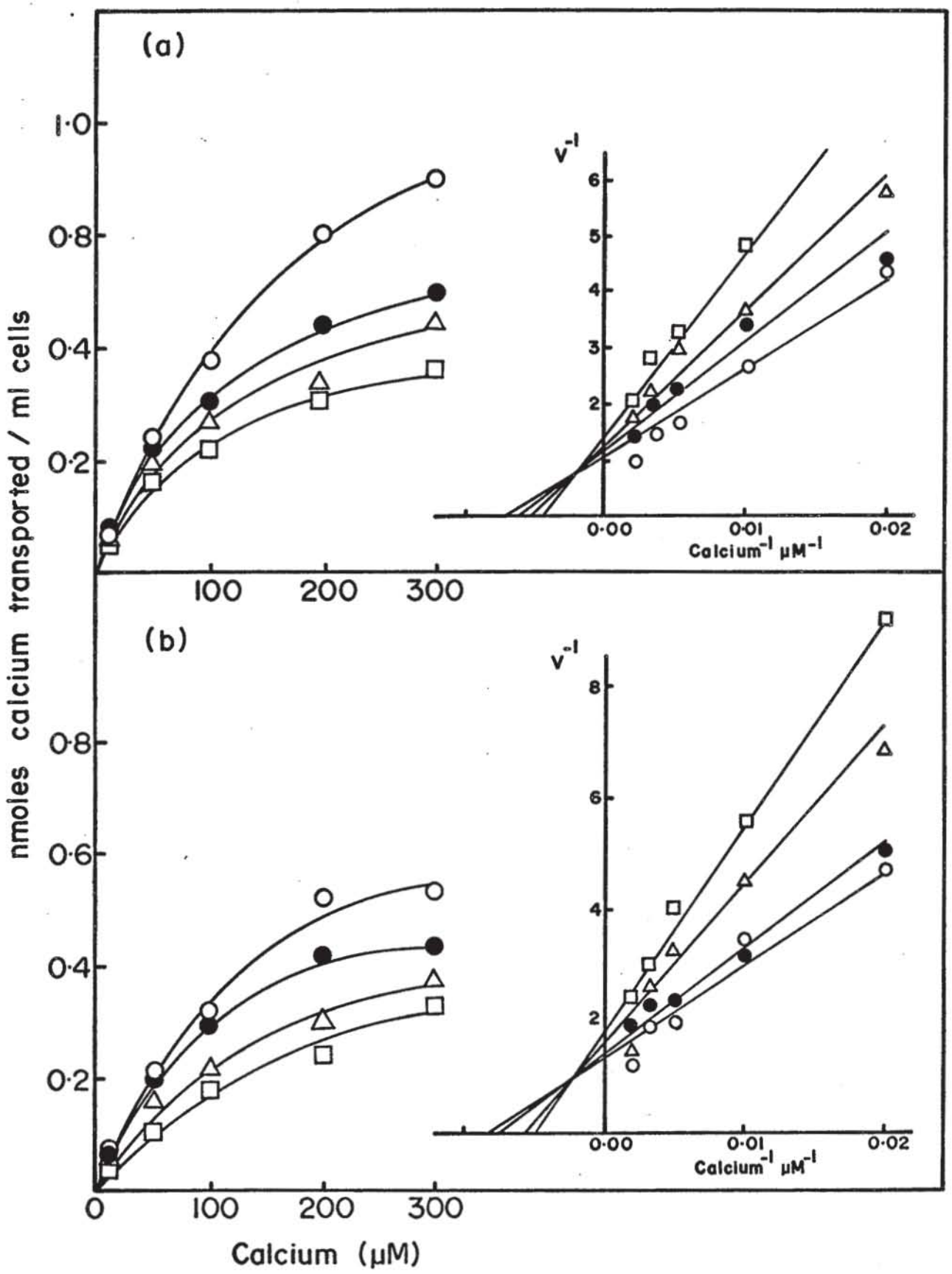
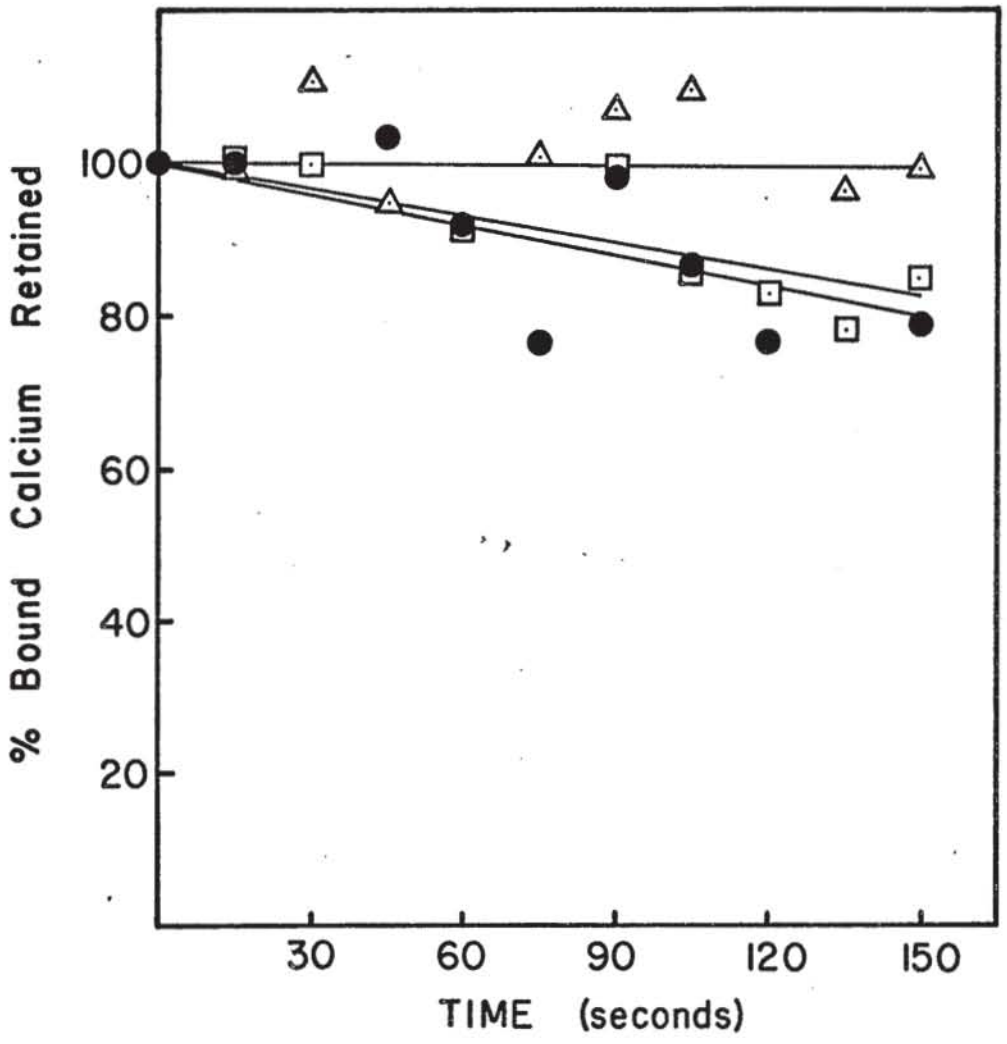

(a)
(b)
nmoles calcium transported / ml cells
Calcium (μM)
0
100
200
300
0·2
0·4
0·6
0·8
1·0
v⁻¹
Calcium⁻¹ μM⁻¹
0·00
0·01
0·02
2
3
4
5
6
8

Figure 7: Calcium efflux as a function of time. The effect of (●), 10 mM Ca^{++}; (⊡), 1 mM azide; and (△), 1 mM 2,4-DNP.

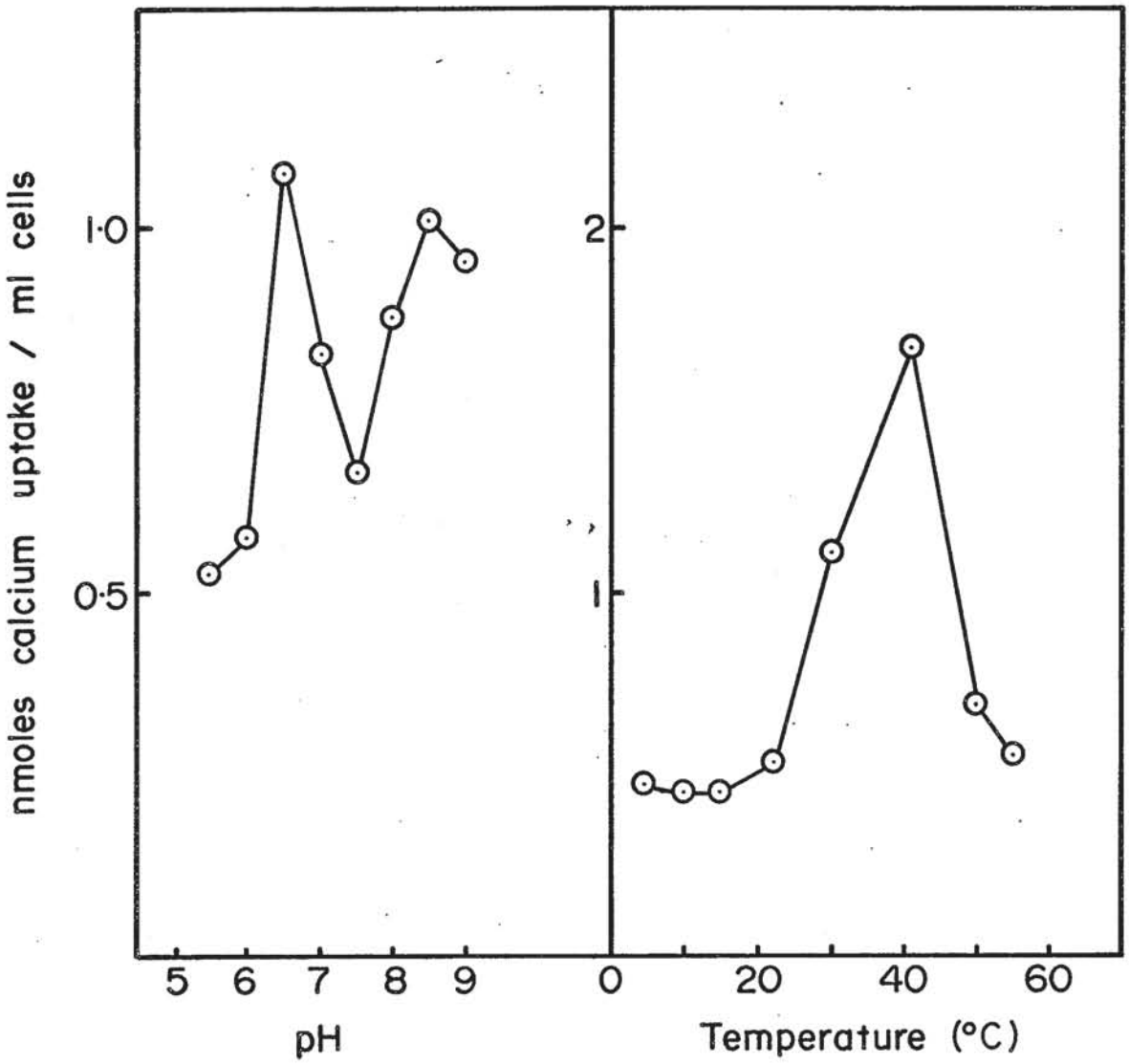

% Bound Calcium Retained
100
80
60
40
20
30
60
90
120
150
TIME (seconds)

turbance of cell energy supply has no apparent effect on calcium release. Results of partial antagonistic effect of metabolic inhibitors on calcium uptake and no effect on calcium efflux suggest that the carrier-mediated calcium transport process is only indirectly connected to cell energy.

pH and temperature effects: Other criteria that are important when characterizing a carrier-mediated transport process is the dependence of initial uptake rates on pH and temperature. The uptake of calcium was found to be dependent on both pH and temperature, as shown in Figure 8, with a temperature optimum in the range of 40°, which is slightly higher than other carrier-mediated transport processes in Achlya, and two pH optima of pH 6.5 and pH 8.5. The pH data added support to the biphasic nature of calcium uptake, since two pH optima also indicate a two component system. Calcium uptake between 5° and 20° was not affected by temperature but from 28° to 40°, calcium uptake was strongly dependent on temperature with a Q_{10} greater than 2 between 22° and 31°, which suggests a high activation energy process may be involved.

Activation by thiol-reacting compounds: During competition studies, $HgCl_2$ was also tested along with other divalent cations to compare antagonistic effect on calcium transport. However, completely opposite results were obtained from those which were expected. By the use of $HgCl_2$ activation effect, additional proof was obtained for the two component calcium transport process.

Figure 8: Influence of (a) pH and (b) temperature on the initial rate of calcium uptake.


nmoles calcium uptake / ml cells
1·0
0·5
2
1
5
6
7
8
9
pH
0
20
40
60
Temperature (°C)

After allowing germinated sporangiospores of Achlya to reach equilibrium in the presence of Ca^{++}, the addition of $HgCl_2$ caused a rapid increase in calcium uptake which reached a new equilibrium condition after 10 min (Fig. 9). This result was unusual not only because $HgCl_2$ should competitively inhibit calcium uptake but also because it normally is an inhibitor of metabolic processes. $HgCl_2$ completely abolished active transport of nucleosides (Stevenson, 1974), amino acids (see later section), and sugars (Goh, 1975). When calcium uptake was studied as a function of $HgCl_2$ concentration, $HgCl_2$ in fact did inhibit calcium uptake but only at high concentration. $HgCl_2$ at 10 μM, produced maximum activation of calcium transport independent of the external calcium concentration (Fig. 10). From the rate-concentration plot and the Lineweaver-Burk form of initial calcium transport (Fig. 11), it was shown that $HgCl_2$ increased the maximum velocity but did not competitively inhibit calcium transport. Calcium transport was competitively inhibited at high concentrations of $HgCl_2$. Other thiol-reacting compounds were tested for ability to activate the calcium transport system, as shown in Figure 12. I_2, Ag^+, HgI_2, thimerosal, and p-hydroxymercuribenzoate stimulated calcium uptake.

To further characterize the effect of sulfhydryls, calcium transport was examined as a function of temperature and pH. Figure 13a shows that $HgCl_2$ activation of calcium uptake is pH dependent with maximum activation at pH 6.5 while at alkaline pH, $HgCl_2$ has no effect on calcium transport. Initial calcium transport assay was carried out as outlined in Methods with the exception that the assay was performed using 12.5 mM Tris-acetate in Buffer A, at a final concentration of

Figure 9: Time-course analysis of the enhancement of Ca^{++} uptake by $HgCl_2$ on germinated sporangiospores of Achlya pre-loaded with $^{45}Ca^{++}$ to equilibrium condition. Effect of $HgCl_2$ at a concentration of (●), 0 mM; and (O), 0.01 mM on calcium transport.

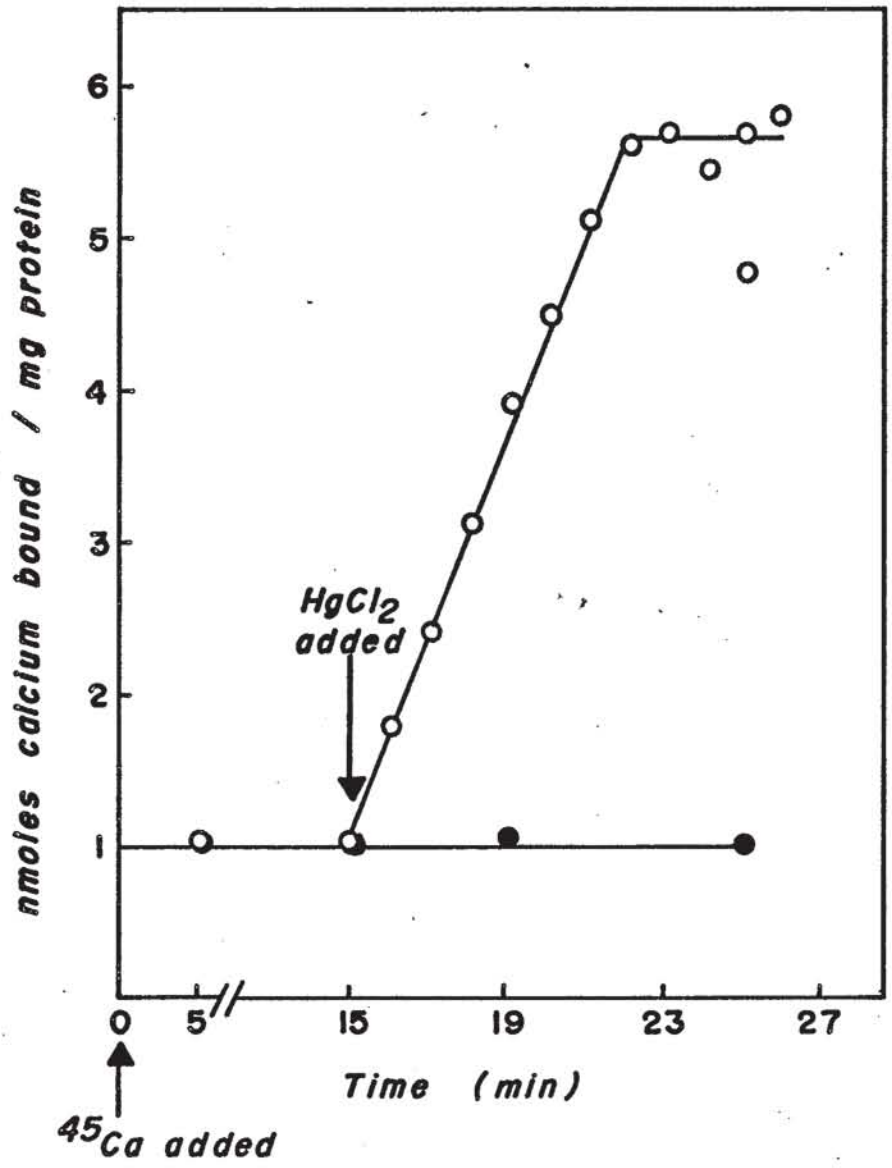

nmoles calcium bound / mg protein
6
5
4
3
2
1
HgCl2 added
0
5
15
19
23
27
Time (min)
45Ca added

Figure 10: Activation plots of calcium transport by $HgCl_2$. $HgCl_2$ effect in the presence of the following fixed calcium concentrations, (O), 0.05 mM; (●), 0.1 mM; and (◑), 0.2 mM.

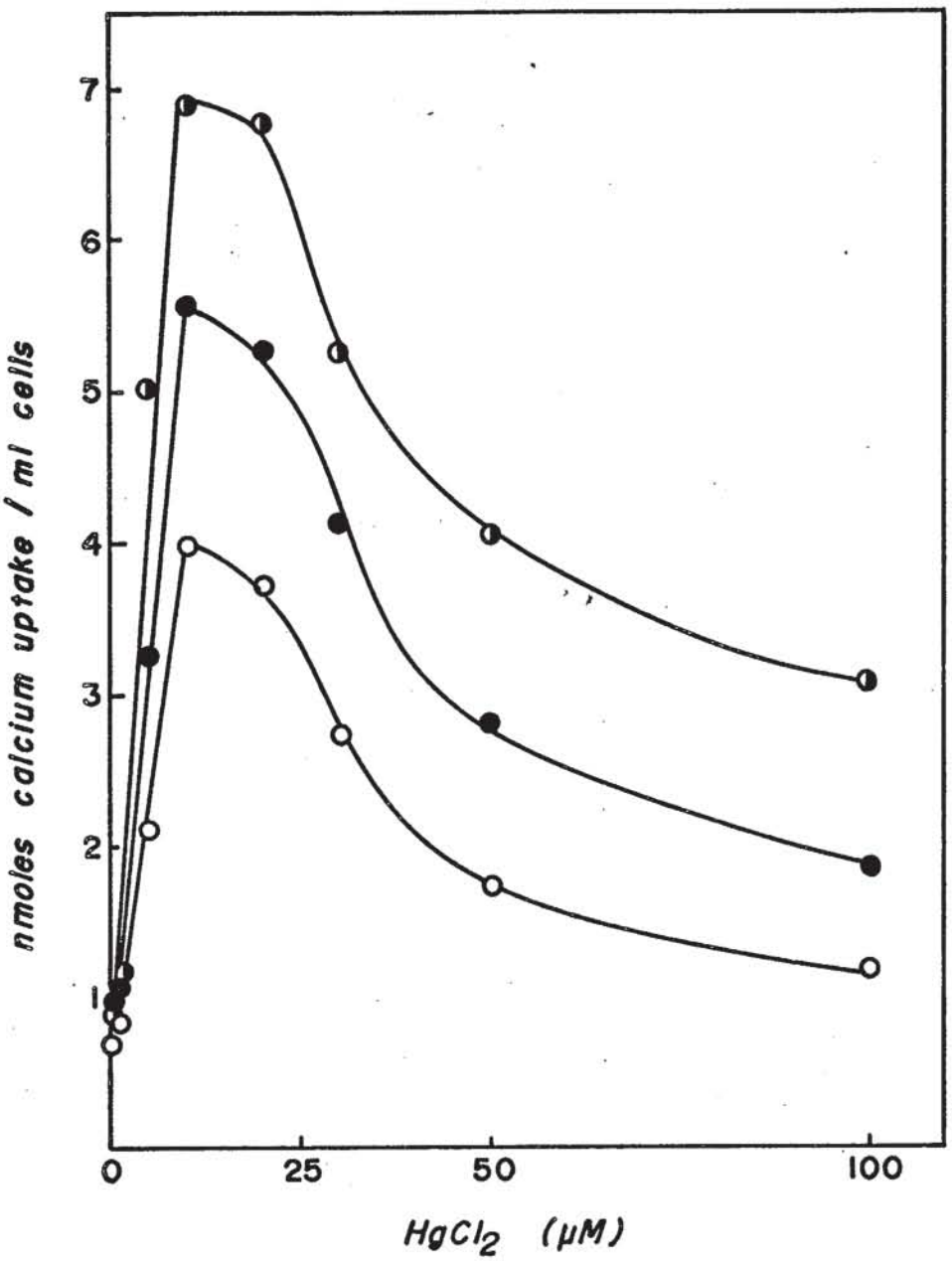

nmoles calcium uptake / ml cells
7
6
5
4
3
2
1
0
25
50
100
HgCl2 (μM)

Figure 11: Rate-concentration plot and Lineweaver-Burk form of calcium transport. The effect of $HgCl_2$ (O), 0 mM; (●), 0.01 mM on calcium transport.

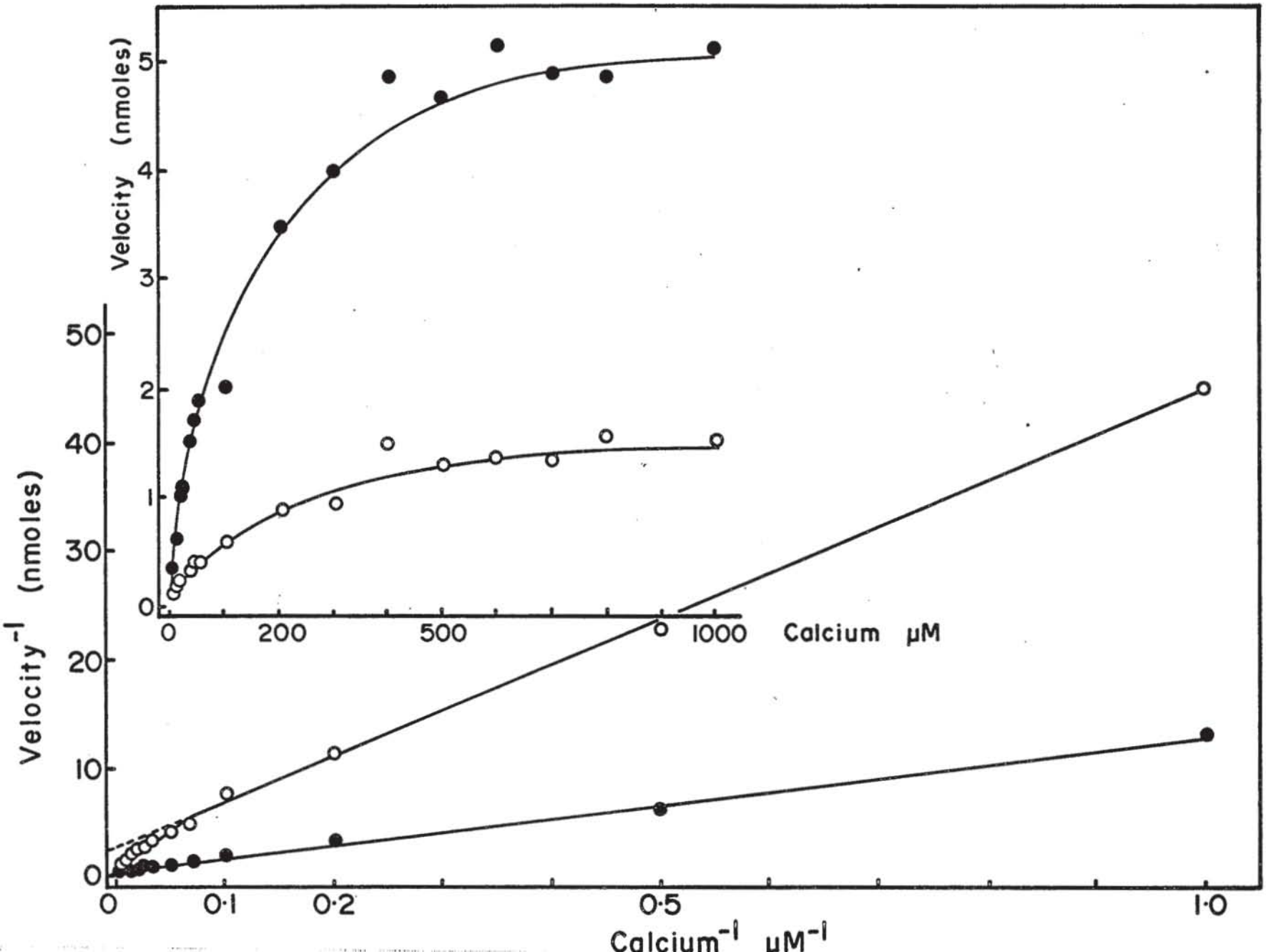

Velocity (nmoles)
0
1
2
3
4
5
0
200
500
1000
Calcium μM
Velocity^{-1} (nmoles)
0
10
20
30
40
50
0
0·1
0·2
0·5
1·0
Calcium^{-1} μM^{-1}

Figure 12: The activation of calcium uptake by thiol-reacting compounds. The effect of (▼), $AgNO_3$; (◑), $HgCl_2$; (▽), thimerosal; (●), I_2; (O), $HgCl_2$ with 0.10 mM L-cysteine.

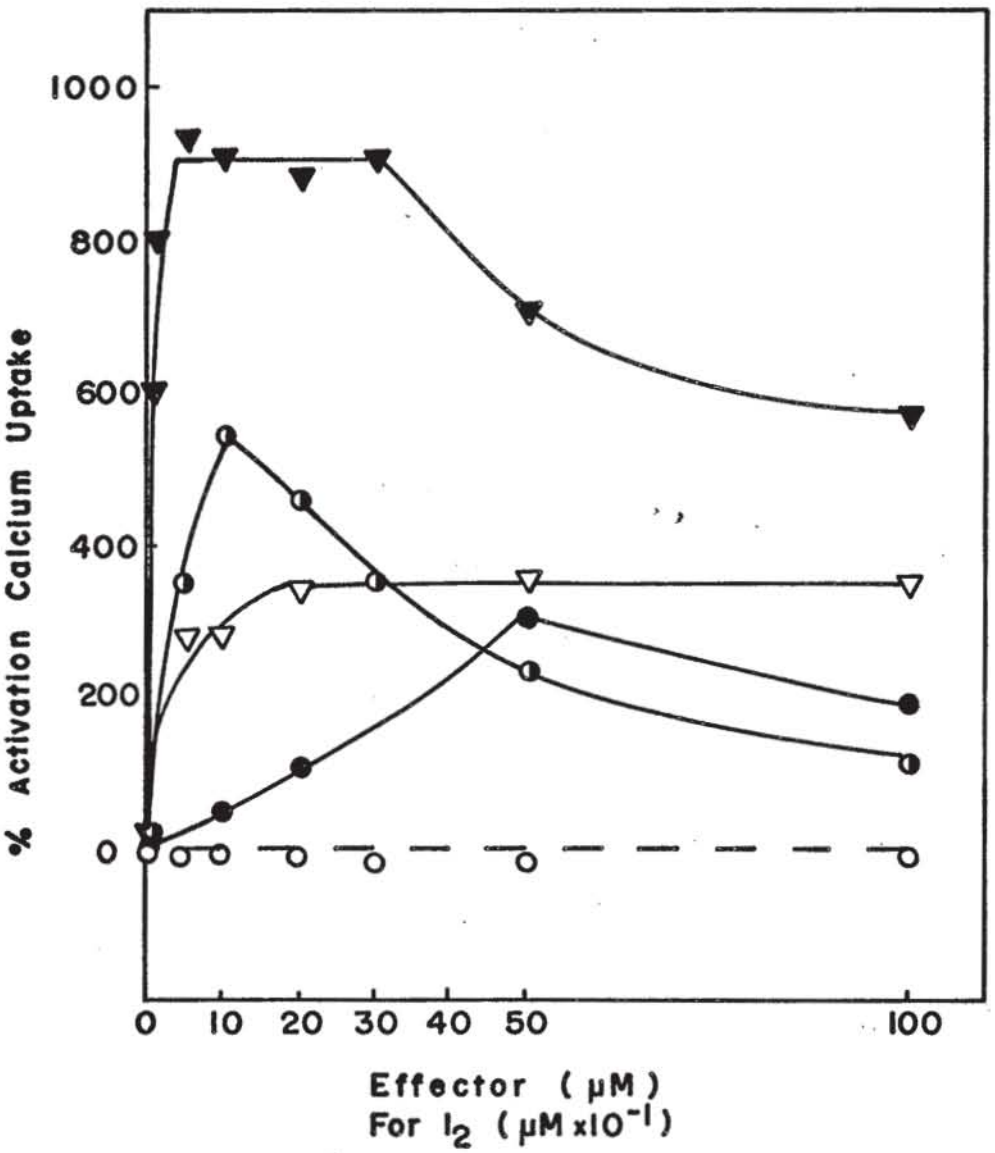
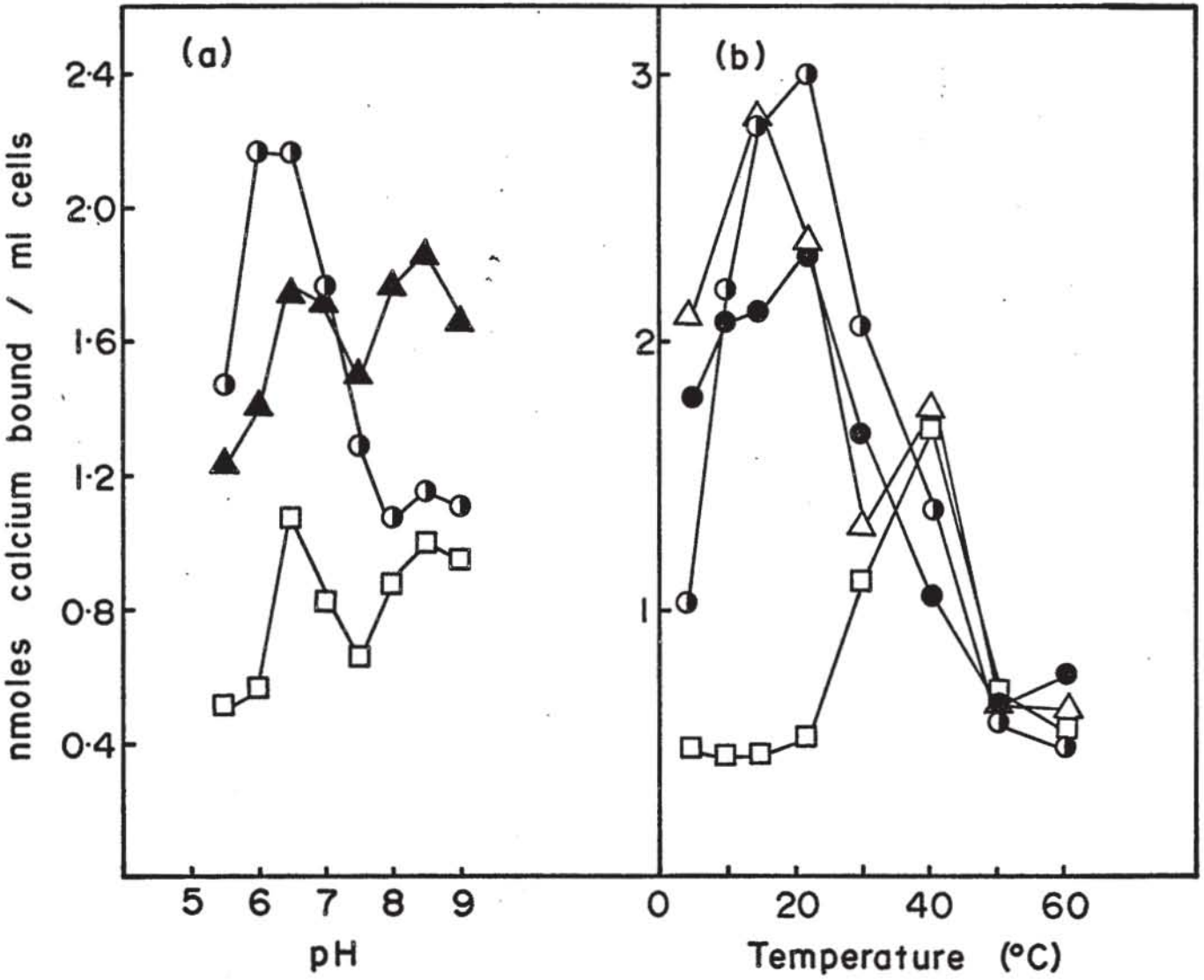

1000
800
600
400
200
0
% Activation Calcium Uptake
0
10
20
30
40
50
100
Effector (μM)
For I_2 (μM x 10^{-1})

Figure 13: (a) The influence of pH on calcium uptake. The effect of (◐), 0.01 mM $HgCl_2$; (▲), 0.005 mM $AgNO_3$; (□), no addition and (b) the influence of temperature on calcium uptake in the presence of (◐), 0.01 mM $HgCl_2$; (△), 0.02 mM thimerosal; (●), 0.5 mM I_2; (□), no addition.

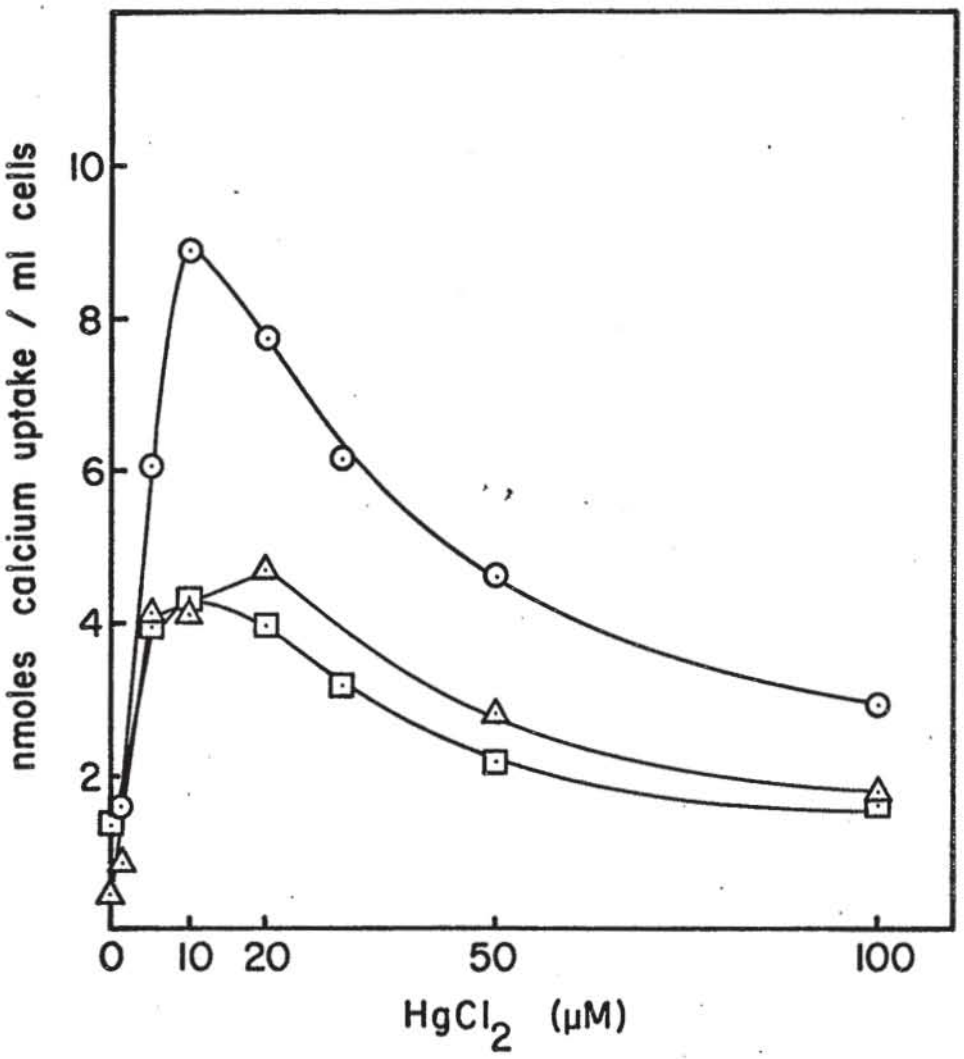

(a)
(b)
nmoles calcium bound / ml cells
2·4
2·0
1·6
1·2
0·8
0·4
3
2
1
5
6
7
8
9
pH
0
20
40
60
Temperature (°C)

250 μM $^{45}Ca^{++}$. Activation of only one calcium uptake pH maximum indicates that $HgCl_2$ does not interact with the complete calcium transport system, and only activates calcium binding to one of the two components. $AgNO_3$ enhanced calcium uptake at both pH maxima. Temperature studies were also instrumental in dissociating the two components of the calcium transport system (Fig. 13b). In the presence of thiol-reacting compounds, calcium transport was optimal at 15°, while the untreated cell optimum of 40° was reduced. The thimerosal-treated cells had in addition to the mercurial optimum of 15°, the untreated cell optimum of 40°. This strongly supported the concept that two components in the Ca^{++} uptake system might be involved.

Germinated sporangiospores were pre-incubated with metabolic inhibitors to examine if energy metabolism was involved in activation of calcium uptake by thiol-reacting compounds. After 15 min incubation with metabolic poison, transport of calcium was studied as a function of mercurial concentration but diluted to a 1:1 ratio with Buffer B (Fig. 14). Both metabolic inhibitors reduced mercurial activation of calcium transport.

Sulfhydryl-agent activation of calcium transport was completely reversed in the presence of an equimolar concentration of the reducing agent, L-cysteine (Fig. 12). After germinated sporangiospores of Achlya had reached a new equilibrium level of calcium in the presence of $HgCl_2$ (see Fig. 9), an efflux experiment with effectors, 0.1 mM cysteine and excess calcium showed that $HgCl_2$ activated calcium transport was quickly reduced in 2 min to the same level of calcium

Figure 14: The activation of 0.2 mM calcium uptake by mercurials with or without pre-treatment with metabolic poisons. Effect of $HgCl_2$ on calcium uptake pre-incubated with (⊙), no addition; (⊡), 0.1 mM 2,4-DNP; (△), 1 mM azide.


nmoles calcium uptake / ml cells
10
8
6
4
2
0
10
20
50
100
HgCl2 (μM)

retained by the cells in the absence of $HgCl_2$ (Fig. 15). The remainder of the bound calcium is apparently either taken up or irreversibly bound by the cell as shown in Figure 16 incubation of $^{45}Ca^{++}$-loaded cells in the presence of excess counterion released only 60% of the calcium taken up. Other cations tested were able to release calcium with varying degrees of effectiveness at 1 mM Ca^{++}: $Ba^{++} = Ca^{++} = Mn^{++} > Mg^{++} > Na^{+} > K^{+}$. The effect of H^{+} on calcium efflux is shown in Figure 17. $^{45}Ca^{++}$ efflux decreased rather sharply between pH 3 and pH 5 and then gradually to pH 7 after which it remained constant. L-cysteine was able to reverse $HgCl_2$ activation of calcium transport added prior to or after a new equilibrium for the calcium transport system had been reached in the presence of $HgCl_2$. Rate-concentration calcium uptake was not activated by cysteine or other reducing agents, such as DTT and β-mercaptoethanol, but cystine did diminish calcium uptake (Fig. 18). As L-cysteine acts to reduce and $HgCl_2$ oxidizes sulfhydryl groups of proteins molecules, the calcium uptake system appears to transport calcium optimally in an oxidized state. The activation of calcium transport by a variety of thiol-reacting compounds, all structurally different, indicates activation is by oxidation of sulfhydryl groups, and the rapidity of reversibility of the mercurial activated calcium uptake by reducing agents indicates the component of the calcium transport system containing the sulfhydryl groups is located on the cell surface. Another compound, iodine, well known for its ability to oxidize macromolecules, also activated calcium uptake (Fig. 12). However, after activation of $^{45}Ca^{++}$ uptake by iodine, subsequent $HgCl_2$ addition reduced calcium uptake to control

Figure 15: Rate of calcium efflux from germinated sporangiospores equilibrated for 15 min with (○), no addition; and (●), 0.10 mM $HgCl_2$.

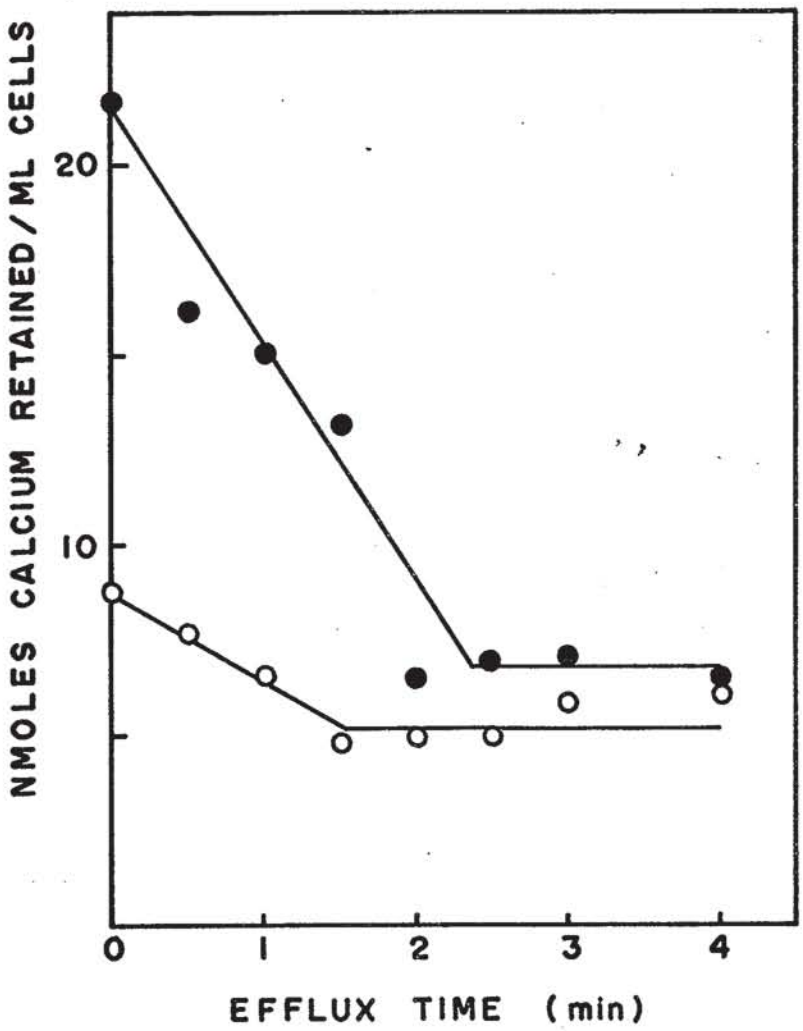

NMOLES CALCIUM RETAINED/ML CELLS
20
10
0
1
2
3
4
EFFLUX TIME (min)

Figure 16: Kinetics of calcium efflux from Achlya mycelial mats just prior to sporangia formation in the presence of competing ions.

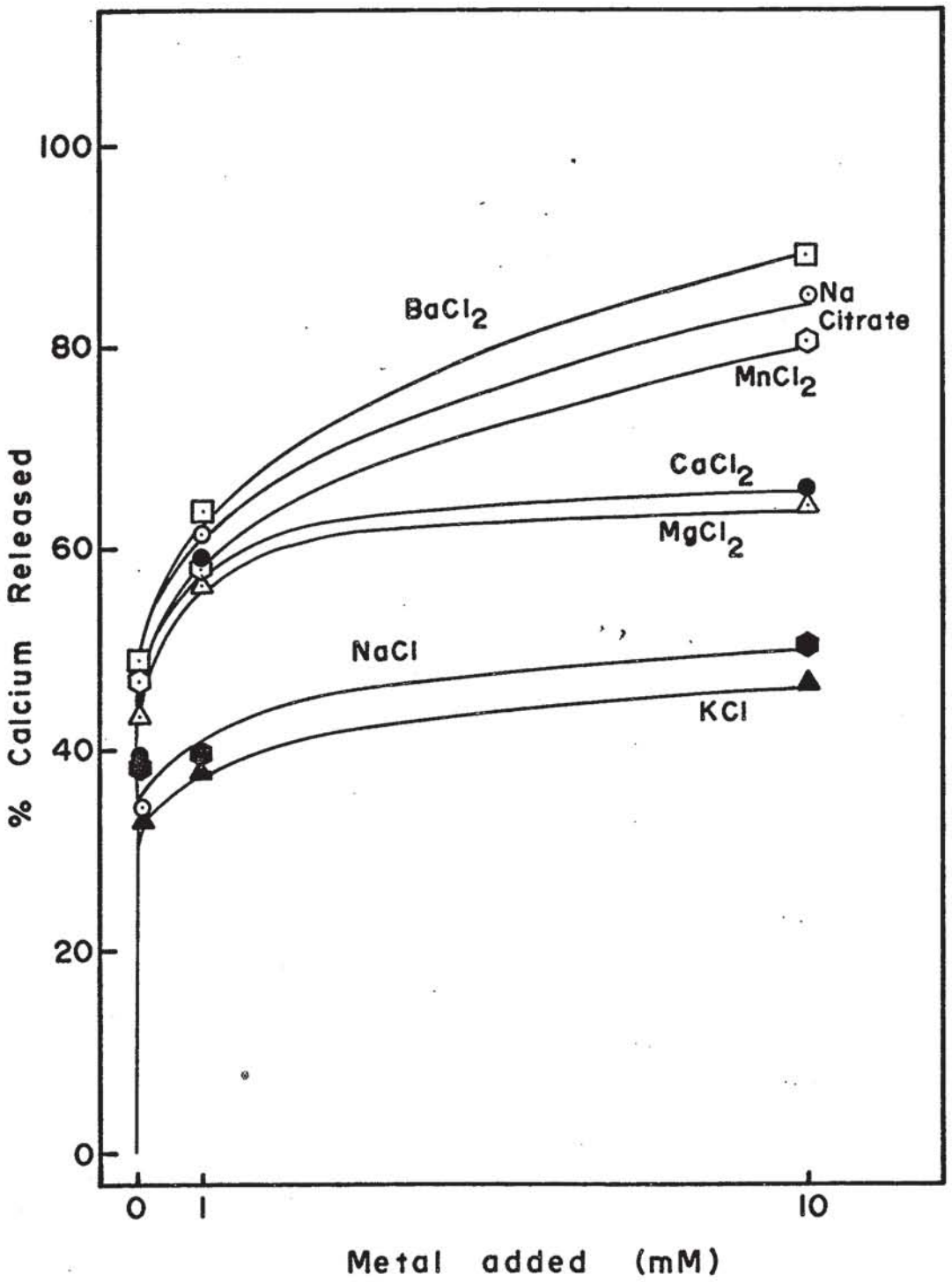

100
80
60
40
20
0
% Calcium Released
0
1
10
Metal added (mM)
BaCl2
Na Citrate
MnCl2
CaCl2
MgCl2
NaCl
KCl

Figure 17: Kinetics of calcium efflux from Achlya mycelial mats as a function of pH.

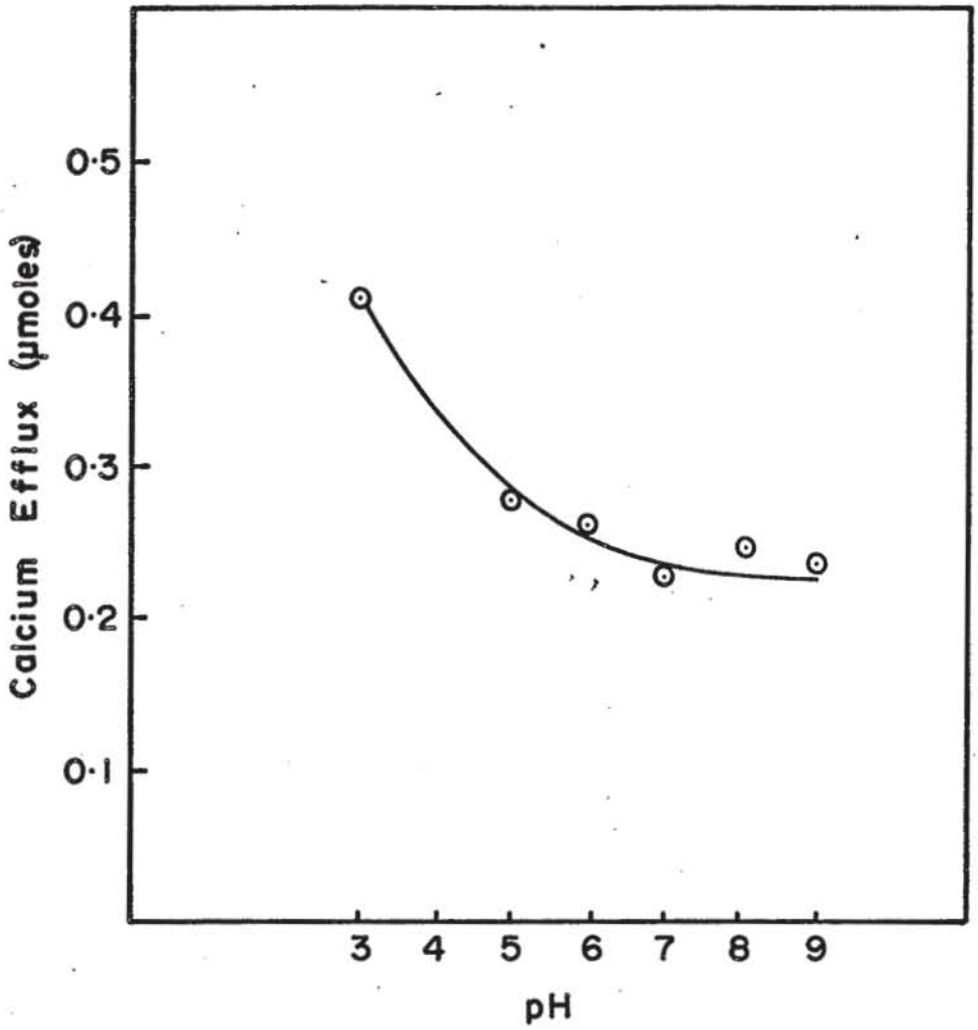
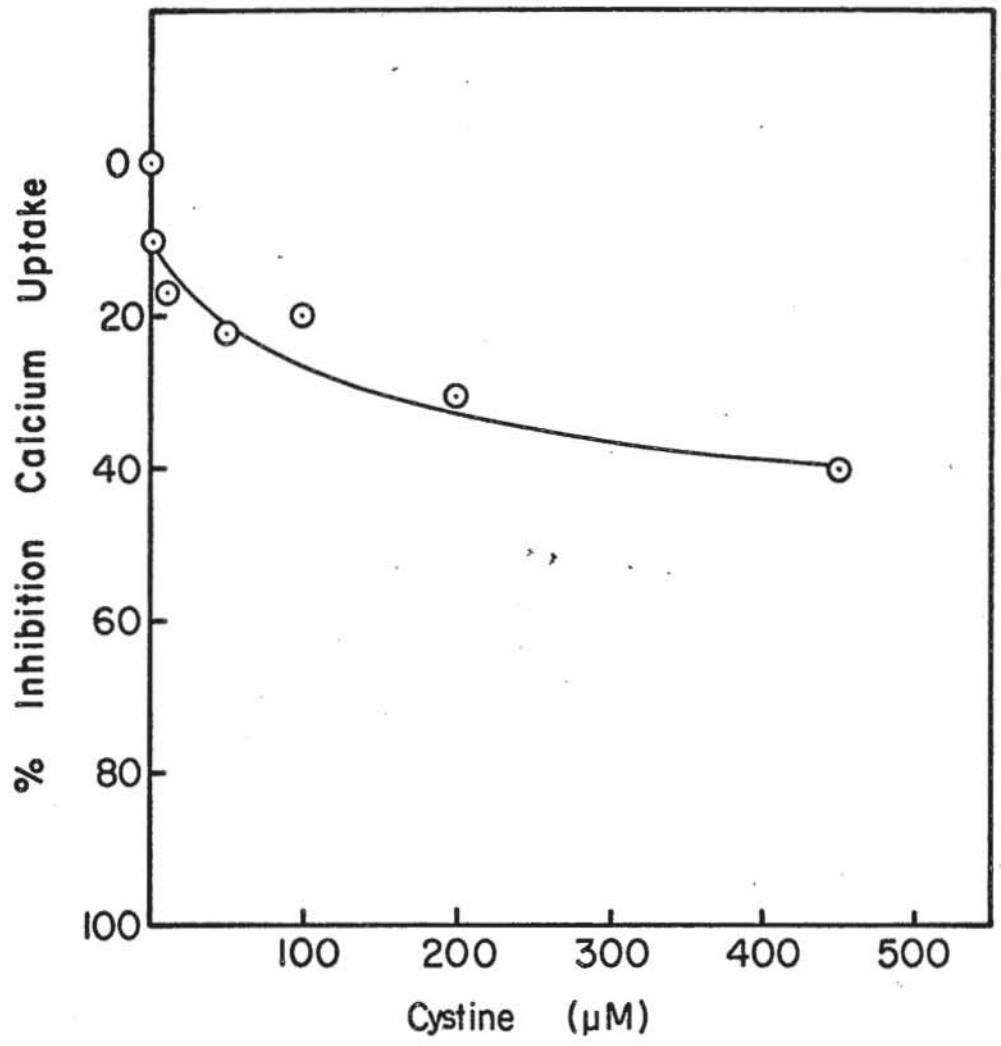

Calcium Efflux (μmoles)
0·5
0·4
0·3
0·2
0·1
3
4
5
6
7
8
9
pH

Figure 18: Inhibition of calcium uptake by cystine.


% Inhibition Calcium Uptake
0
20
40
60
80
100
100
200
300
400
500
Cystine (μM)

level (Fig. 19). When cells are pretreated with iodine before the addition of $HgCl_2$, the resulting inhibition is probably due to $HgCl_2$ binding to calcium binding sites similar to inhibition in the presence of high $HgCl_2$ concentration.

B. Intrinsic Calcium Transport Component

Cold osmotic shocked cells: In germinated sporangiospores of Achlya which have been cold osmotically shocked as described under Methods, calcium is still taken up by the cells but with a reduced transport capacity. Osmotic shocked cells remain intact and viable, having the ability to resume growth after an 8 to 10 h lag period in growth medium. In contrast to normal cells, the rate-concentration plot followed normal Michaelian form with an apparent K_m value of 4×10^{-5} M (Fig. 20), which agrees well with the low affinity calcium binding component of whole cells. To affirm that the calcium transport process was an intrinsic part of the plasma membrane, calcium transport was examined in membrane vesicles. Membrane fragments were isolated from cold osmotically shocked Achlya cells by the method of repeated sonication, washing, and centrifugation. A small quantity of cells on ice were sonicated with an ultra-sonic probe for 30 s, washed with 2 mM Tris-acetate, pH 7, and centrifuged at 5,000 x g for 10 min. This process was repeated numerous times until all the membrane fragments were cleaned of cytoplasmic material as determined by microscopic examination. The fragmented membrane preparation was suspended at a final concentration of 2 mM Tris-acetate, pH 7. The

Figure 19: Effect of sulfhydryl-reacting compounds on calcium transport. $HgCl_2$ activation pre-treated with iodine (O), and not pre-treated (Δ).

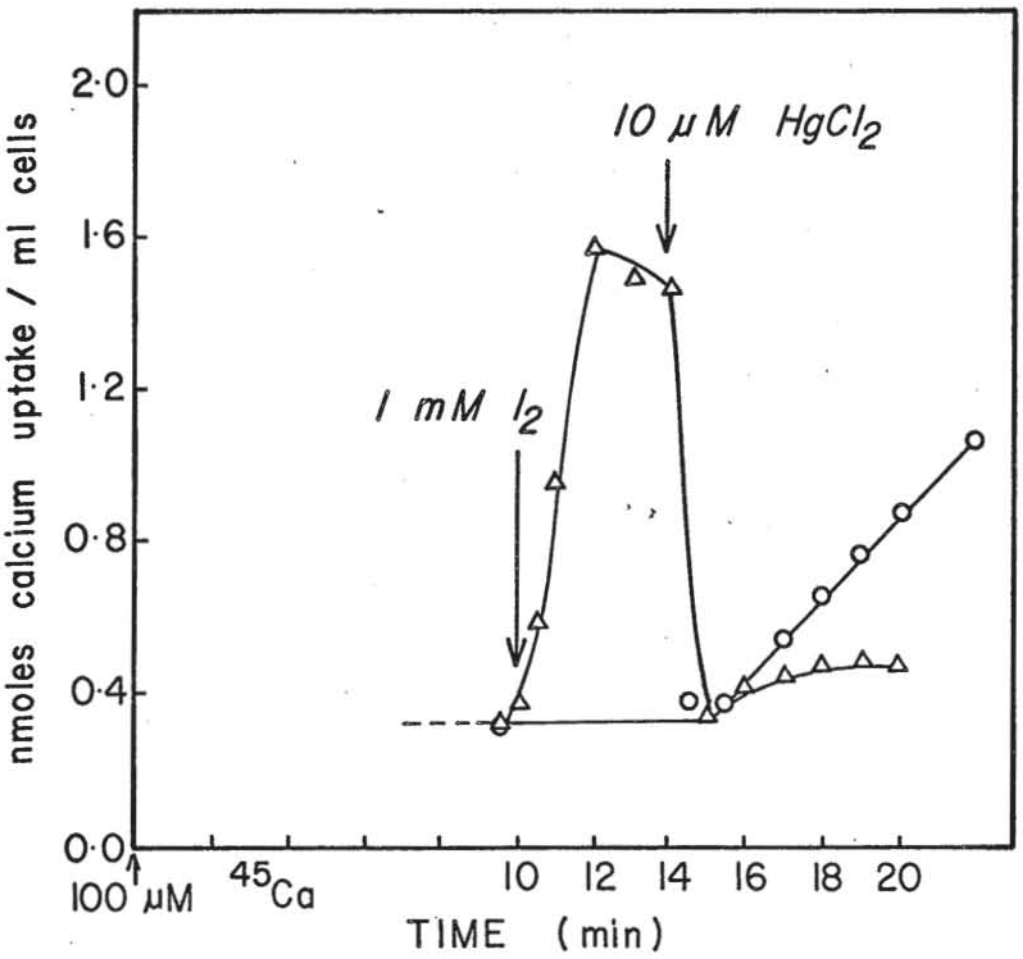
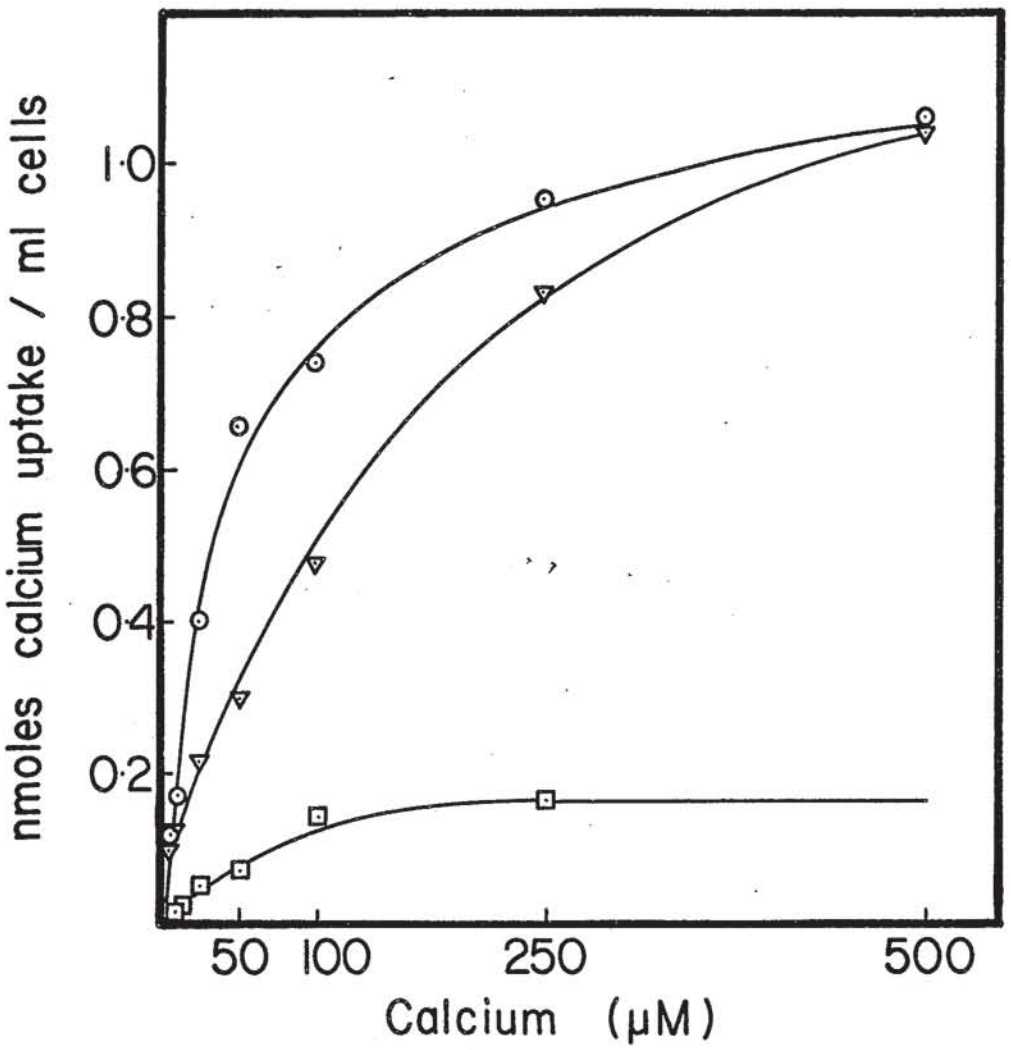

nmoles calcium uptake / ml cells
2·0
1·6
1·2
0·8
0·4
0·0
10 μM HgCl2
1 mM I2
100 μM 45Ca
10
12
14
16
18
20
TIME (min)

Figure 20: Rate-concentration plot of calcium transport in cold osmotically shocked germinated sporangiospores. The effect of $HgCl_2$ (⊙), 0 mM; (▽), 0.01 mM; and ruthenium red (⊡), 0.25 mM.


nmoles calcium uptake / ml cells
1·0
0·8
0·6
0·4
0·2
50
100
250
500
Calcium (μM)

rate-concentration plot and Lineweaver-Burk form of calcium uptake in the resulting vesicles is shown in Figure 21. Calcium transport followed normal Michaelis-Menten kinetics and was transported with an apparent K_m of 5.1×10^{-5} M which is in agreement with both the low affinity transport process of normal cells and apparent K_m value for transport in osmotically shocked cells.

Table XV lists the effect of some important compounds on the calcium transport system of shocked cells. In general, the effects are similar to those found for normal cells, except that sulfhydryl-reacting compounds no longer activate calcium transport. Moreover, sulfhydryl-reacting compounds were slightly inhibitory at low calcium concentration, as shown for $HgCl_2$ in Figure 20 and 21.

Support for the conclusion that only one component of the calcium transport process remained in shocked cells came from pH and temperature studies (Fig. 22). There was only one pH optimum at alkaline pH (pH 8.5), and mercurial compounds no longer differentially activated calcium uptake. Cold osmotic shocked cells had a temperature optimum of 40° that resembled the normal cell temperature optimum, but sulfhydryl-reacting compounds no longer stimulated calcium uptake at an optimal of 15°. In agreement with the negative mercurial effect on shocked cells, cysteine was found to have no effect on calcium transport or $^{45}Ca^{++}/^{40}Ca^{++}$ exchange efflux whether the cold osmotic shocked cells were incubated in the absence or presence of $HgCl_2$.

Figure 21: Rate-concentration plot and Lineweaver-Burk form of calcium binding by membrane vesicles. The effect of (○), no addition; (△), 1 mM $MgCl_2$; and (□), 0.01 mM $HgCl_2$.

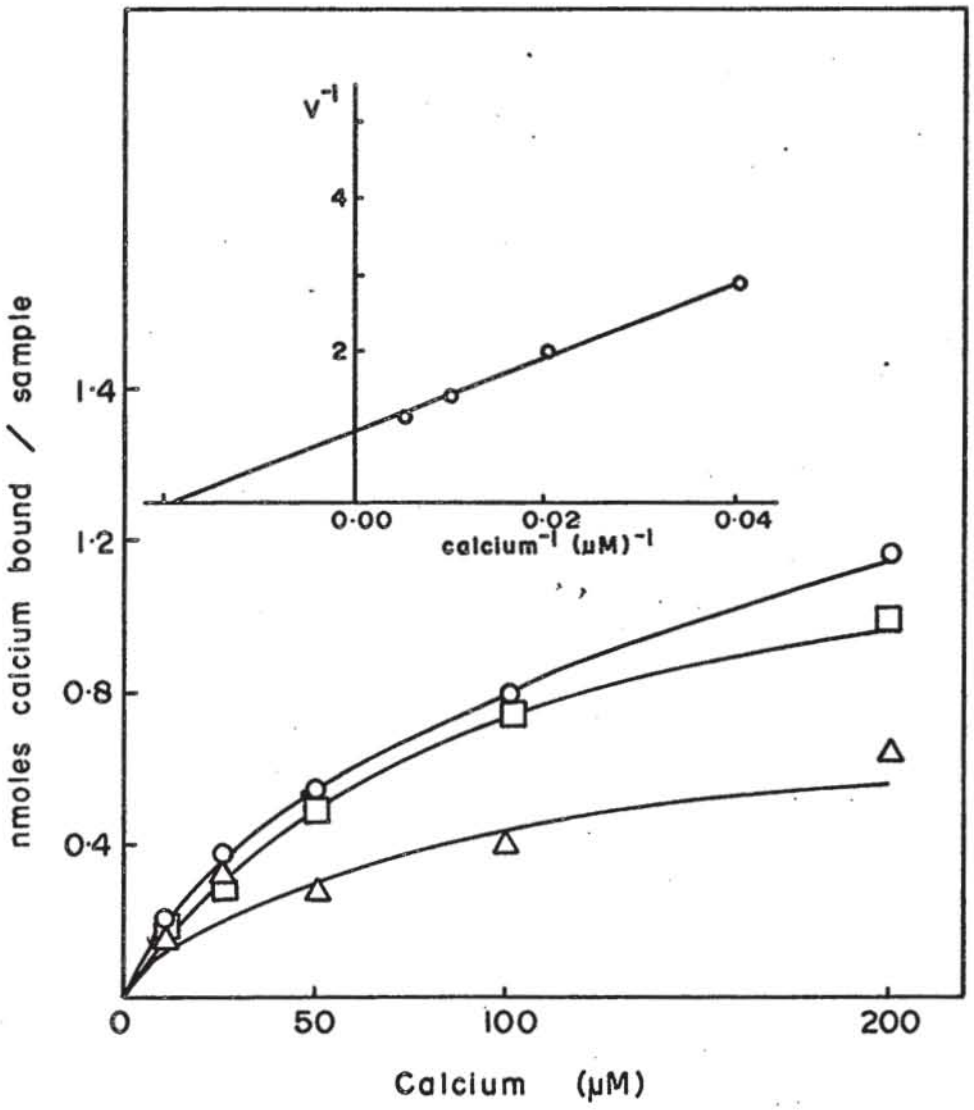
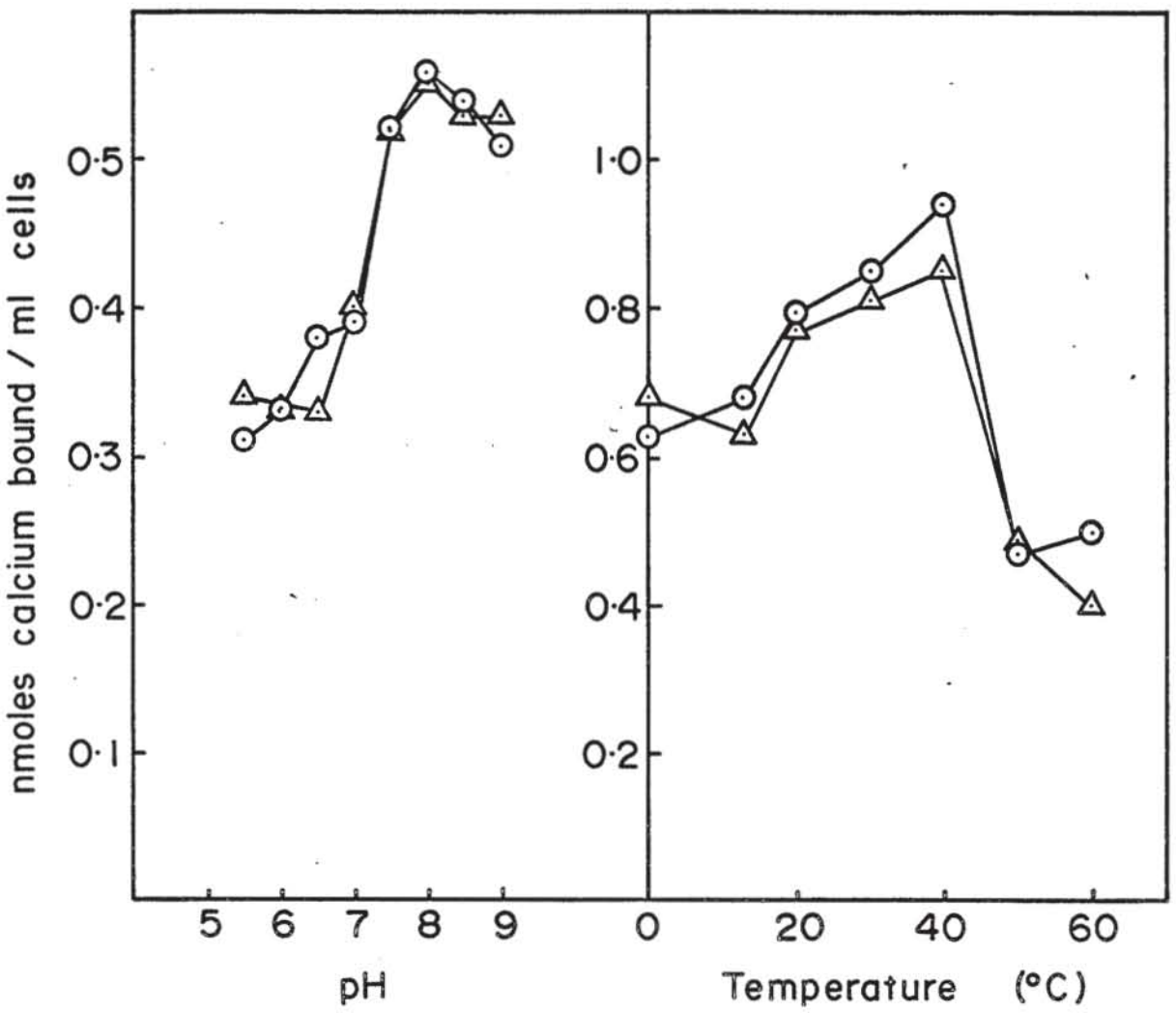

V⁻¹
4
2
0·00
0·02
0·04
calcium⁻¹ (μM)⁻¹
nmoles calcium bound / sample
1·4
1·2
0·8
0·4
0
50
100
200
Calcium (μM)

TABLE XV

Inhibition of calcium transport in osmotically shocked germinated sporangiospores of Achlya by a variety of compounds.

Effector	Concentration (μM)	Percent calcium transported (200 μM)
-	-	100%
2,4-DNP	100	82.2
	500	93.8
	1000	85.2
ruthenium red	50	28.6
	100	24.5
	500	8.8
citrate	50	87.6
	100	82.2
	500	73.4
$Sr(NO_3)_2$	100	70.3
	500	46.7
	1000	34.5
$La(NO_3)_3$	100	17.5
	500	9.0
	1000	8.4
$HgCl_2$	10	96.7
	50	69.4
	100	52.7
iodine	100	53.0
	500	83.2
	1000	83.8
$MnCl_2$	100	52.2
$MgCl_2$	500	51.1

Figure 22: Influence of (a) pH and (b) temperature on calcium uptake in cold osmotically shocked germinated sporangiospores in the presence of 0.01 mM $HgCl_2$ (△), and in the absence (⊙).


nmoles calcium bound / ml cells
0·5
0·4
0·3
0·2
0·1
5
6
7
8
9
pH
1·0
0·8
0·6
0·4
0·2
0
20
40
60
Temperature (°C)

C. Surface Calcium Binding Component

Kinetic values and specificity: After cold osmotic shock treatment less than 0.1% of the total cellular protein was released from the cell. In bacteria, both the sulfate (Pardee, 1968) and the phosphate (see Oxender, 1972) transport systems have associated binding proteins that can be released by osmotic shock. In Achlya, kinetic studies, reversibility of sulfhydryl activation, and reduced transport in cold osmotically shocked cells indicated that a calcium binding glycopeptide may be associated with the calcium transport process. Osmotic shock fluid of Achlya when assayed by equilibrium dialysis as described under Methods was found to bind calcium. Equilibrium dialysis experiments because of high affinity binding activity at low calcium concentration were employed for kinetic analysis of calcium binding activity. A maximum calcium binding equilibrium was reached within 3 h at 4^{o} under experimental conditions described in Methods, and the equilibrium conditions was maintained at this level for 12 h, after which a gradual decrease in binding occurred (Fig. 23b). Figure 23a shows the almost instantaneous reaction time of $^{45}Ca^{++}$ binding as assayed by the anionic exchange resin Dowex 2-X8 assay, however, maximum binding was 4 times less than could be obtained by the equilibrium dialysis procedure. The rate-concentration plot and reciprocal form (inset) of calcium binding activity as measured by anionic Dowex exchange resin assay are shown in Figure 24. This assay method was inadequate for kinetic analysis due to the problem of poor binding in the presence of low external calcium concentration. Only one type of binding activity was discerned by this method, which had a

Figure 23: Reaction time of calcium binding activity by glycopeptide as measured by (a) anionic exchange resin Dowex 2-X8 assay and (b) equilibrium dialysis assay.

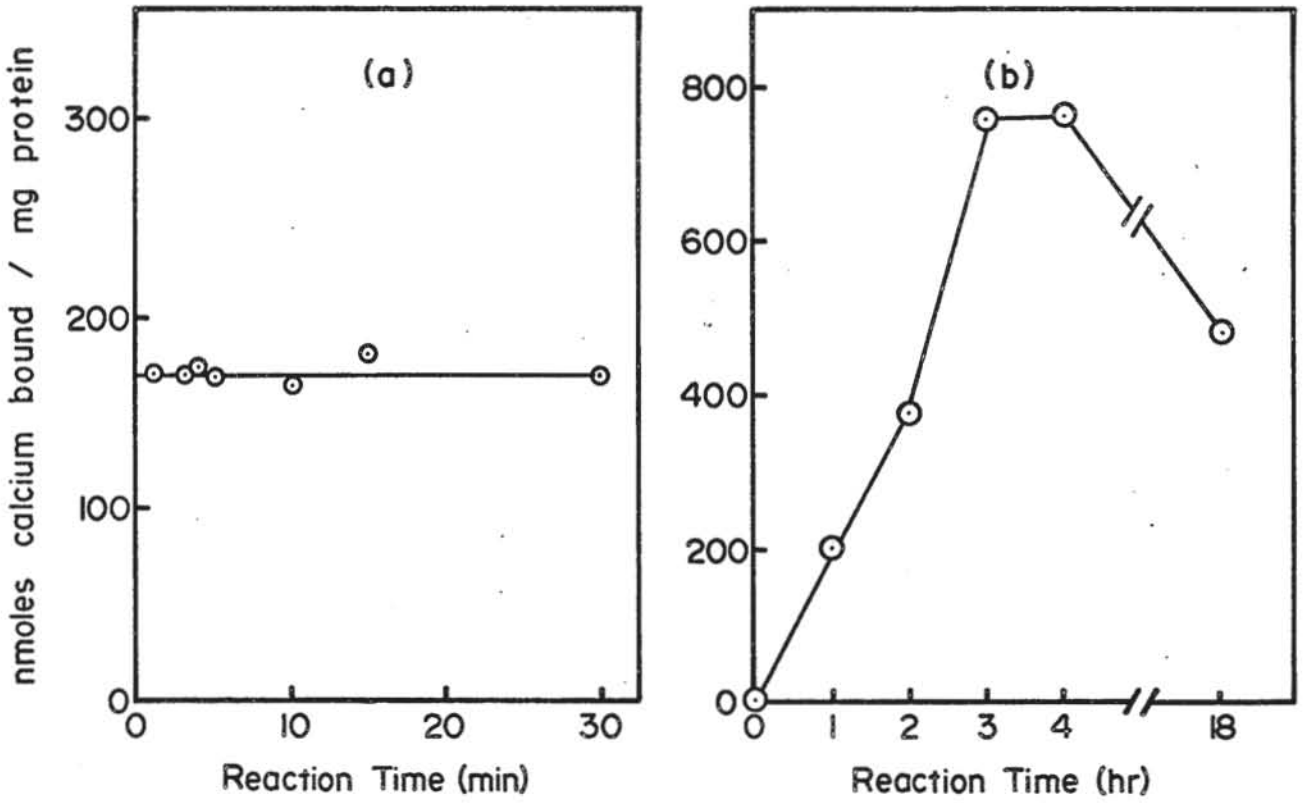
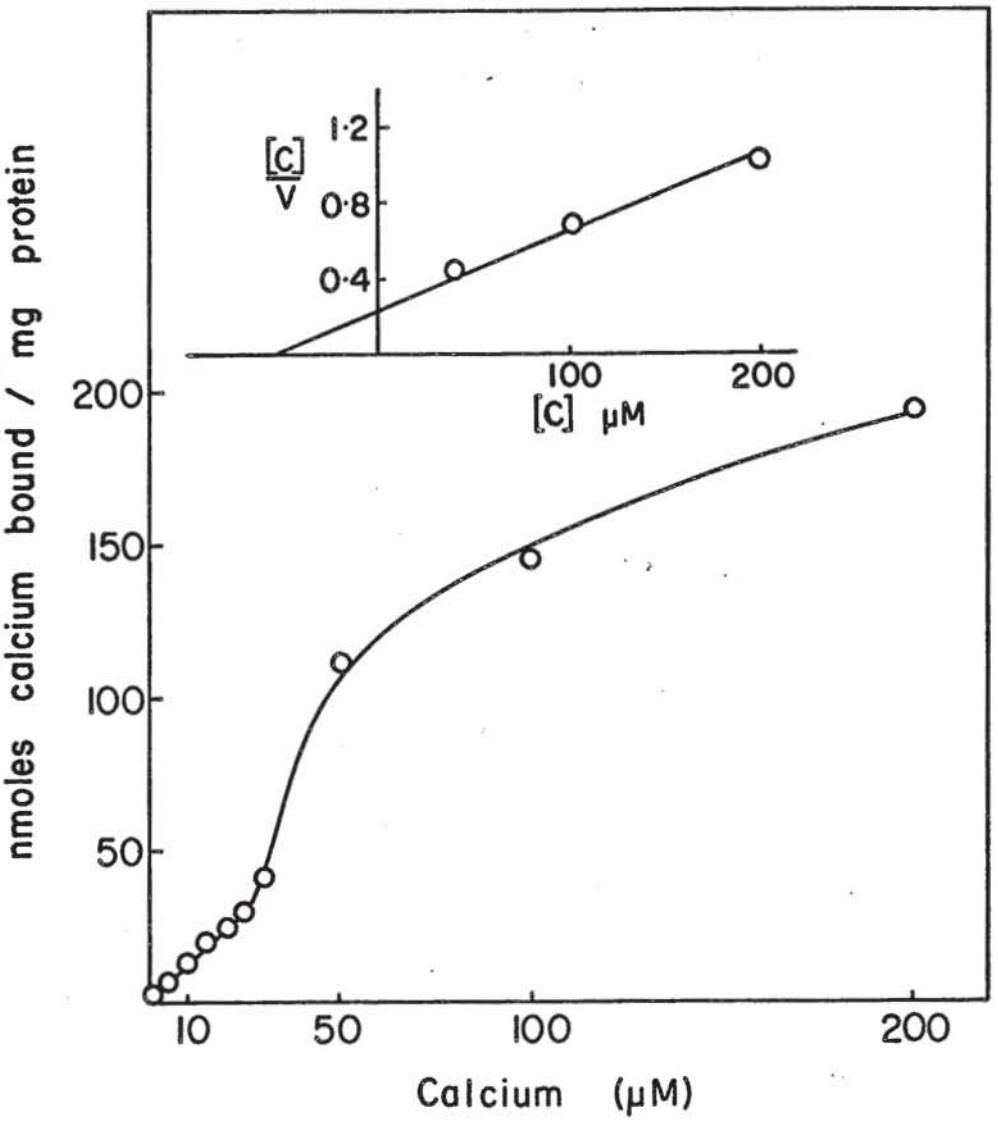

(a)
(b)
nmoles calcium bound / mg protein
0
100
200
300
0
10
20
30
Reaction Time (min)
0
200
400
600
800
0
1
2
3
4
18
Reaction Time (hr)

Figure 24: Rate-concentration plot and reciprocal form (inset) for glycopeptide calcium binding activity assayed by anionic exchange resin: Dowex 2-X8 (50 - 100 mesh).


nmoles calcium bound / mg protein
200
150
100
50
10
50
100
200
Calcium (μM)
[C]/V
1·2
0·8
0·4
100
200
[C] μM

dissociation constant of 1.5×10^{-5} M.

In subsequent studies, the surface calcium binding component will be denoted calcium binding glycopeptide due to chemical constitution as characterized in a later section. The rate-concentration plot and the Scatchard form of calcium binding activity as measured by equilibrium dialysis are shown in Figure 25. Futhermore calcium binding activity was unaffected by the concentration of glycopeptide for the concentration range: 20 µg protein/ml to 20 mg protein/ml. When calculations were based on the Scatchard plot, one mole of glycopeptide bound a total of 7 - 14 moles of calcium of which approximately one half were bound with a $K_d = 5.52 \times 10^{-6}$. A dissociation constant equivalent to the apparent affinity constant for calcium uptake in whole cells. The Scatchard plots of calcium binding activity for glycopeptide isolated from two other water-moulds are presented in Figure 26. The Scatchard plot form for Pythium and Blastocladiella are linear, not biphasic as suggested for Achlya. The biphasic Scatchard plot of Achlya can be interpreted as (i) two different calcium binding afffinity sites, (ii) negative co-operative calcium binding, that is, the more calcium bound, the more difficut it becomes to bind calcium, or (iii) different affinity binding of calcium by monomer glycopeptide and aggregate glycopeptide. A summary of the dissociation constants are presented in Table XVI. Basically, the dissociation constants of the three fungal species were the same.

To establish the specificity of calcium binding by the glycopeptide, other cations were examined for their ability to interfere with calcium binding. The effects of various cations on calcium binding by the glycopeptide are listed in Table XVII. Other divalent cations reduced glycopeptide binding activity with the following degree of binding specificity: $Mn^{++} > Ba^{++} > Sr^{++} > Mg^{++}$. Since

Figure 25: Rate-concentration plot and Scatchard form of calcium binding by Achlya glycopeptide.

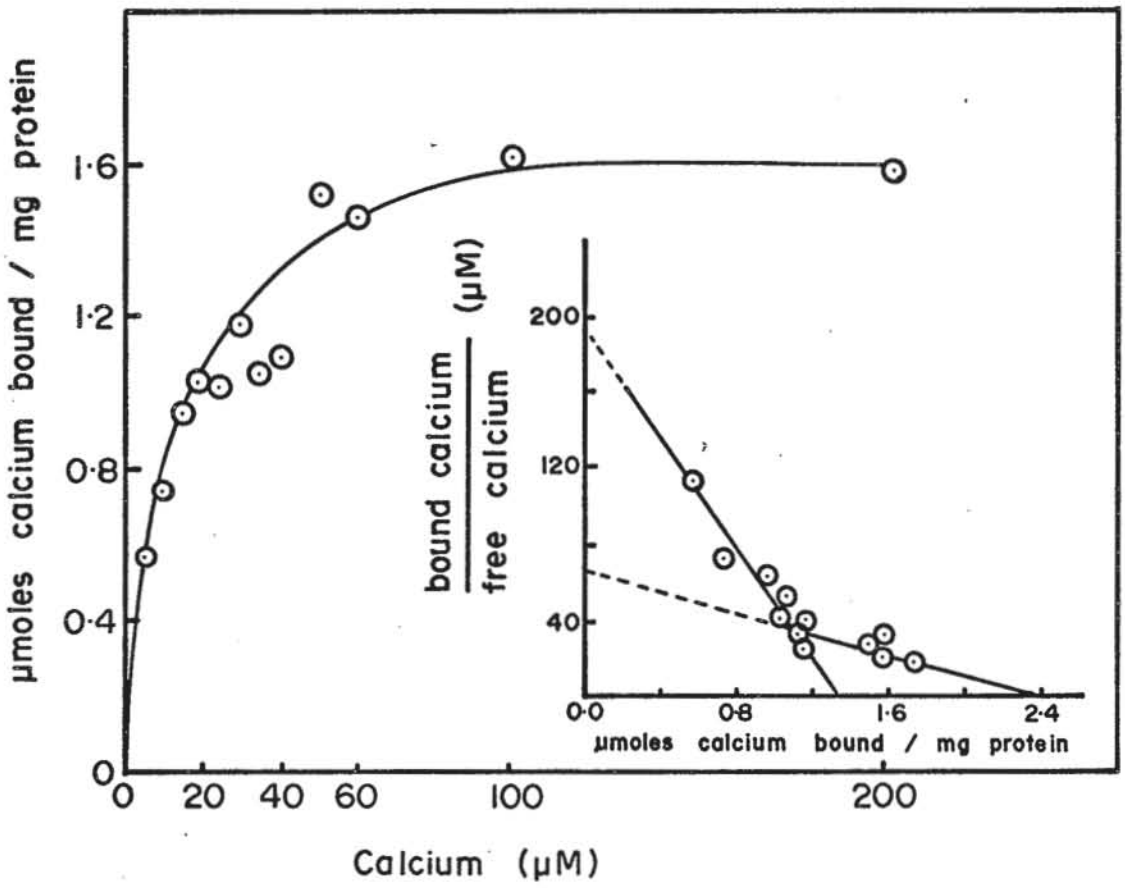

µmoles calcium bound / mg protein
1·6
1·2
0·8
0·4
0
0
20
40
60
100
200
Calcium (µM)
bound calcium / free calcium (µM)
200
120
40
0·0
0·8
1·6
2·4
µmoles calcium bound / mg protein

Figure 26: Scatchard plots of calcium binding activity for glycopeptides isolated from three fungi. (a) Achlya, (b) Pythium and (c) Blastocladiella.

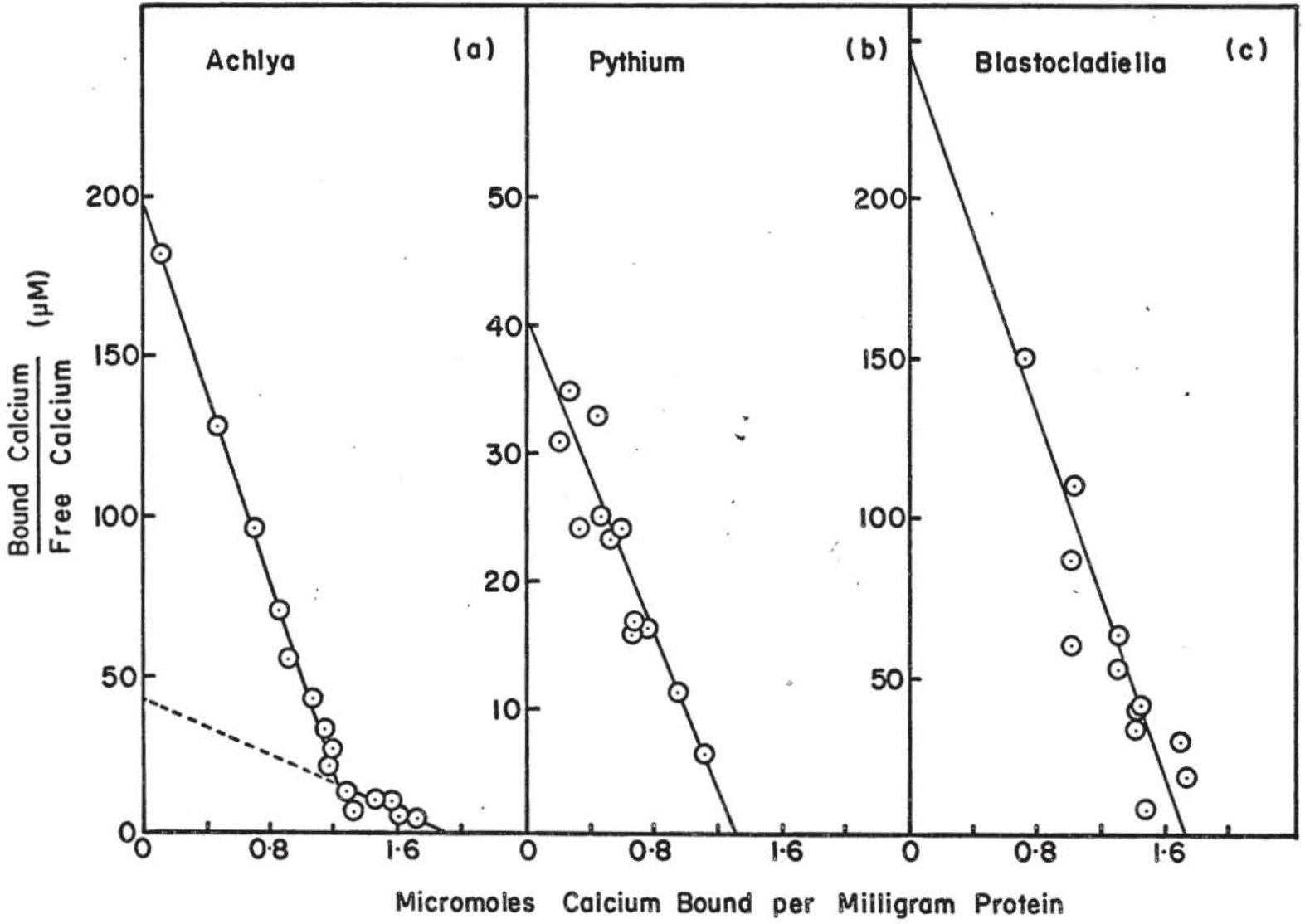

Achlya
(a)
Pythium
(b)
Blastocladiella
(c)
Bound Calcium / Free Calcium (μM)
200
150
100
50
0
50
40
30
20
10
0
0·8
1·6
Micromoles Calcium Bound per Milligram Protein

TABLE XVI

A summary of kinetic constants for three fungal glycopeptides. Calcium binding was assayed by equilibrium dialysis as described under Methods. Glycopeptide was treated with enzyme at a concentration of 1 mg enzyme/mg protein for 30 min at 30° prior to equilibrium dialysis. Enzymes were inactivated by boiling and the mixture used to study glycopeptide binding.

Source of glycopeptide	Enzyme treatment (1 mg/mg protein)	K_D (M) Ca^{++}	Mn^{++}
Achlya sp.	-	5.52×10^{-6}	5.8×10^{-6}
		2.41×10^{-5}	-
	lysozyme	1.15×10^{-5}	8.9×10^{-6}
		3.47×10^{-5}	-
	crude pronase	2.81×10^{-5}	3.1×10^{-5}
Blastocladiella emersonii	-	5.8×10^{-6}	-
Pythium heterothallicum	-	3.42×10^{-5}	-

TABLE XVII

Chemical and physical effects on the calcium binding activity of Achlya glycopeptide. Assay was carried out by equilibrium dialysis of 4°C for 4 hr against 1 mM tris-acetate, pH 7, with 10 μM $^{45}Ca^{++}$.

Treatment		Per cent of control
A. Nil		100
B. Cations added		
$MnCl_2$	(10^{-4}M)	8
$MgCl_2$	(10^{-4}M)	78
$BaCl_2$	(10^{-4}M)	35
$Sr(NO_3)_2$	(10^{-4}M)	56
KCl	(50 mM)	4
NaCl	(50 mM)	3
C. Enzyme action		
Ribonuclease	(0.5 mg/mg glycopeptide)	103
Neuraminidase	(0.5 mg/mg glycopeptide)	105
Pronase	(0.5 mg/mg glycopeptide)	48
Lysozyme	(0.5 mg/mg glycopeptide)	45
D. Miscellaneous		
Ruthenium red	(2.5×10^{-4}M)	22
$HgCl_2$	(2.5×10^{-8}M)	220
	(1.0×10^{-6}M)	122
	(1.0×10^{-5}M)	70
Iodine	(2.5×10^{-5}M)	300
100°C for 5 min		97
24°C for 4 hr		125
Citrate (10 mM)		45

manganese readily competed with calcium binding activity, the ability of the glycopeptide to bind Mn^{++} was studied. The rate-concentration plot and Scatchard form for $^{54}Mn^{++}$ binding are shown in Figure 27. Unlike glycopeptide Ca^{++} binding activity, a linear Scatchard relationship for glycopeptide Mn^{++} binding activity was obtained.

Of particular interest was the ability of $HgCl_2$ to activate calcium binding by the glycopeptide when present at a very low concentration. Enhansed calcium binding by the glycopeptide was quantitatively 4 fold less than $HgCl_2$ activated calcium uptake in whole cells. This discrepancy is apparently due to the in vitro assay system for calcium binding by the glycopeptide. This indicates that the calcium binding glycopeptide released by osmotic shock treatment is associated with the transport system in intact cells and appears to be indentical to the component that is selectively activated by mercurials in whole cells which has an apparent $K_m = 5 \times 10^{-6}$ M. In agreement, iodine was found to activate calcium binding activity. The glycopeptide probably binds calcium maximally when oxidized.

The influence of citrate and ruthenium red on calcium binding activity was checked as shown in Table XVII. Inhibition of calcium binding activity by these two compounds, both specific antagonists of the calcium transport process (see later section), confirms that the calcium binding glycopeptide is associated with the calcium transport process.

Calcium binding glycopeptide and ontogenic cycle: The ability of Achlya cells to release calcium binding glycopeptide throughout the growth cycle was studied. Spores were unable to release any material with calcium binding property. In fact, little proteinaceous material was released from the spores. Table XVIII lists the dissociation

Figure 27: Rate-concentration plot and inset Scatchard plot of manganese binding activity.

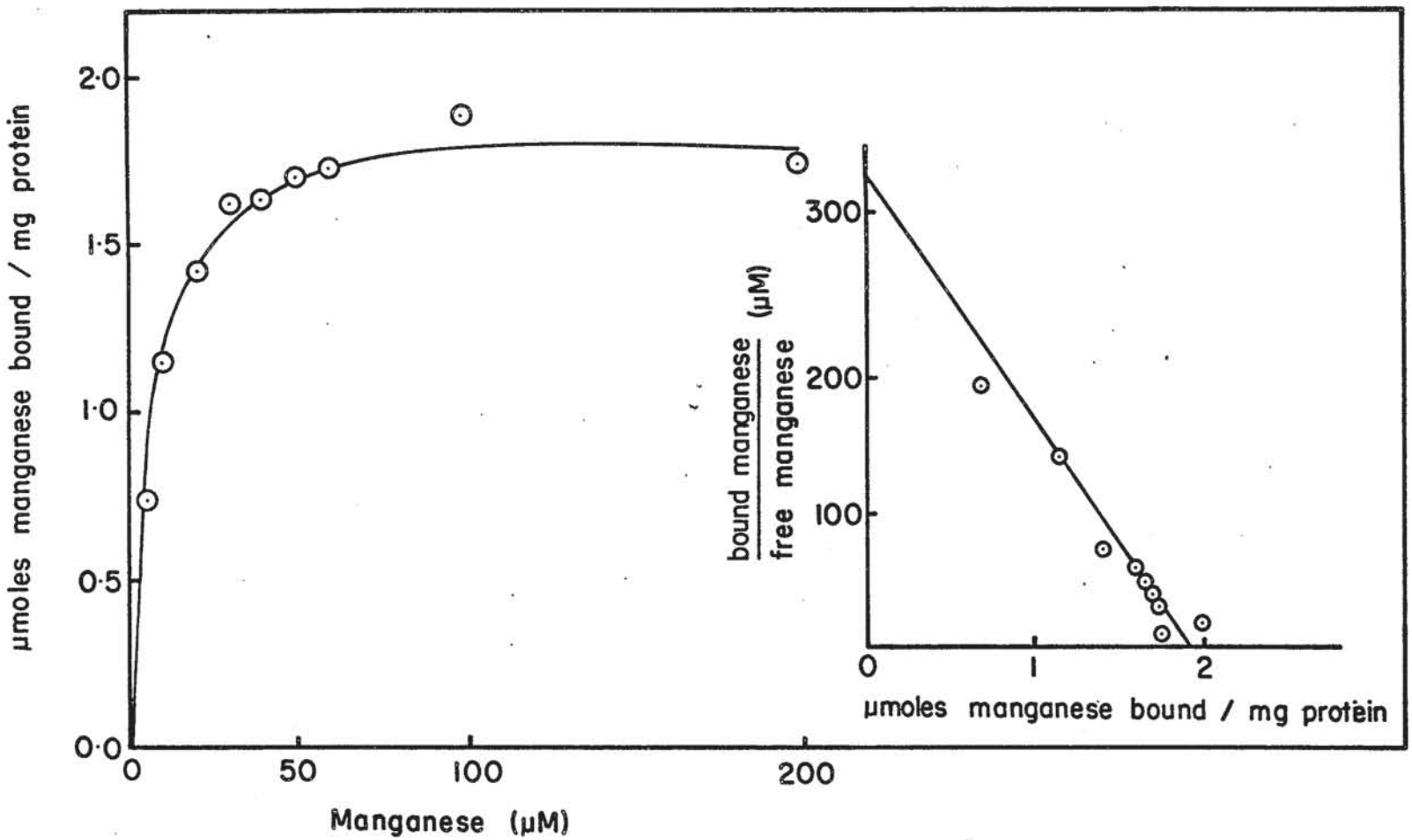

2·0
1·5
1·0
0·5
0·0
µmoles manganese bound / mg protein
0
50
100
200
Manganese (µM)
300
200
100
bound manganese / free manganese (µM)
0
1
2
µmoles manganese bound / mg protein

TABLE XVIII

Relationship between ontogeny and Ca^{++} binding affinity of glycopeptide of Achlya.

Age of organism (hr)	K_D (M) of Ca^{++} binding
2	5.52×10^{-6} and 2.41×10^{-5}
6	5.52×10^{-6} and 2.41×10^{-5}
8	3.50×10^{-5}
12	2.85×10^{-5}
16	2.62×10^{-5}
24	3.12×10^{-5}

constant values of calcium binding at time intervals throughout the growth cycle. There was little change in low affinity K_d values throughout the growth cycle, however, higher affinity binding property was lost after six hours. The reason why a change in calcium binding affinity occurs is unknown, but the change indicates that a relationship between glycopeptide and differentiation possibly exists.

The effect that the external concentration of calcium has on the amount of calcium binding glycopeptide released and its specific activity was examined, but both remained the same whether in the absence of calcium or in the presence of 0.01 mM to 1.0 mM calcium.

Influence on the release of calcium binding protein was of interest as a possible means of determining its association with the cell surface. When spores were first germinated in defined medium of Barksdale (1963) prior to cold osmotic shock treatment, osmotic shock procedure was almost impossible due to cell clumping. No glycopeptide was released from these cells. Evidence that the cells were affected in some manner came from studies on growth. Unlike GY medium-grown osmotic shocked germinated sporangiospores, defined medium grown osmotic shocked germinated sporangiospores were unable to resume growth. The reason why this occurs is unknown as no protein was lost and cells remained intact although severely clumped.

Synthesis of calcium binding glycopeptide: It was of interest to determine the regeneration time of the calcium binding glycopeptide

by Achlya cells after cold osmotic shock treatment. The selective property of $HgCl_2$ activation of only normal cell calcium uptake was useful for the study of glycopeptide resynthesis after cold osmotic shock treatment. At selected time intervals, cell samples were tested for their ability to be activated by 20 μg $HgCl_2$, the concentration where maximum activation occurred in GY medium. Figure 28 shows the time dependent resynthesis of calcium binding glycopeptide. Significant synthesis of glycopeptide had been initiated by 10 h, which correlates with the time of resumption of growth, again implicating a possible role of glycopeptide in growth functions.

Osmotic shock treatment had no lasting detrimental effect on the resynthesis of glycopeptide. Upon re-isolation of calcium binding glycopeptide from twice-osmotically shocked cells, the Scatchard plot and dissociation constant values for calcium binding activity was identical to initial glycopeptide isolated (Fig. 25 and Table XVI). It was important to immediately resuspend cold osmotically shocked cells in growth medium. When osmotic shocked cells were incubated at $0^{\circ}C$ (ice bath) overnight, neither growth nor resynthesis of glycopeptide occurred when cells were resuspended in growth medium at 22°.

D. Characterization of Calcium Binding Glycopeptide

Purification: The calcium binding glycopeptide was isolated and purified from the crude osmotic shock fluid as summarized in Table XIX. The procedures used during purification are described under

Figure 28: Difference plot of calcium uptake as a function of time: calcium uptake in the presence of 0.02 mM $HgCl_2$ minus uptake in the absence.

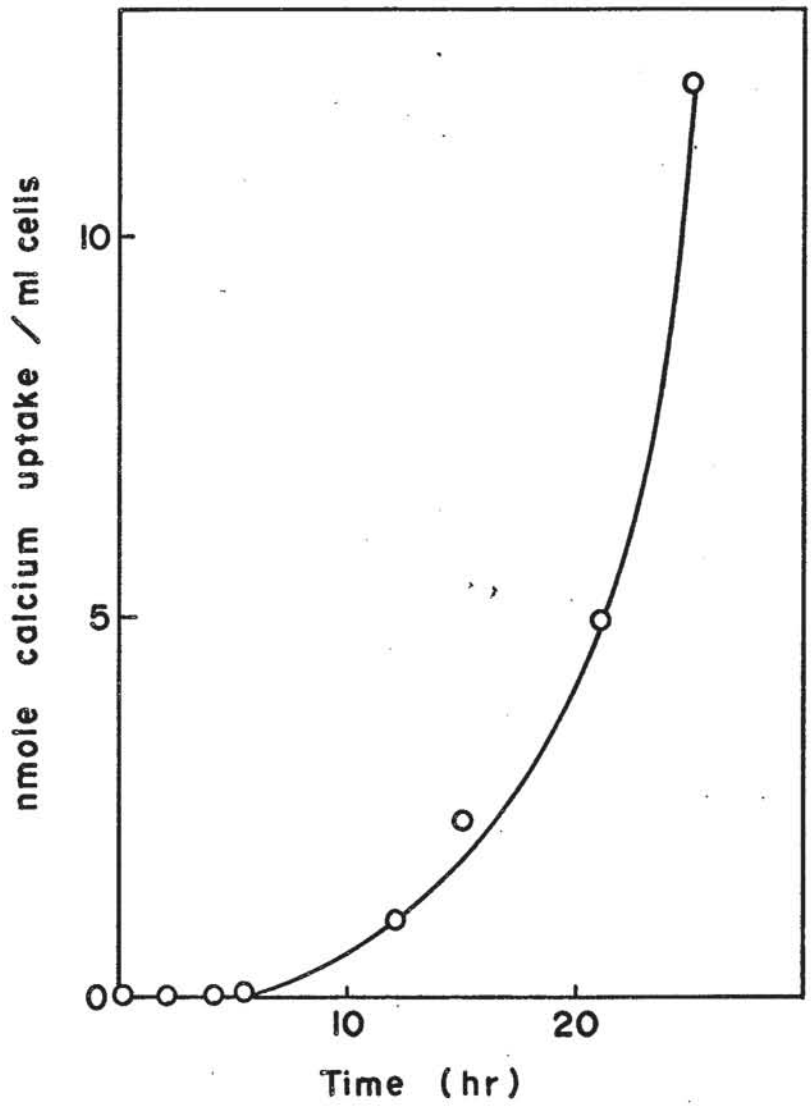

nmole calcium uptake / ml cells
10
5
0
10
20
Time (hr)

TABLE XIX

Recovery of protein and $^{45}Ca^{2+}$ binding capacity during purification of Achlya Ca^{2+} binding glycoprotein. For the preparation of this table, the assay system employed at every stage was equilibrium dialysis at 4° for 3 hr against 100 μM Ca^{2+} in 1 mM Tris-acetate buffer, pH 7.0

Fraction	Total protein (mg)	Total binding capacity (nmols $^{45}Ca^{2+}$)	(%)	Specific binding capacity nmols Ca bound/mg protein	Increase in glycoprotein activity
1. Osmotic shock fluid	20.25	7515	100	371	1.0
2. Ultrafiltration	6.07	3782	50	623	1.68
3. CM-cellulose and Ultrafiltration	1.55	1539	20.4	993	2.68
4. Sephadex-G50	0.33	409.4	5.4	1240.6	3.34

Methods. After isolation of purified glycopeptide, there was a 3.3 fold increase by equilibrium dialysis assay (Table XIX) or a 2.8 fold increase by anionic exchange resin assay (Fig. 29) of calcium binding activity which accounted for 1.6% of the protein initially present in the crude extract. As a result, the calcium binding glycopeptide represents only .00016% of the total cell protein.

For each stage of purification of the calcium binding glycopeptide Tris-barbital, pH 8 gel electrophoresis was used to determine the purity of the glycopeptide. The gel electrophoretic Coomassie blue staining pattern showed that at step 3, CM-cellulose and ultrafiltration removed the majority of contaminating proteins. The electrophoretic patterns of (B) crude shock fluid and (A) step 3 of purification (CM-cellulose and ultrafiltration) are shown in Figure 30. A short electrophoresis running time and 10% polyacrylamide gels were used to prevent the acidic glycopeptide from running near the bottom. This electrophoretic analysis indicated that the glycopeptide was strongly acidic. The electrophoretic gels were scanned using a Joyce Loebel chromoscan set at $A_{660\,nm}$ (Fig. 31). Fig. 31a shows the glycopeptide peak and a small contaminant running slightly behind it. This was similar to Coomassie brilliant blue staining pattern of the step 3 stage of purification, CM-cellulose and ultrafiltration, with one major band and a small contaminant running above it (Fig. 30). In graph (b) of Figure 31, a chromoscan of the first step of purification, osmotic shock fluid, shows numerous peaks.

Figure 29: Calcium binding activity as function of calcium concentration during purification procedure for osmotic shock fluid (◑), ultrafiltration (●), and CM-cellulose and ultrafiltration (○). Assayed by anionic exchange resin Dowex 2-X8 (50 - 100 mesh) assay.

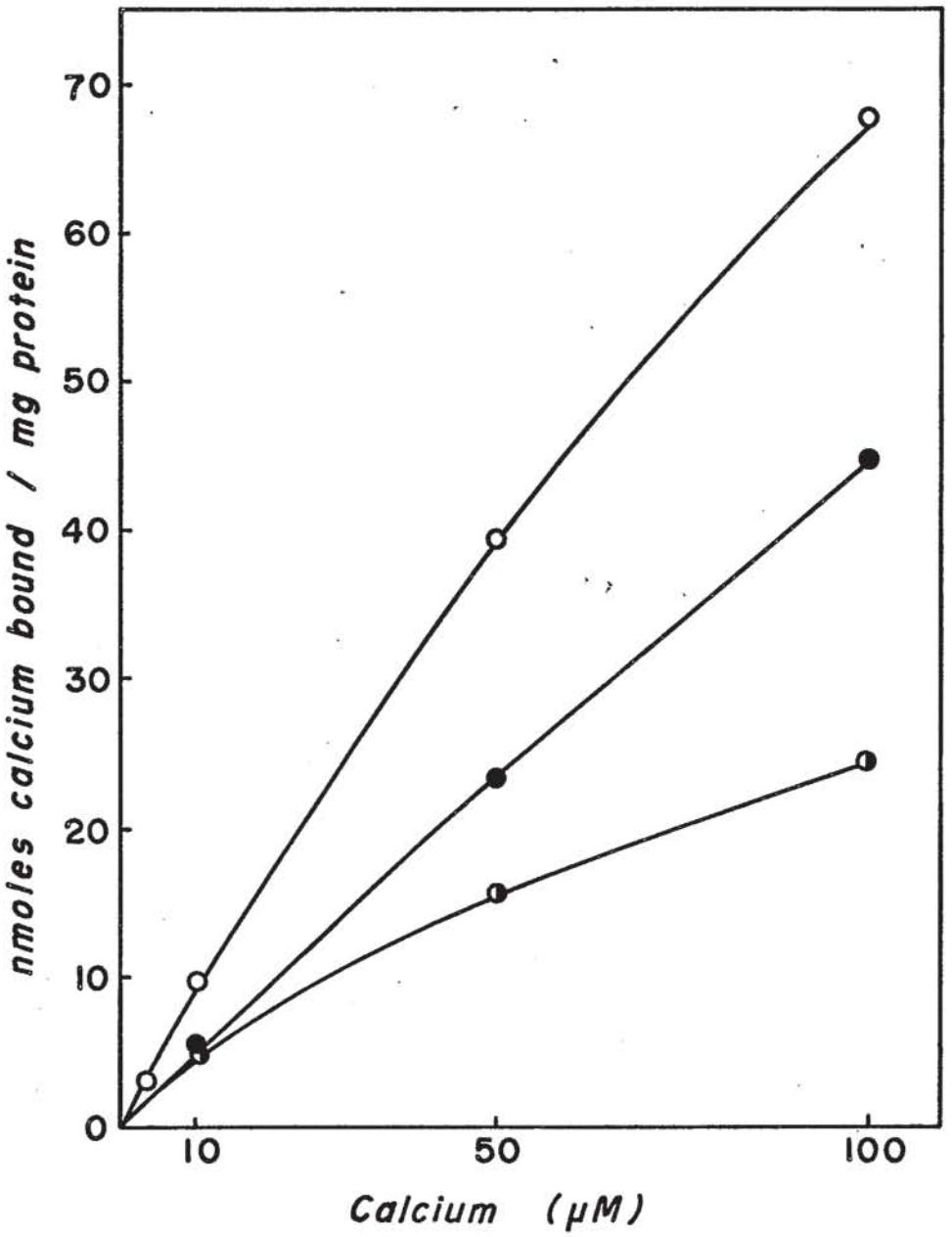

70
60
50
40
30
20
10
0
nmoles calcium bound / mg protein
10
50
100
Calcium (μM)

Figure 30: Polyacrylamide gel electrophoretic patterns of ($_B$) crude shock fluid, and ($_A$) CM-cellulose-ultrafiltration purification step stained by Coomassie brillant blue.

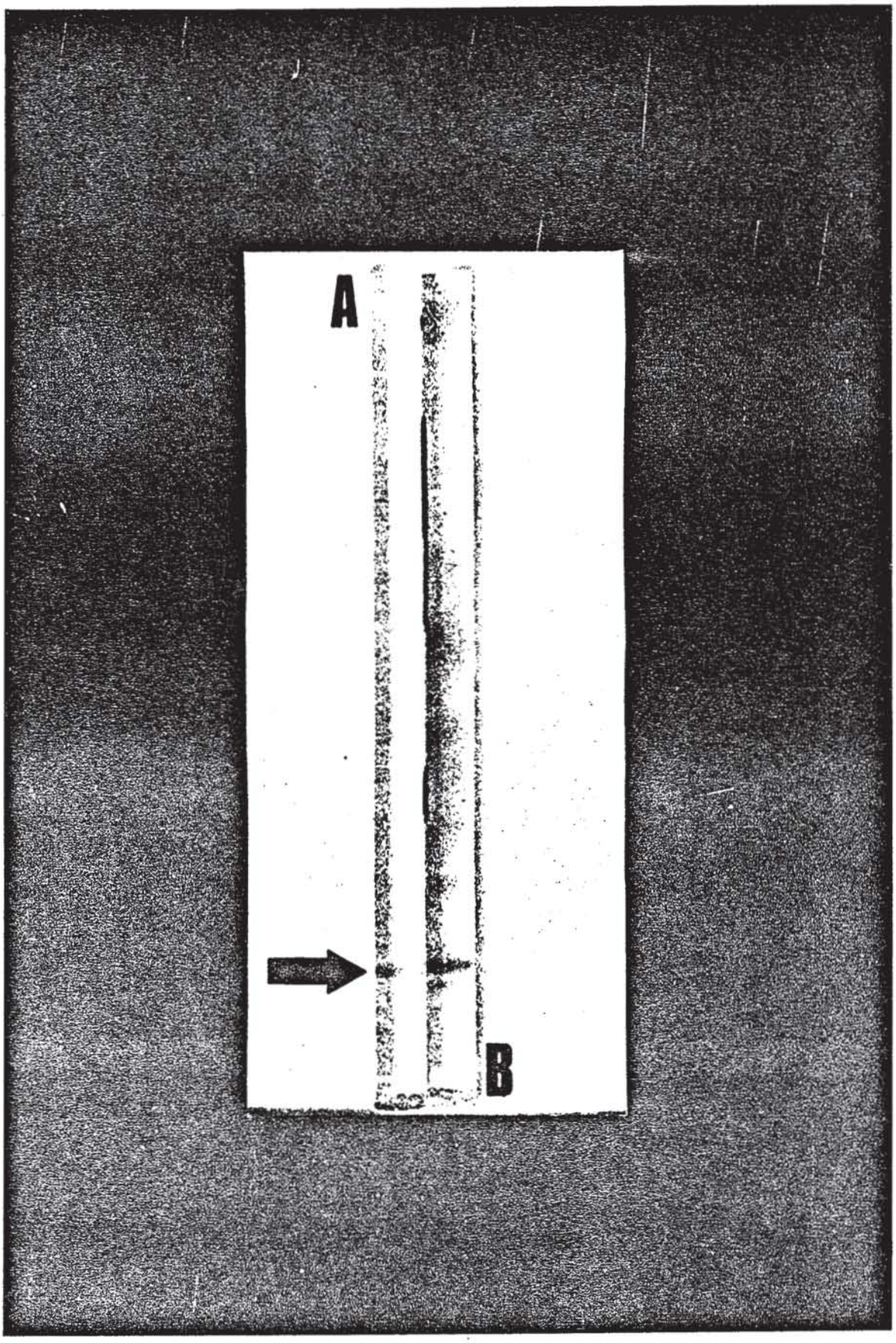
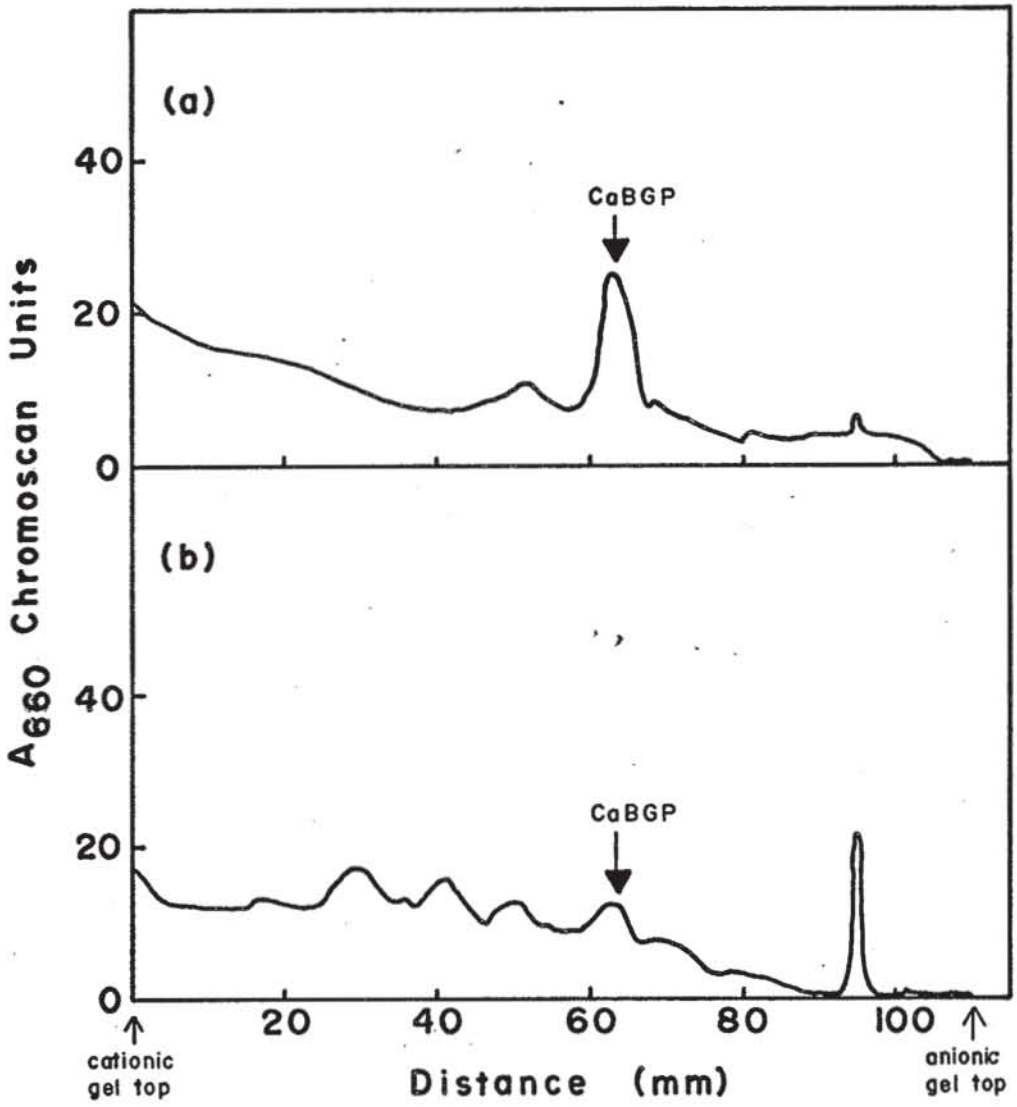

A
B

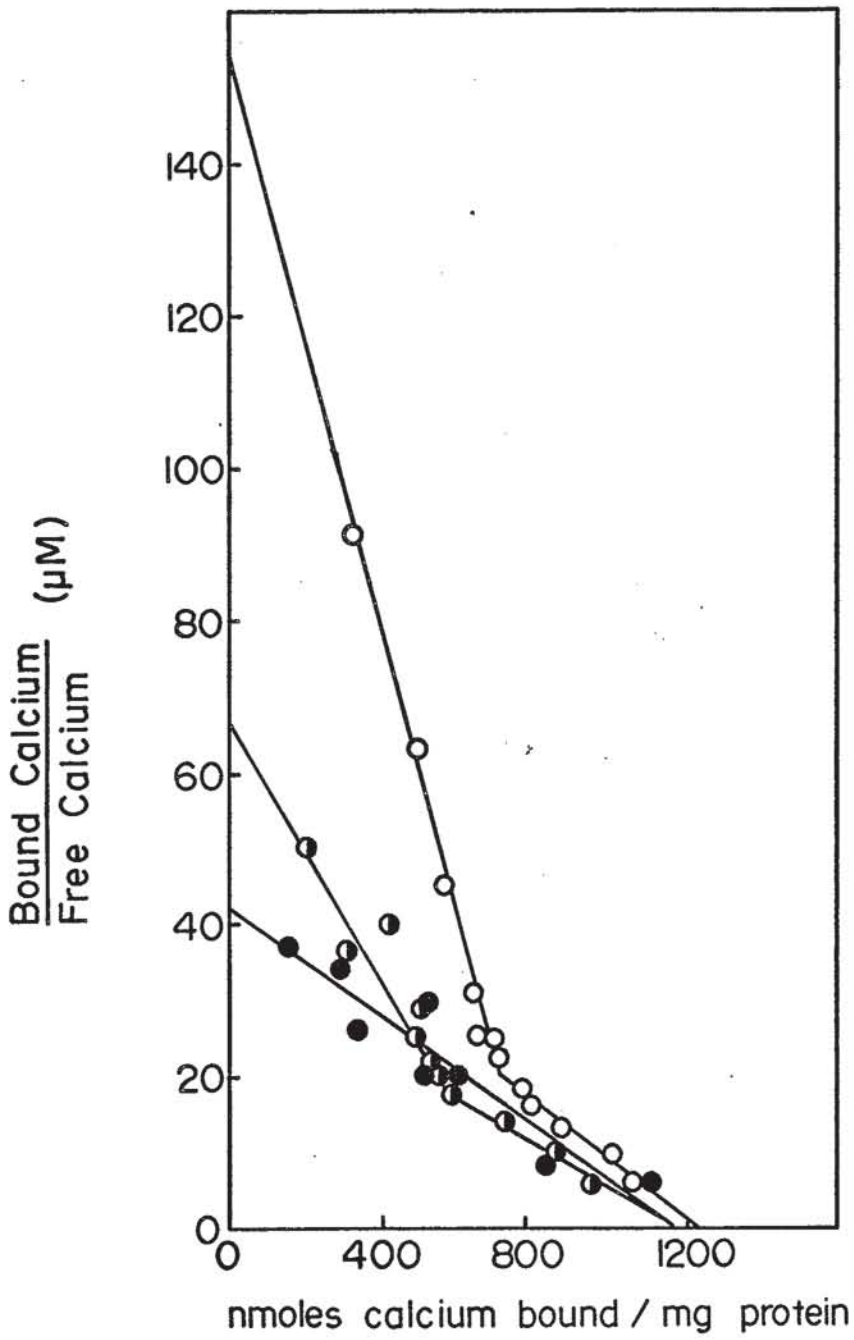
Figure 31: Chromoscan ($A_{660\ nm}$) of glycopeptide in 7.5% polyacrylamide gel for (a) fraction 3, CM-cellulose-ultrafiltration and (b) fraction 1, osmotic shock fluid.


(a)
40
20
0
CaBGP
(b)
40
20
0
CaBGP
A_{660} Chromoscan Units
20
40
60
80
100
cationic gel top
anionic gel top
Distance (mm)

Degradative enzyme effects: Ribonuclease and neuraminidase had no effect on calcium binding, indicating that there was no ribonucleic acid or sialic acid in the glycopeptide (Table XVIII). Although both pronase and lysozyme were found to be inhibitory as shown in Figure 32, this result was contradictory to later results that showed that the majority of amino acids were the D-isomer form. Lamport (1973) was unable to degrade the 'extensin' glycopeptide with either purified pronase, trypsin, or other proteolytic enzymes, but by using a crude preparation of pronase he was able to degrade the plant cell wall (extensin) glycopeptide. In Achlya, crude pronase produced little change in low affinity calcium binding, while almost completely removing the high affinity calcium binding (Table XVI). Lysozyme which hydrolyzes 1 to 4 glycosidic linkages, slightly decreased the calcium binding affinity of both component sites. Both degradative enzymes only slightly diminished moles calcium bound per mole glycopeptide. These results suggest that the high affinity binding sites appear to be of a porteinaceous nature and the low affinity binding sites possibly are carbohydrate associated. Why these enzymes do not diminish the moles calcium bound per glycopeptide is unknown. When these enzymes were tested for diminishing effect on molecular weight as assayed by $^{45}Ca^{++}$-equilibrium binding on Sephadex G-50 column, no major differences were discerned. There was less binding in the presence of either pronase or lysozyme but little change in size of glycopeptide. Glycopeptide samples were pretreated with enzymes prior to equilibrium binding in the same manner as for equilibrium dialysis.

Studies of $^{54}Mn^{++}$ binding by the glycopeptide in the presence of the hydrolytic enzymes seemed pertinent since the glycopeptide binds

Figure 32: Scatchard plot of the calcium binding activity of glycopeptide untreated (○), or treated with either crude pronase (●) or lysozyme (◑).


Bound Calcium / Free Calcium (μM)
140
120
100
80
60
40
20
0
0
400
800
1200
nmoles calcium bound / mg protein

manganese linearly with only one dissociation constant. In Figure 33, $^{54}Mn^{++}$ binding in Scatchard plot form shows that in the presence and absence of the enzymes the proteinaceous nature of the high affinity binding site was evinced since lysozyme produced little effect on manganese binding affinity while crude pronase strongly reduced high affinity binding (see Table XVI). However lysozyme reduced the moles calcium bound per mole glycopeptide by 40% while crude pronase reduced binding in a slight manner. The question of whether the two glycopeptide calcium binding affinity sites are synonymous with distinct protein and carbohydrate type sites can not be determined from this data, but it is apparent that both protein and carbohydrate units play an important part in calcium binding by the glycopeptide.

Ionic strength effects: The calcium binding activity of the fungal glycopeptide was found to be dependent on ionic strength (Fig. 34). When the Tris-acetate buffer concentration was increased from 1 mM to 5 mM, there was a 50% decrease in calcium binding to the glycopeptide, and, at a concentration of 25 mM, the maximum inhibition (90%) occurred. High concentrations (50 mM) of either NaCl or KCl abolished calcium binding completely (Table XVII). Futhermore, high concentration of $MgCl_2$ considerably reduced calcium binding activity, as shown in Figure 35, even through $MgCl_2$ at low concentration relative to other competing divalent cations previously tested had little effect (see Table XVII). In the same figure, the inset Scatchard plot shows that calcium binding was almost completely abolished at 2 mM $MgCl_2$ with a high K_d of 1.5×10^{-4} M.

pH effects: Calcium binding activity was found to be dependent on pH changes (Fig. 36). At low ionic strength (1 mM Tris-acetate)

Figure 33: Scatchard plot of manganese binding activity of glycopeptide untreated (○), or treated with either crude pronase (●) or lysozyme (◑).

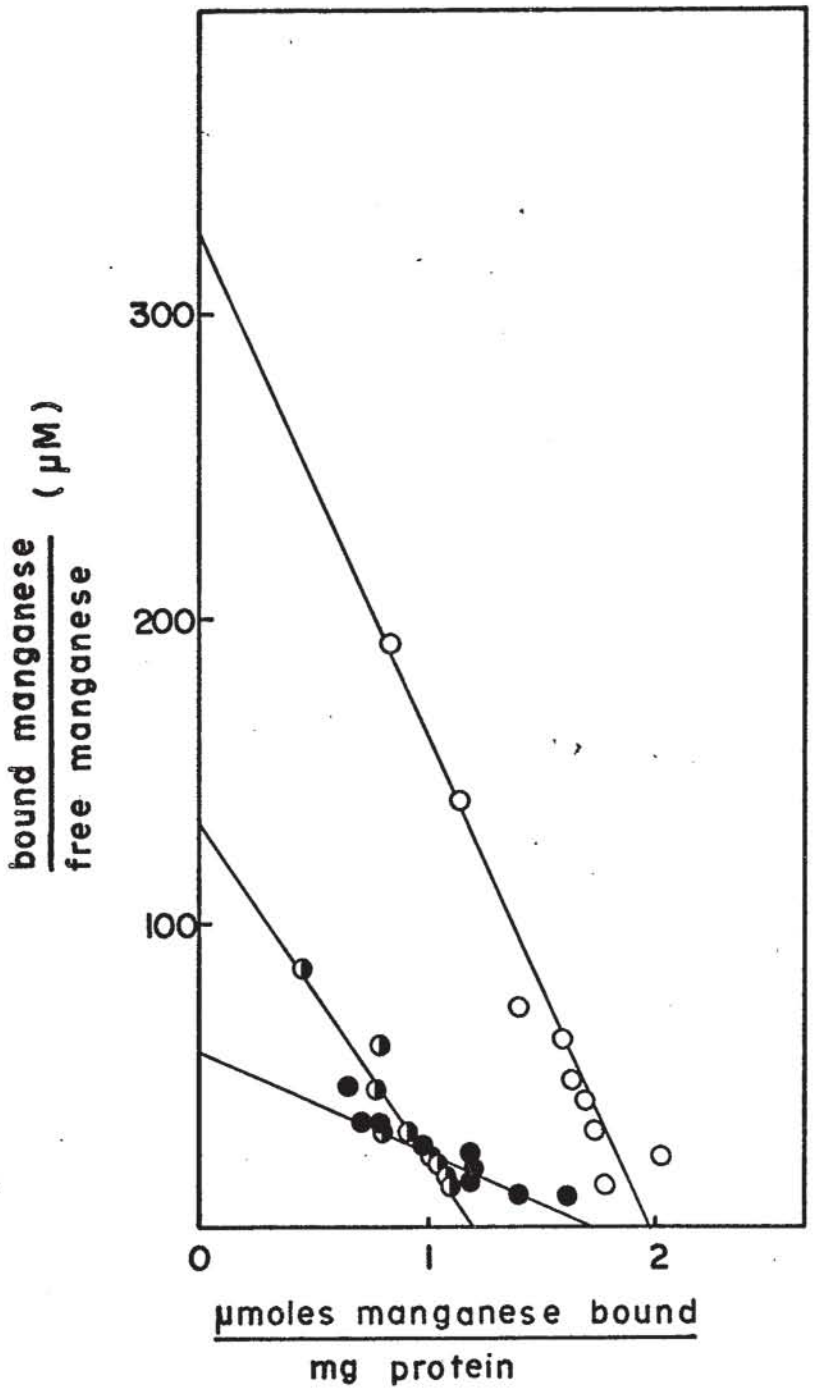

300
200
100
0
1
2
bound manganese / free manganese (μM)
μmoles manganese bound / mg protein

Figure 34: Effect of ionic strength on glycopeptide calcium binding activity. The inset plot is the semi-log form of rate $^{45}Ca^{++}$ bound as a function of Tris-acetate ionic strength.

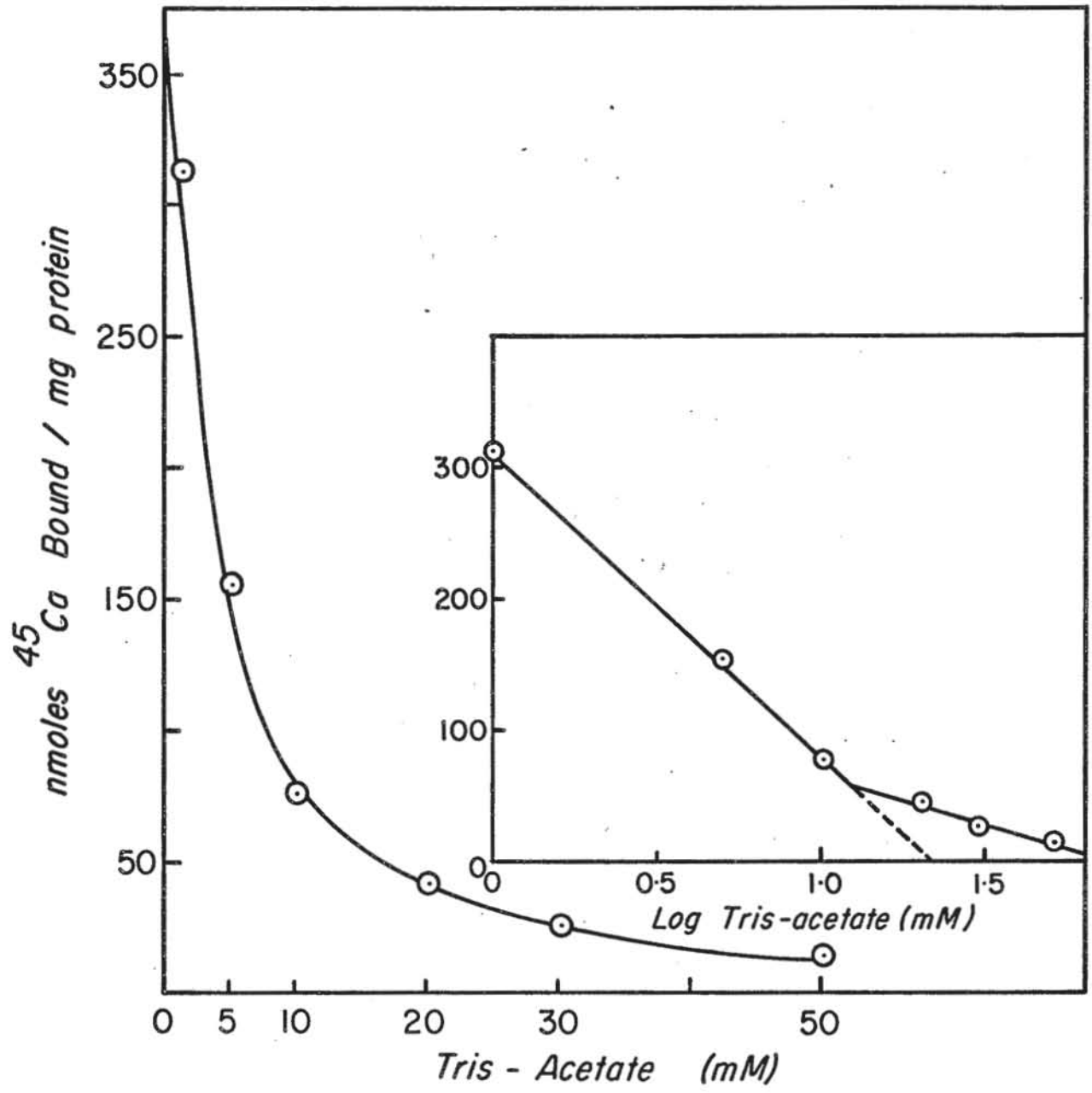

350
250
150
50
nmoles 45Ca Bound / mg protein
0
5
10
20
30
50
Tris - Acetate (mM)
300
200
100
0
0
0·5
1·0
1·5
Log Tris-acetate (mM)

Figure 35: Effect of magnesium on calcium binding to glycopeptide. Inset: Scatchard plot of calcium binding activity in the presence of 2 mM $MgCl_2$ (○) and in its absence (◒).

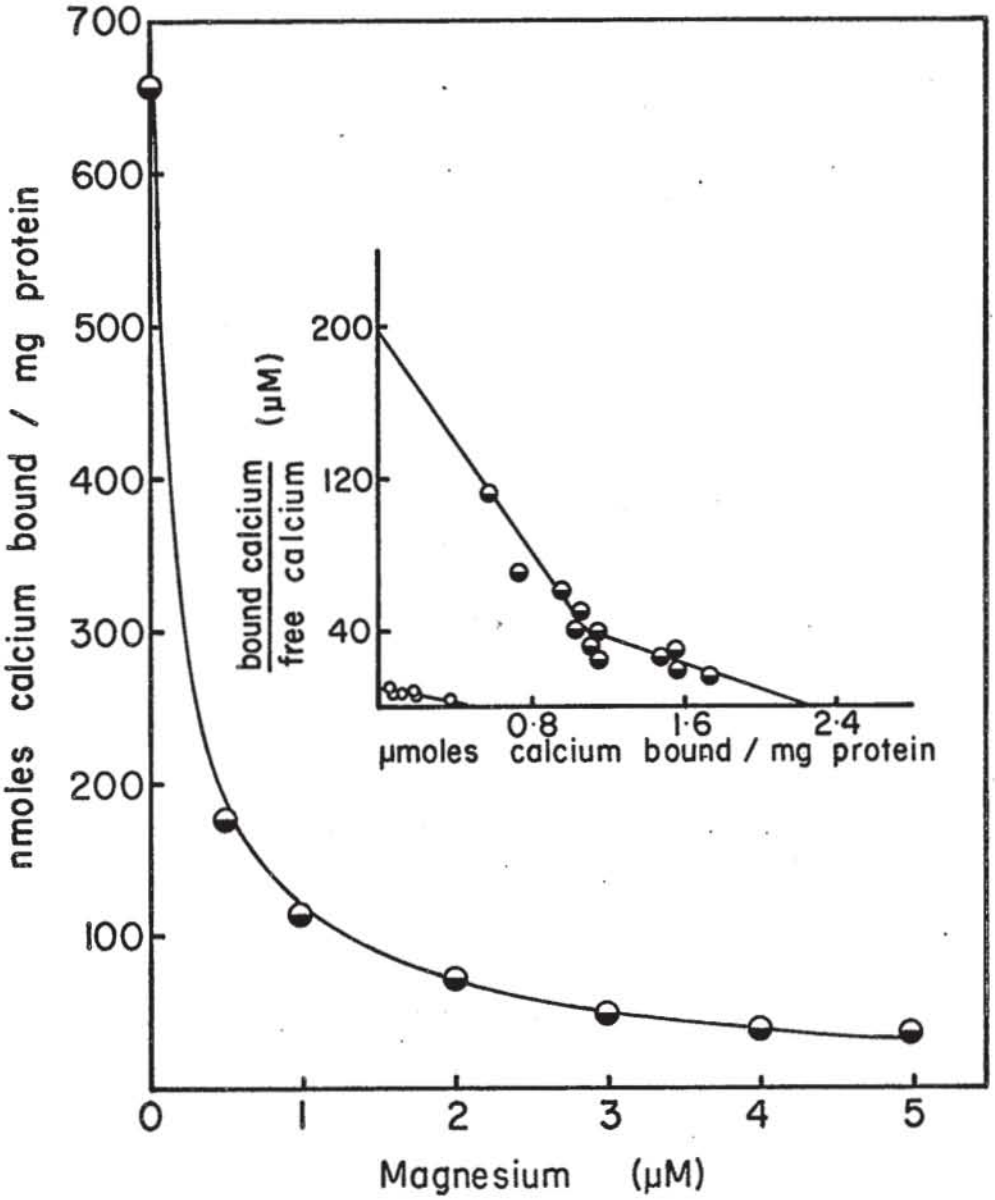
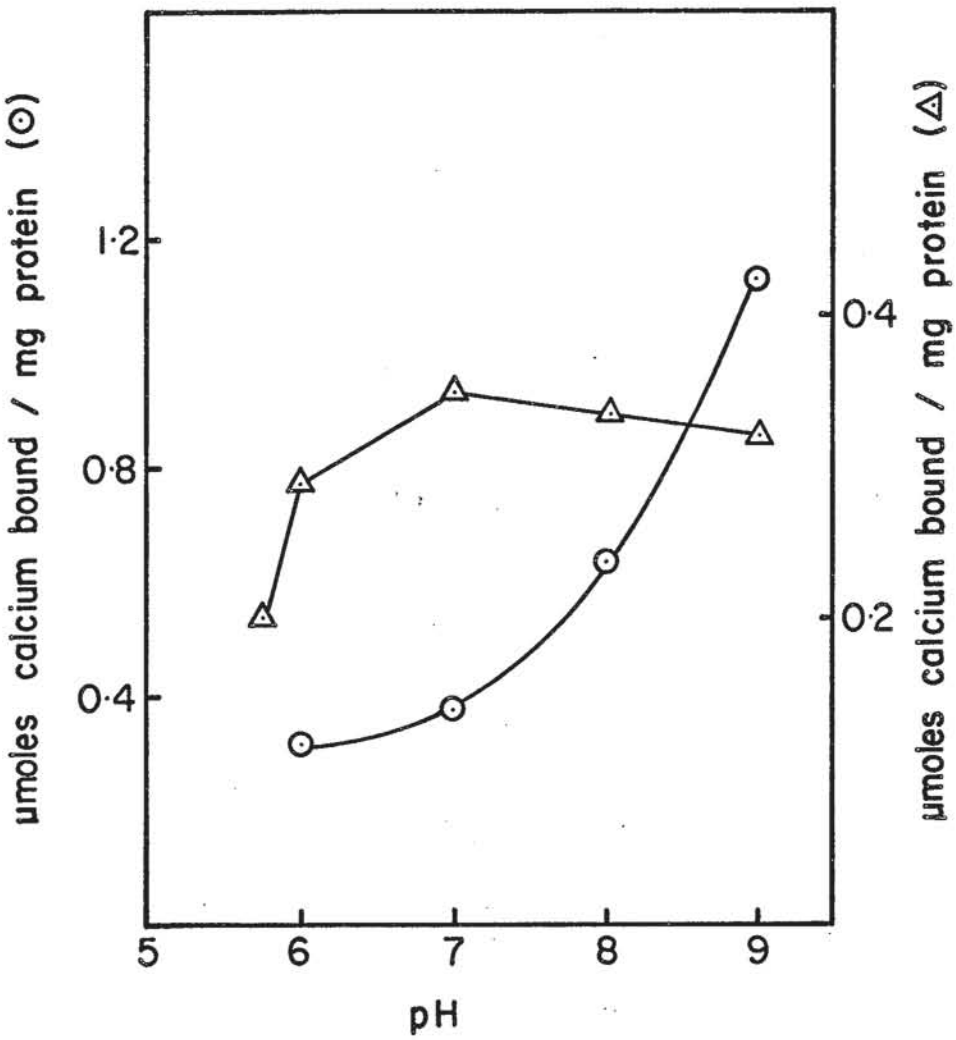

700
600
500
400
300
200
100
nmoles calcium bound / mg protein
0
1
2
3
4
5
Magnesium (μM)
200
120
40
bound calcium / free calcium (μM)
0·8
1·6
2·4
μmoles calcium bound / mg protein

Figure 36: The influence of pH on glycopeptide calcium binding activity. Buffer used was Tris-acetate at (⊙), 1 mM and (△), 12.5 mM.


μmoles calcium bound / mg protein (⊙)
μmoles calcium bound / mg protein (△)
1·2
0·8
0·4
0·4
0·2
5
6
7
8
9
pH

increasing pH enhanced calcium binding activity. As the ionic strength (12.5 mM Tris-acetate) was increased the alkaline pH effect was lessened, with an ill-defined optimum between pH 6 and pH 8. It was difficult to correlate pH characterization of the glycopeptide with the $HgCl_2$ enhanced (pH 6.5 optimum) component of the calcium transport system, but dissimilarity of results may be due to ionic strength differences as 25 mM Tris-acetate buffer was used for *in vivo* transport studies.

Chemical composition. Prior to analysis of glycopeptide chemical constituents, additional purification steps were implemented to ensure that only momomer glycopeptide molecules were being analyzed, and to eliminate the problem of analysis of carbohydrate molecules that were ionically linked to the glycopeptide. After obtaining the purified glycopeptide of *Achlya*, the glycopeptide was again collected as a single peak on a Sephadex G-50 column, but in the presence of 100 mM Tris-acetate, pH 7. The pooled glycopeptide peak was extensively dialyzed against de-ionized H_2O and concentrated by the Diaflo UM-2 Amicon membrane filter system. A second method used to ensure that only the monomeric form of the glycopeptide was present was by Sephadex filtration in the presence of 8 M urea. Chemical analysis of these lyophilized pure glycopeptide samples produced identical results to glycopeptide samples that had been purified by the method outlined in Table XIX confirming that only monomeric glycopeptide molecules probably were present.

TABLE XX

Chemical composition of Achlya calcium binding glycopeptide.

Residue	Moles/100 moles glycopeptide
Glutamic acid	19.2
Glycine	6.9
Aspartic acid	2.9
Alanine	1.6
Lysine	1.5
Serine	1.7
Cystine	1.2
Glucose	50.2
Ribose	14.8

Verification of the covalent linkage between carbohydrate and peptide components of the glycopeptide came from ionic charge separation studies of doubly radioactively labelled (sugar and peptide) glycopeptide (Fig. 37). After fractionation of Tris-barbital, pH 8 electrophoretic gels, both labels ran almost coincident. The possibility that the label as precursors were broken down into other compounds prior to synthesis of glycopeptide was eliminated since glycopeptide labelled with ^{14}C-glucose ran coincident with $^{45}Ca^{++}$ equilibrium binding peak on Sephadex as determined by the phenolsulfuric acid test for glucose. After acid hydrolysis and chromatography of the glycopeptide products (amino acids) from ^{3}H-glycine-labelled glycopeptide the use of unlabelled glycine sample for co-chromatographic analysis revealed that the ^{3}H counts could be detected in the glycine position on the paper. Furthermore, the double labelled glycopeptide was shown to be the calcium binding glycopeptide as it coincided in gel electrophoresis with $^{45}Ca^{++}$ equilibrium binding peak (Fig. 38).

Chemical analysis of glycopeptide after 8 M urea treatment on Sephadex gel (based on dry weight) showed that the purified glycopeptide consisted of 35% protein as determined by the method of Lowry et al (1951) and 65% carbohydrate as described in Methods. The peptide component consisted of 7 major amino acids, as listed in Table XX. At least half of the amino acids present was found to be glutamic acid and the remainder consisted of glycine, aspartic acid, alanine, lysine, serine, and cystine. Since the glycopeptide contained no aromatic amino acids, the validity of basing glycopeptide data on protein as determined by the method of Lowry was questioned. The micro-

Figure 37: Gel electrophoretic pattern of ^{14}C-glucose and ^{3}H-glycine doubly labelled calcium binding glycopeptide.

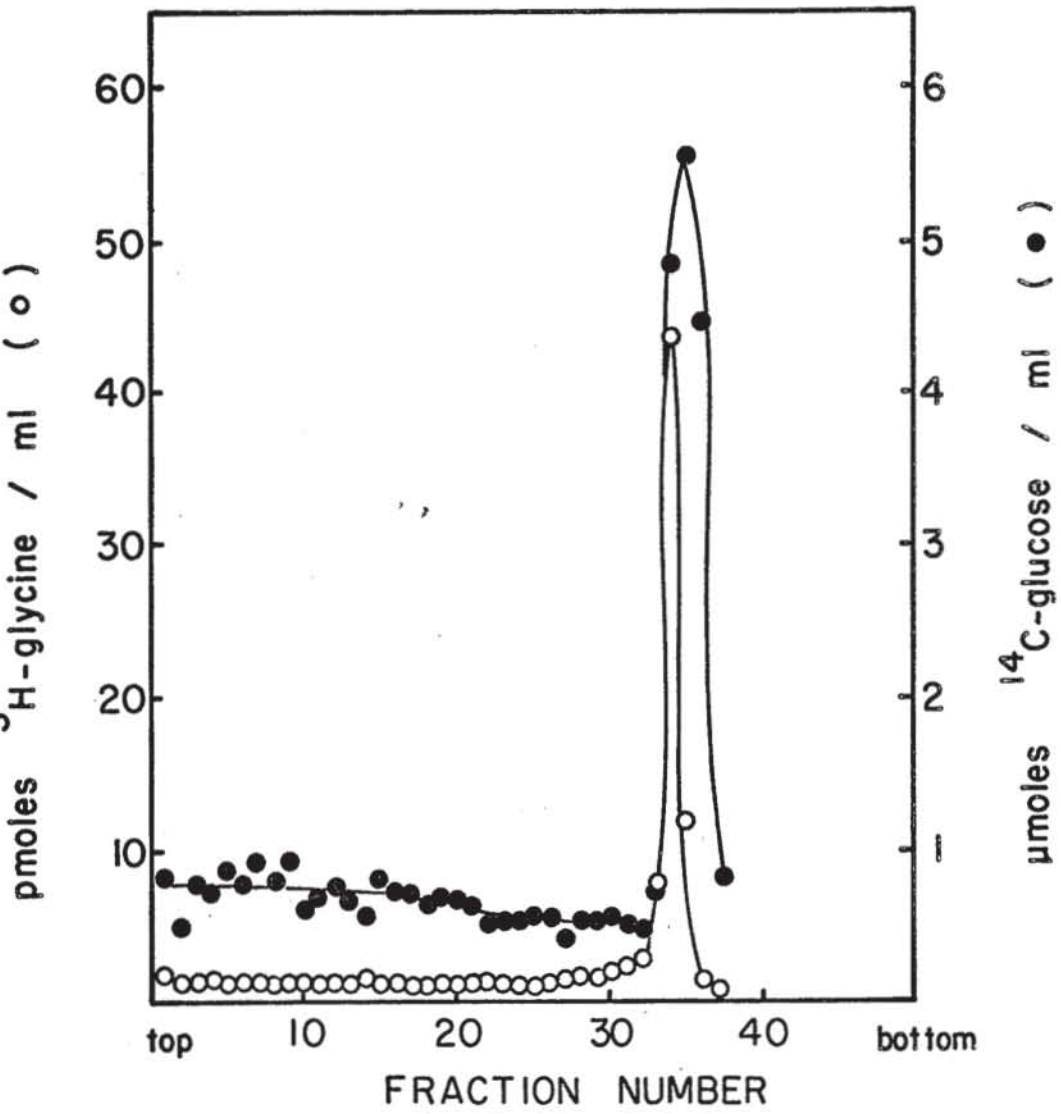
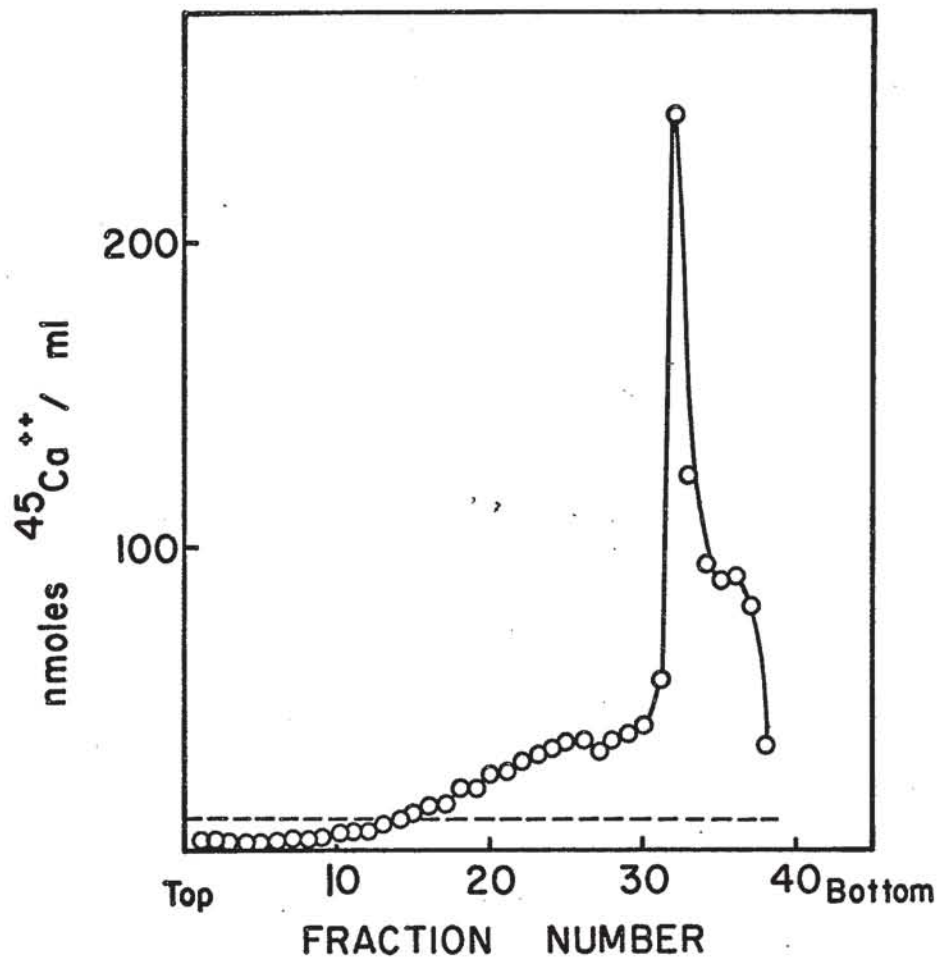

60
50
40
30
20
10
6
5
4
3
2
1
pmoles ^{3}H-glycine / ml (o)
µmoles ^{14}C-glucose / ml (•)
top
10
20
30
40
bottom
FRACTION NUMBER

Figure 38: Glycopeptide $^{45}Ca^{++}$ -equilibrium binding in Tris-acetate pH 8 gel by the electrophoretic procedure (see Methods).


200
100
nmoles $^{45}Ca^{++}$ / ml
Top
10
20
30
40
Bottom
FRACTION NUMBER

biuret assay was employed to check protein content of the glycopeptide (Zamenhoff & Chargaff, 1957) because the color production of this assay is based on the general amino acid linkage while the color production of the Lowry assay is based on the ring structure of tryptophan, phenylalanine, and tyrosine in addition to peptide linkage. Neglible difference was discerned between the two assay techniques, assuring the authenticity of the protein determination by Lowry.

Since a large percentage of the glycopeptide was found to be carbohydrate, the possibility that the glycopeptide may be associated with cell wall arose which in turn presented the question of the isomeric form of the amino acids present in the glycopeptide. After treatment of amino acid hydrolysate samples with either L- or D- amino acid oxidase prior to chromatography as described under Methods, it was found that only a small amount of glutamic acid was resistant to enzyme hydrolysis and this was broken down by L- amino acid oxidase. This result indicates that some of the glutamic acid present may be L-isomers and all other amino acids would be D-isomers.

The carbohydrate component of the glycopeptide was isolated and chromatographed as described under Methods, and was found to contain two types of neutral sugars, glucose and ribose. Glucose formed the majority of the sugar present in the glycopeptide. The presence of glucose was affirmed by a glucostat test. The glucostat test indicated that 80% of the carbohydrate component was glucose. The remainder of the neutral sugar component seemed to be ribose, although another possibility is that it is fucose, due to the close chromatographic R_f

values of these sugars (Partridge & Westall, 1948). A small amount of uronic acid appeared to be also present. Although sialic acid is usually associated with acidic surface glycoproteins, the glycopeptide of Achlya was found to contain no sialic acid.

Each glycopeptide molecule apparently contains 19 amino acids and 22 sugar residues (Table XX). Calculation of percentage composition based on the amino acid and sugar residues present agreed well with chemical analysis estimate. The molecular weight of the peptide component was calculated to be 2134 based on amino acid composition, which represented 37.5% of the total molecular weight of the glycopeptide. This agrees well with the peptide composition analysis by Lowry of the complete glycopeptide.

Molecular Weight: The molecular weight of Achlya glycopeptide was determined by a variety of methods due to problems that occur because of small size and high carbohydrate percentage. First, an estimate of the glycopeptide molecular weight (<10,000 daltons) was determined by ultrafiltration technique as described under Methods (Fig. 39). The discrepancy between the two curves can be accounted for by the measurement of calcium binding activity that is based on protein since retention of large molecular weight proteins would lower apparent specific calcium binding activity.

The molecular weight of fungal glycopeptide was determined by Sephadex gel filtration and the filtration standard curve was prepared as described under Methods (Fig. 40). The molecular weight values of three fungal species are shown in Figure 41, for Pythium,

Figure 39: Calcium binding activity as a function of calcium concentration for efluent (O) and concentrate (□) of Diaflo ultrafiltration using Amicon UM-10 membrane filter, and assayed by anionic exchange resin Dowex 2-X8 (50-100 mesh).

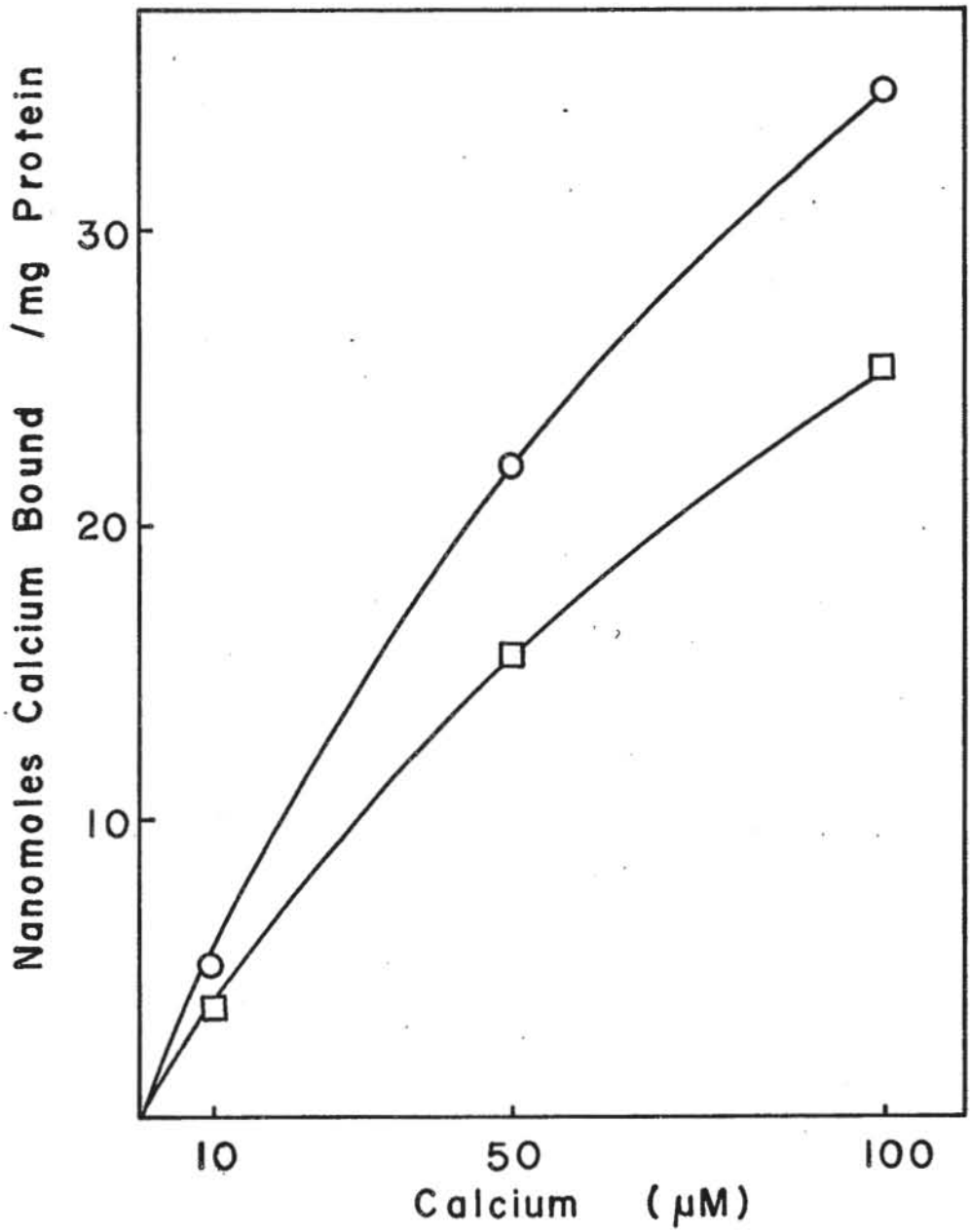

Nanomoles Calcium Bound /mg Protein
30
20
10
10
50
100
Calcium (μM)

Figure 40: Standard curve for Sephadex G-50 gel filtration determination of molecular weight of fungal calcium binding glycopeptides.

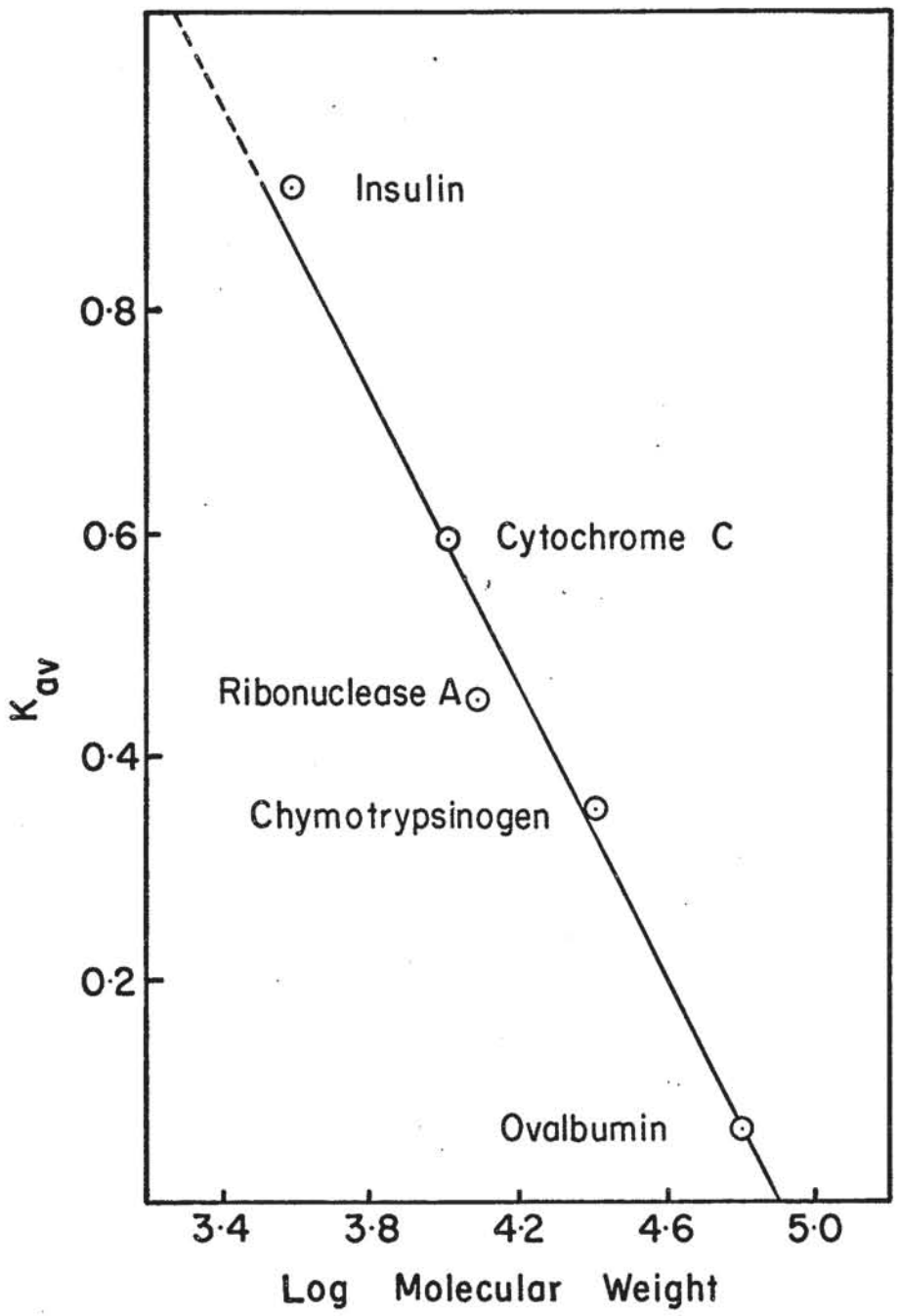
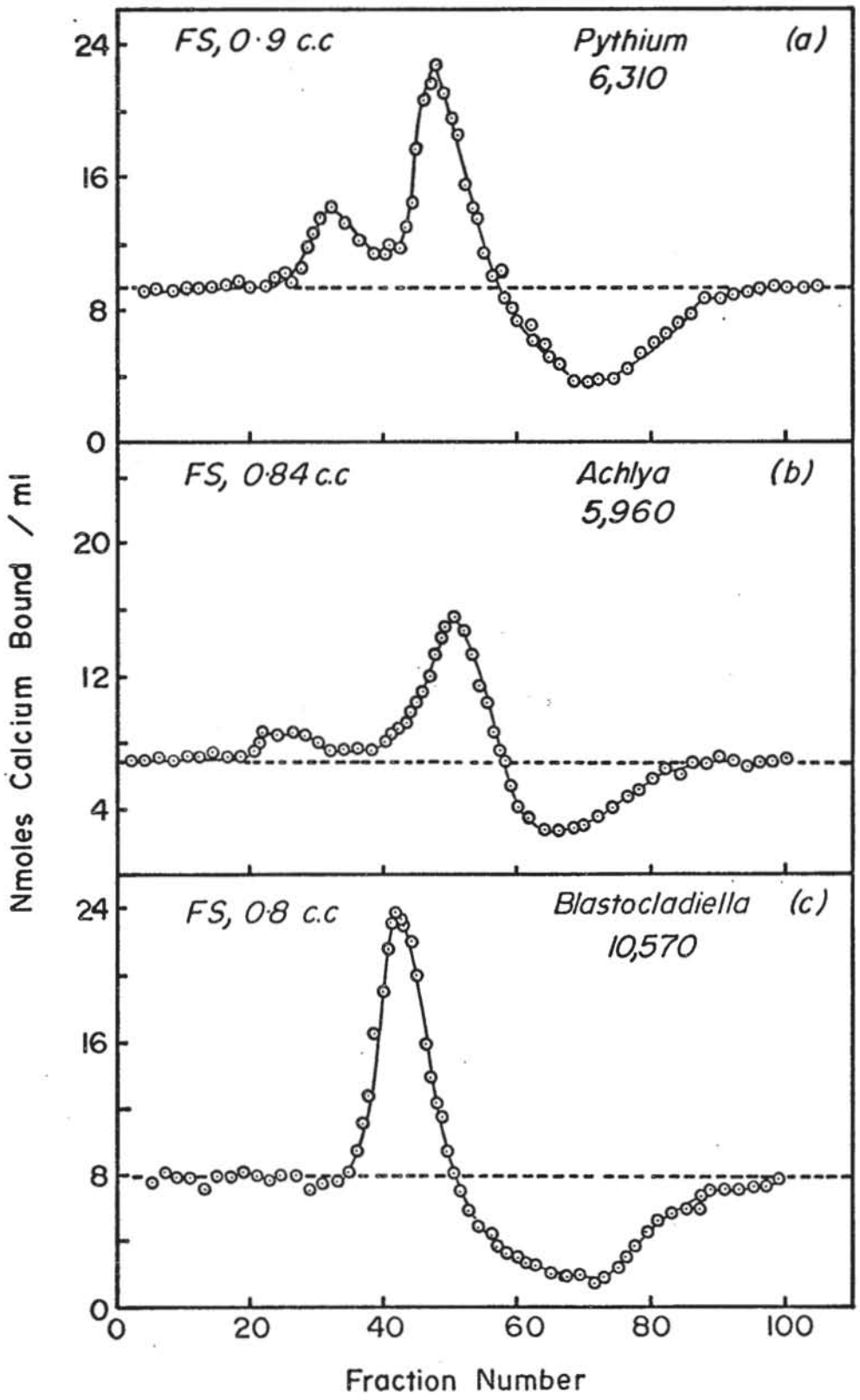

Insulin
Cytochrome C
Ribonuclease A
Chymotrypsinogen
Ovalbumin
0·8
0·6
0·4
0·2
K_{av}
3·4
3·8
4·2
4·6
5·0
Log Molecular Weight

Figure 41: Sephadex G-50 gel filtration determination of molecular weight by $^{45}Ca^{++}$ equilibrium binding. FS is the abbreviation for fraction size. The concentration of Ca^{++} used was 0.01 mM.


FS, 0·9 c.c
Pythium
6,310
(a)
FS, 0·84 c.c
Achlya
5,960
(b)
FS, 0·8 c.c
Blastocladiella
10,570
(c)
Nmoles Calcium Bound / ml
Fraction Number
0
4
8
12
16
20
24
0
20
40
60
80
100

6,310 daltons; for Achlya, 5,960 daltons; and for Blastocladiella, 10,570 daltons. Protein and sugar analysis confirmed that the $^{45}Ca^{++}$ equilibrium peak of Achlya was indeed the purified calcium binding glycopeptide (Fig. 42).

The glycopeptide molecular weight determined by gel filtration was checked by SDS gel electrophoretic determination of molecular weight. The SDS gel electrophoresis standard curve and the electrophoretic mobility of the glycopeptide are shown in Figure 43. The percentage acrylamide was varied (2.5%, 5.0%, 10.0%) to alleviate the problem created by the high carbohydrate content in the glycopeptide. The molecular weight of Achlya glycopeptide was found to be 6,200 daltons, which was in close agreement with the molecular weight value determined by sephadex gel filtration.

The molecular weight based on chemical composition was found to be 5,920 daltons, verifying the results of gel filtration and elctrophoretic analysis. An attempt to determine the molecular weight by sedimentation equilibrium using the meniscus depletion method (Yiphantis, 1964) was unsuccessful. Even after a prolonged period of ultracentrifugation at maximum speed, an equilibrium condition could not be obtained. This method indirectly showed that the weight of the glycopeptide had to be less than 10,000 daltons as that is the lower limit of this physical characterization method.

E. Plant Hormone Regulation of Calcium Transport

Cytokinin effects in intact cells: Concurrent studies by

Figure 42: Calcium binding glycopeptide peak of Achlya on Sephadex G-50 as measured by $^{45}Ca^{++}$ equilibrium binding (O); protein at $A_{280\ nm}$ (△), and by Lowry (●); and sugar content (■).

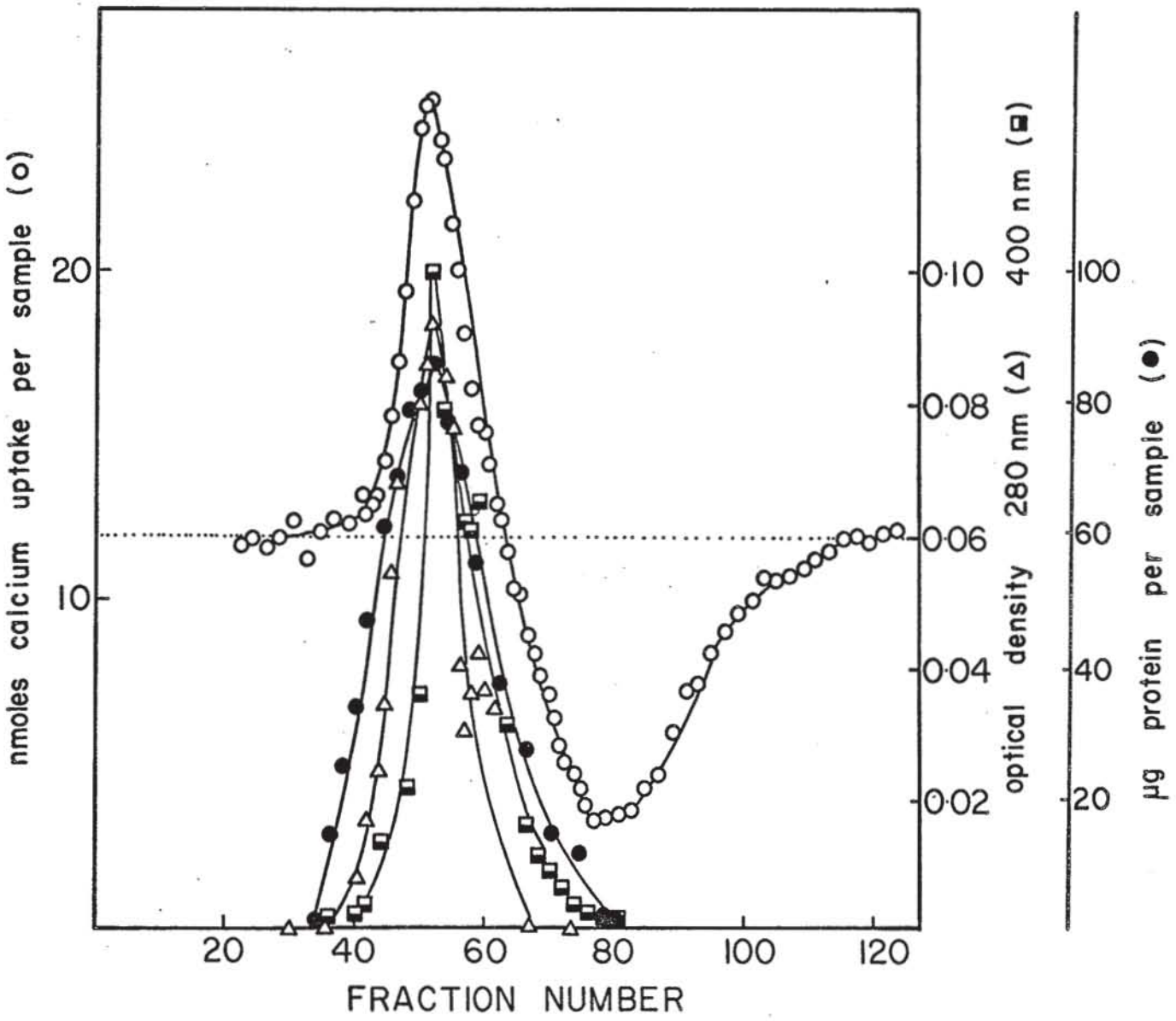
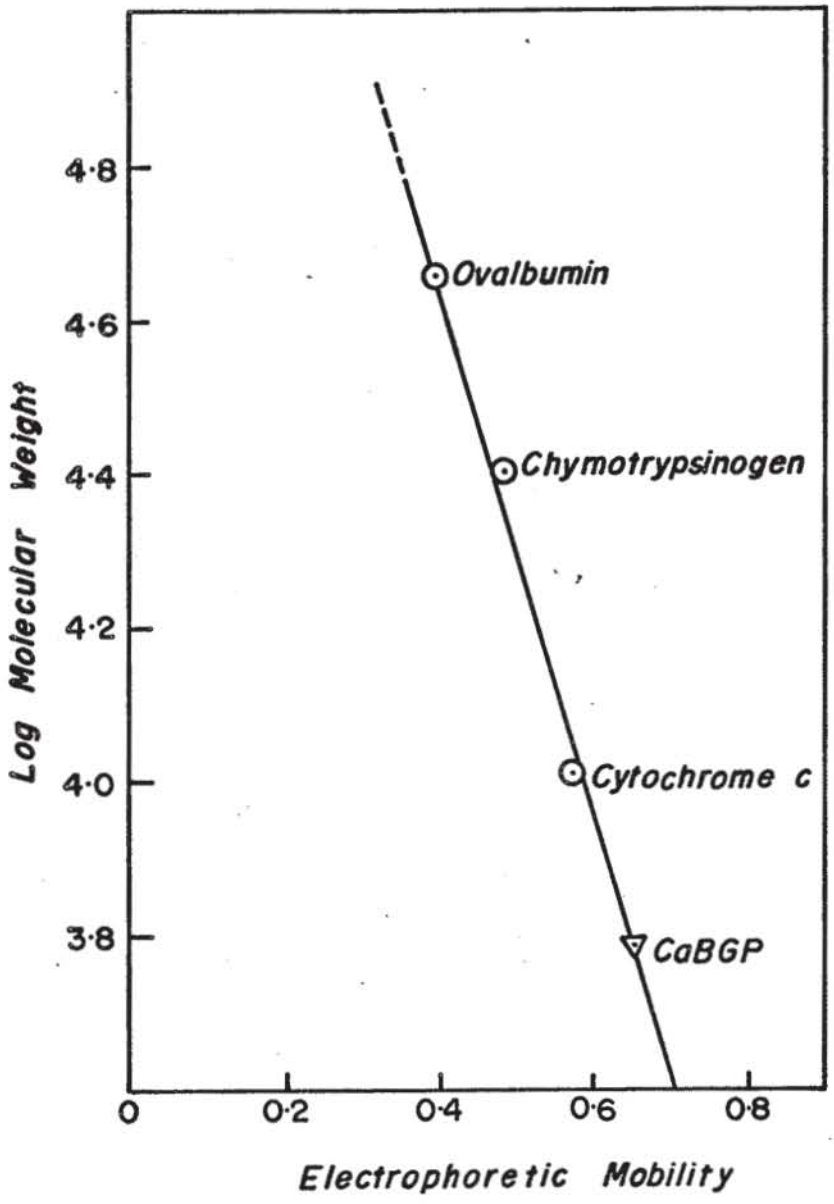

nmoles calcium uptake per sample (○)
optical density 280 nm (△) 400 nm (□)
μg protein per sample (●)
FRACTION NUMBER
20
10
0·10
0·08
0·06
0·04
0·02
100
80
60
40
20
20
40
60
80
100
120

Figure 43: Determination of the molecular weight of Achlya calcium binding glycopeptide.


Log Molecular Weight
4·8
4·6
4·4
4·2
4·0
3·8
Ovalbumin
Chymotrypsinogen
Cytochrome c
CaBGP
0
0·2
0·4
0·6
0·8
Electrophoretic Mobility

Stevenson (1974) showed that $HgCl_2$ stimulated cytokinin N^6-(substituted) adenine and purine but only adenine uptake in germinated sporangiospores of Achlya. Adenine was used to demonstrate cytokinin effect frequently throughout these studies because of its ability to function as a cytokinin, and because of the difficulty in handling cytokinin compounds which had to be dissolved in DMSO or if DMSO was omitted, alkaline pH effects had to be considered. In addition, xanthine and hypoxanthine could mimic cytokinin effect but other nucleic acid bases were ineffective. Moreover, when specificity of cytokinin effect was important only physiologically active cytokinin compounds would produce an effect, with the extent of effectiveness correlating with results obtained with a cytokinin bioassay (LeJohn, 1975). It was of interest to determine if cytokinins had any effect on calcium transport as $HgCl_2$ appeared to activate only the binding of cytokinins and Ca^{++}.

When germinated sporangiospores were incubated in Barksdale's (1963) defined medium, 6-benzyladenine stimulated the uptake of calcium, but this occurred only after a 30 min incubation time, as shown in Figure 44. At 30 min 6-benzyladenine stimulation of calcium uptake was maximum at a concentration of 10^{-6} M. With increasing incubation time, maximal stimulation by 6-benzyladenine of calcium uptake occurred at decreasing concentrations, and at 240 min $\geq$ 10^{-9} M 6-benzyladenine maximally stimulated calcium uptake. Calcium transport, however, was significantly affected by adenine in the presence of 10 µM $HgCl_2$ (Fig. 45). Adenine reduced $HgCl_2$-activated calcium transport almost to normal level of calcium uptake in the

Figure 44: Stimulation of calcium uptake in normal germinated sporangiospores by 6-benzyladenine at the following fixed time of pre-incubation with cytokinin in defined medium, 30 min (⊙), 90 min (◒), 120 min (⊡), 210 min (△), and 240 min (●).

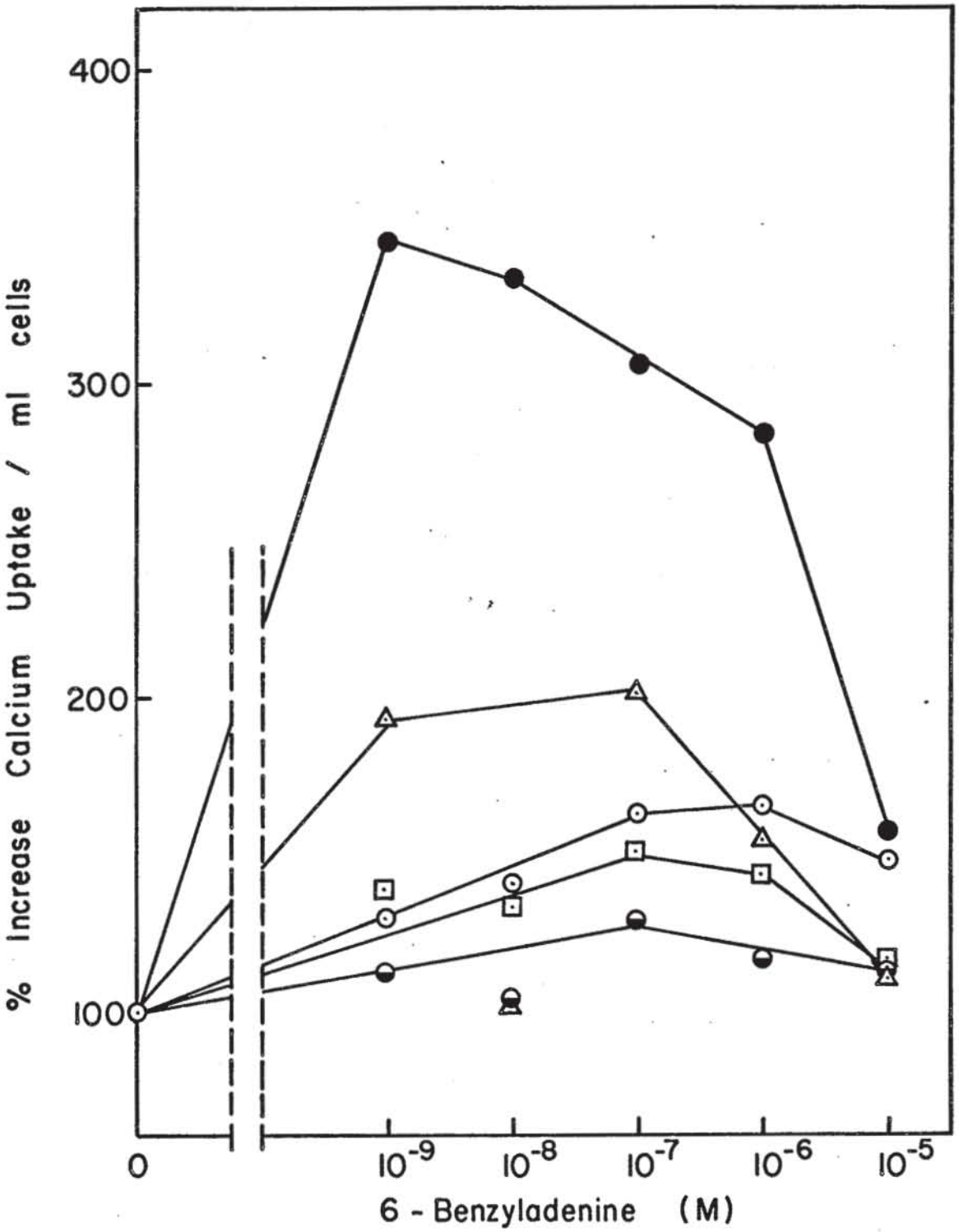
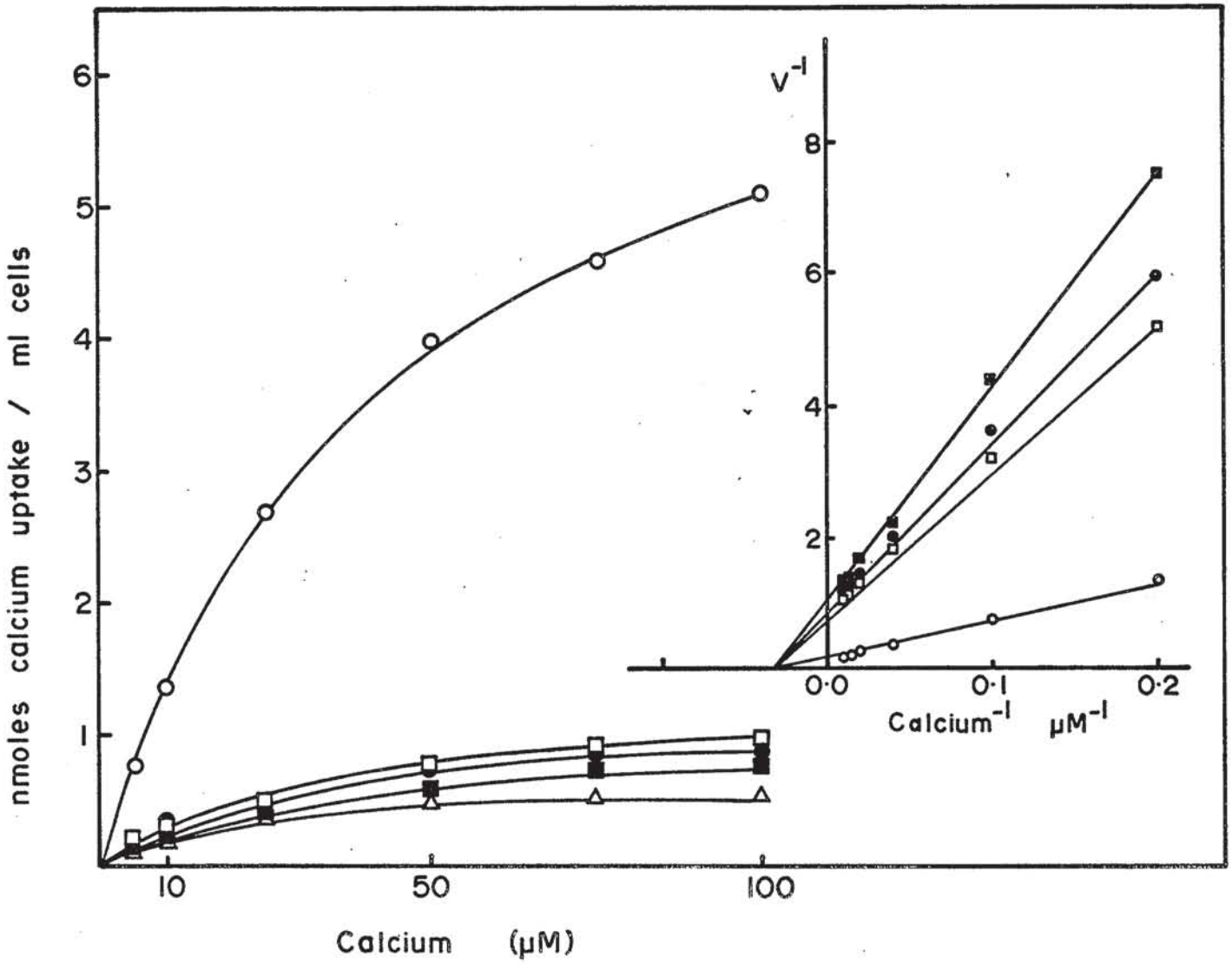

400
300
200
100
% Increase Calcium Uptake / ml cells
0
10-9
10-8
10-7
10-6
10-5
6 - Benzyladenine (M)

Figure 45: Rate-concentration plot and Lineweaver-Burk form of calcium uptake for normal cells in the presence of 0.01 mM $HgCl_2$. The effect of adenine (○), 0 mM; (□), 0.5 mM; (●), 1.0 mM; (■), 2.0 mM, and (△), no addition in the absence of $HgCl_2$.


nmoles calcium uptake / ml cells
6
5
4
3
2
1
10
50
100
Calcium (μM)
V⁻¹
8
6
4
2
0·0
0·1
0·2
Calcium⁻¹ μM⁻¹

absence of $HgCl_2$. The Lineweaver-Burk form indicated that adenine reduced the maximum velocity, but had no effect on the apparent binding affinity. Furthermore, noncompetitive inhibition indicated that these two ligands appear not to be binding to the same site.

Other cytokinins tested also caused release of calcium from $HgCl_2$ stimulated cells. After the cells were stimulated to take up $^{45}Ca^{++}$ in the presence of $HgCl_2$, a variety of cytokinin compounds were tested for their ability to release $^{45}Ca^{++}$ from the cells, as shown in Figure 46. The specificity of the cytokinin action was affirmed by the inability of adenine sugar and adenine monophosphate to stimulate calcium release from the cells. In Figure 46b, the majority of metabolically active cytokinins were efficient antagonist of calcium bound by the cells. Other agents were tested for calcium-releasing ability in addition to active cytokinin compounds, but only cytokinins and analogues were found to be effective as strong calcium releasing agents. A sampling of agents tested are summarized in Table XXI.

Cytokinin effects in osmotically shocked cells: Further studies of cytokinin effect on calcium transport after separation of the transport system clarified the nature of the cytokinin effect. Cytokinins enhanced the uptake of calcium when fixed at 100 μM in osmotic shocked cells, however, at low cytokinin concentration, maximum activation of calcium uptake only occurred in the presence of 10 μM $HgCl_2$, as shown in Figure 47. Reconciliation of this plot with the normal hyperbolic

Figure 46: Stimulation of $^{45}Ca^{++}$ release from germinated sporangiospores (a) by AMP (⊙), adenosine (⊡), adenine (△), hypoxanthine (◒), and xanthine (●) in the presence of 0.01 mM $HgCl_2$ and adenine (---) in the absence of 0.01 mM $HgCl_2$ and (b) by 6-amino-3-dimethylallylpurine (⊙), N^6-(trans-4-hydroxy-3-methylbut-2-enyl)adenine (△), 6ipAde (●), 6-methylaminopurine (⊡), and 6-dimethylaminopurine (◑) in the presence of 0.01 mM $HgCl_2$. In the experiments with 6ipAde and N^6-(trans-4-hydroxy-3-methylbut-2-enyl)adenine the pH of the reaction was 7.3 rather than 7.0.

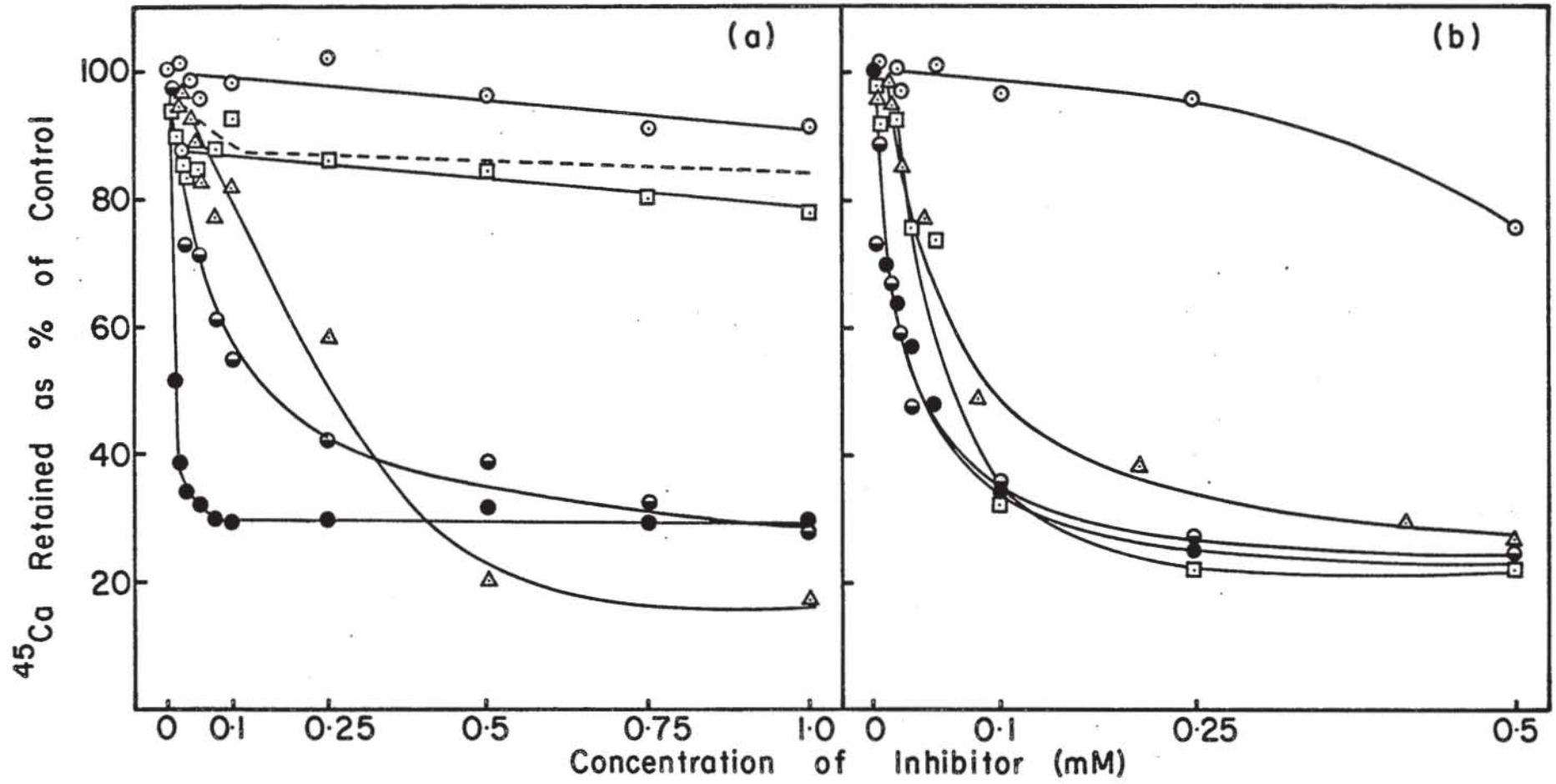
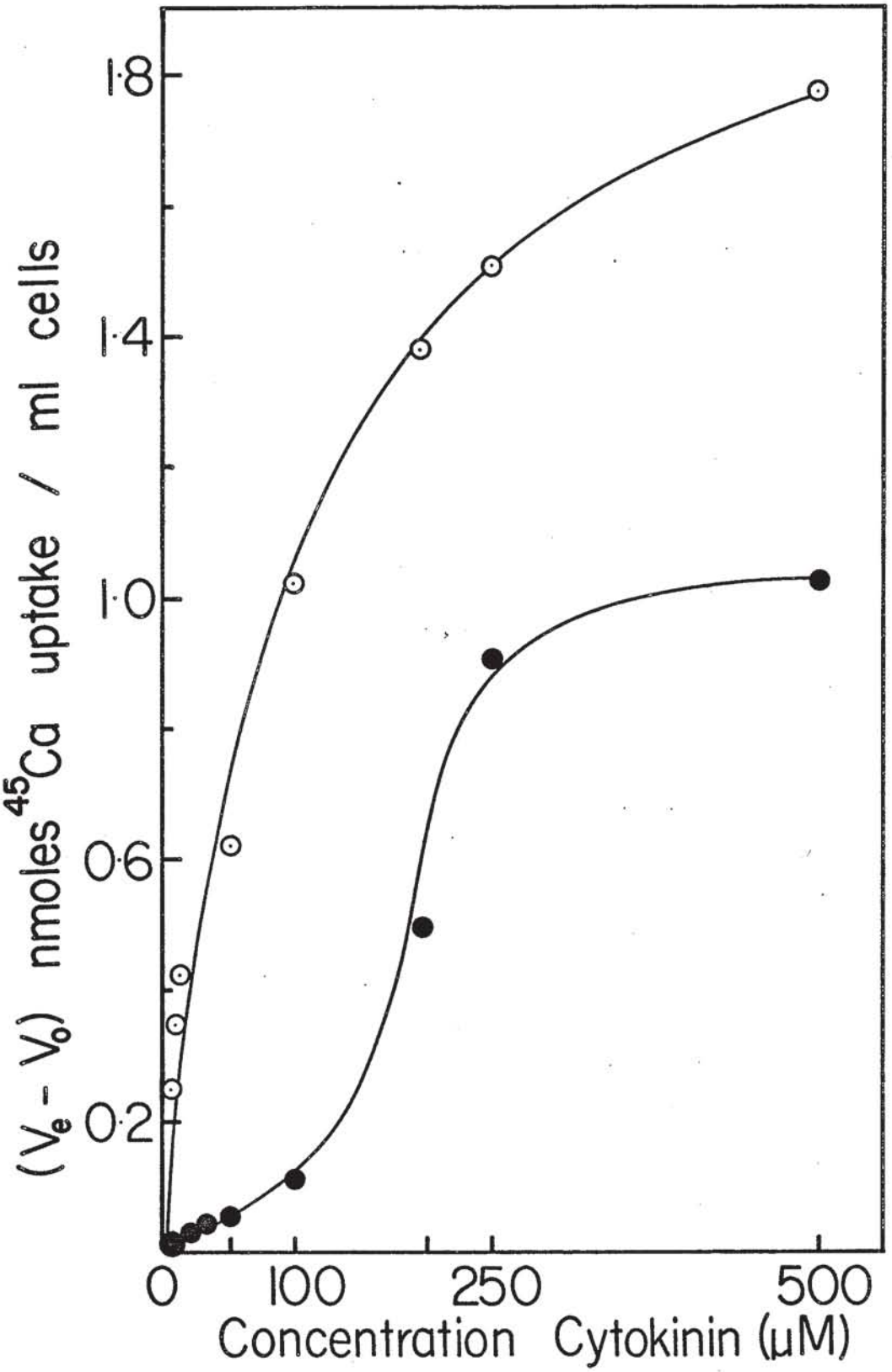

(a)
(b)
100
80
60
40
20
^{45}Ca Retained as % of Control
0
0·1
0·25
0·5
0·75
1·0
Concentration of Inhibitor (mM)

Figure 47: Activation of calcium uptake by osmotically shocked germinated sporangiospores of Achlya by the cytokinin 6ipAde in the presence (○) and absence (●) of 0.01 mM $HgCl_2$. V_e is the reaction rate in the presence of 6ipAde and V_o the rate in its absence.


1·8
1·4
1·0
0·6
0·2
$(V_e - V_o)$ nmoles ^{45}Ca uptake / ml cells
0
100
250
500
Concentration Cytokinin (μM)

uptake rate-concentration plot of calcium in osmotically shocked cells at first seemed improbable. However, sigmoidal uptake of calcium in osmotically shocked cells readily occurred in the presence of $MgCl_2$ or $MnCl_2$ (Fig. 48). When isopentenyladenine was added in the presence of divalent cation, calcium uptake resumed hyperbolic uptake kinetic pattern. Apparent sigmoidal activation by cytokinin of calcium transport was in fact due to $MgCl_2$ contaminating the buffer system.

Of particular interest was the ability of isopentenyladenine to stimulate calcium uptake maximally in the presence of $HgCl_2$. As was shown in Figure 20, $HgCl_2$ reduced calcium transport in shocked cells in the absence of cytokinin. Therefore, in addition to neutralizing the inhibitory effect of $MgCl_2$ or $MnCl_2$, isopentenyladenine also antagonized the inhibitory action of the mercurial. Table XXI lists the activation constants ($S_{0.5}$) of other cytokinin and cytokinin-like agents for calcium uptake in osmotically shocked cells. In a separate study, Stevenson (1974) showed adenine was bound by osmotic shocked cells and thiol-reacting agents, $AgNO_3$ and iodine enhanced adenine binding.

Cytokinin effect on calcium binding glycopeptide: If indeed, calcium uptake is antagonized by cytokinins in intact cells and seemingly activated in osmotic shocked cells, it was of particular interest to study the effect of cytokinin on the calcium binding glycopeptide as a possible site of calcium-releasing action of cytokinin. Calcium binding activity of the glycopeptide was shown

Figure 48: Effect of the interaction between divalent cations and 6ipAde on calcium transport in osmotically shocked cells. (a) $MgCl_2$ effect at (⊙), 0 mM; (⊡), 5 mM; (△), 5 mM with 0.5 mM 6ipAde, and (b) $MnCl_2$ effect at (⊙), 0 mM; (⊡), 0.1 mM; (△), 0.1 mM with 0.5 mM 6ipAde.

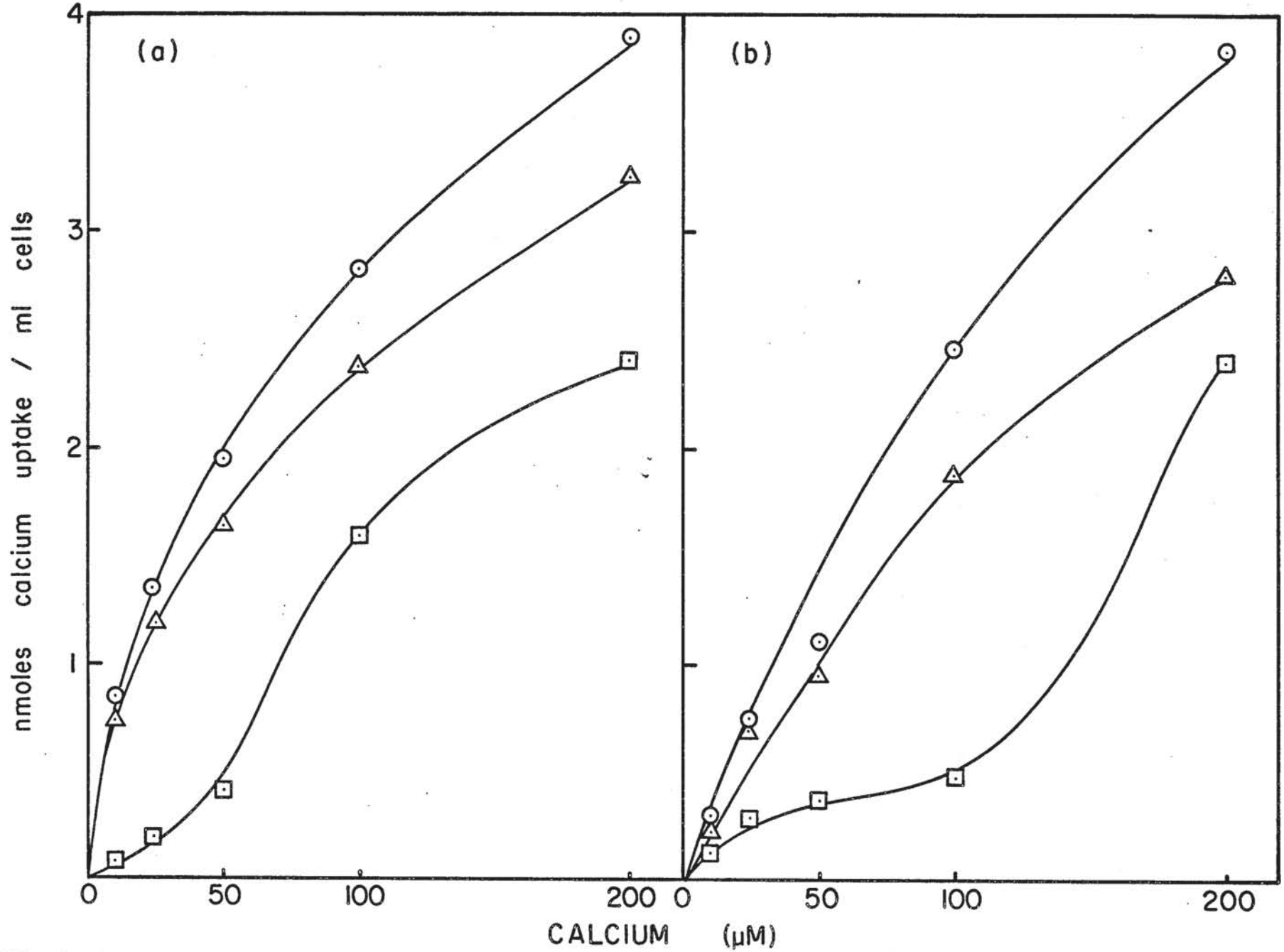

(a)
(b)
nmoles calcium uptake / ml cells
CALCIUM (μM)
0
50
100
200
1
2
3
4

TABLE XXI

A summary of the activation ($S_{(0.5)}$) and inhibition ($I_{(0.5)}$) constants of various agents that influence Ca^{++} release from germinated sporangiospores and Ca^{++} uptake into osmotically-shocked germlings of Achlya. Compounds that were totally ineffective are cyclic AMP, cyclic GMP, AMP, ADP, TMP, TDP, CMP, CDP, UMP, UDP, IMP, IDP, guanine, cytosine, uracil, thymine, guanosine, cytodine, uridine, thymidine, inosine, xanthosine, indole acetic acid, naphthalene acetic acid, proflavin, acriflavin, kinetin, kinetin riboside, triacanthine, 6-isopentenyladenosine, abscisic acid, caffeine and benzimidazole.

Several compounds that are close analogues of cytokinin-active agents (e.g. 6-n-propylmercaptopurine, 6-n-isopropoxypurine, 6-propoxypurine, 6-n-butoxypurine, and 6-n-histaminopurine) stimulate Ca^{++} release but their activating properties are not yet determined.

Agent	$S_{(0.5)}$ M	$I_{(0.5)}$
Adenine	x	1.6×10^{-4}
Hypoxanthine	x	8.0×10^{-5}
Xanthine	5.0×10^{-5}	8.3×10^{-6}
Theophylline	x	2.5×10^{-4}
6-Benzyladenine	x	5.0×10^{-5}
6-Methylaminopurine	5.0×10^{-5}	4.0×10^{-5}
6-Dimethylaminopurine	2.5×10^{-5}	2.0×10^{-5}
2,6-Diminopurine	x	7.5×10^{-5}
Zeatin	x	5.0×10^{-5}
2-Aminobenzimidazole	x	1.0×10^{-4}
6-Isopentenyladenine	4.6×10^{-6}	6.67×10^{-6}

to be dramatically reduced by N^6-(substitued)-adenine derivatives such as N^6-(Δ^2-isopentenyl)adenine, N^6-dimethyladenine, N^6-methyl-adenine, xanthine, hypoxanthine, N^6-(trans-4-hydroxy-3-methylbut-2-enyl)adenine, adenine, and 6-amino-3-(3-methyl-2-butenyl)purine ((tricanthine)). Generally, the extent of inhibition correlated with cytokinin effectiveness in plant growth cytokinin bioassay systems. In contrast, tricanthine is usually not an active cytokinin, but is believed to be an inactive form of the natural growth factor, isopentenyladenine formed by a rearrangement of the hydrocarbon chain from the 3 to the 6 position. The reason why tricanthine strongly reduced glycopeptide calcium binding is unknown. These results are summarized in Table XXII. Furthermore, mercurial activated calcium binding by the glycopeptide was strongly inhibited by cytokinin (Fig. 49). Adenine inhibition of calcium binding activity was measured by anionic exchange resin Dowex 2-X8 assay. The location of cytokinin action in whole cells was confirmed as the calcium binding glycopeptide because analogous results were obtained as to extent of effectiveness of cytokinin. Here again, the nucleosides of purines and pyrimidines were inactive calcium releasers. The possibility of simple chelation of calcium by cytokinin was eliminated by the specificity of the system and the inability of nucleosides to function as calcium releasers. The cytokinin action on the calcium transport system seems to be predominantly at the calcium binding glycopeptide causing the release of calcium from its storage site and subsequent uptake into the cell via the intrinsic calcium

TABLE XXII

Influence of cytokinins and purine compounds on the $^{45}Ca^{++}$ binding activity of Achlya glycoprotein. Activity was assayed by equilibrium dialysis at 4° for 4 hr against 1 mM Tris-acetate at the specified pH and containing 50 μM Na^{+} (determined from the amount of NaOH used to solubilize cytokinins and some purine compounds). The concentration of Ca ions added was kept fixed in all experiments at 10 μM. Because different preparations of binding protein were used in these analyses, the age and purity of the glycoprotein are specified. Experimental pH could not be normalized at low ionic strength buffer because of solubility problems.

Expt. No.	Agent	Concentration (μM)	Age/Purity of glycoprotein	pH	nmols $^{45}Ca^{2+}$ bound/mg protein
1.	nil	-	2 day-old crude* isolate	7.0	332.3
	Adenine	500			162.7
	Adenosine	500			309.4
	Triacanthine[a]	500			69.1
2.	nil	-		8.1	320.2
	Zeatin[b]	500			152.0
3.	nil	-		9.9	548
	6-Dimethylaminopurine	500			348.3
	6-Methylaminopurine	500			202.2
4.	nil	-	1 day-old crude* isolate	7.0	450.8
	Inosine	500			450.8

Table XXII. Continued.

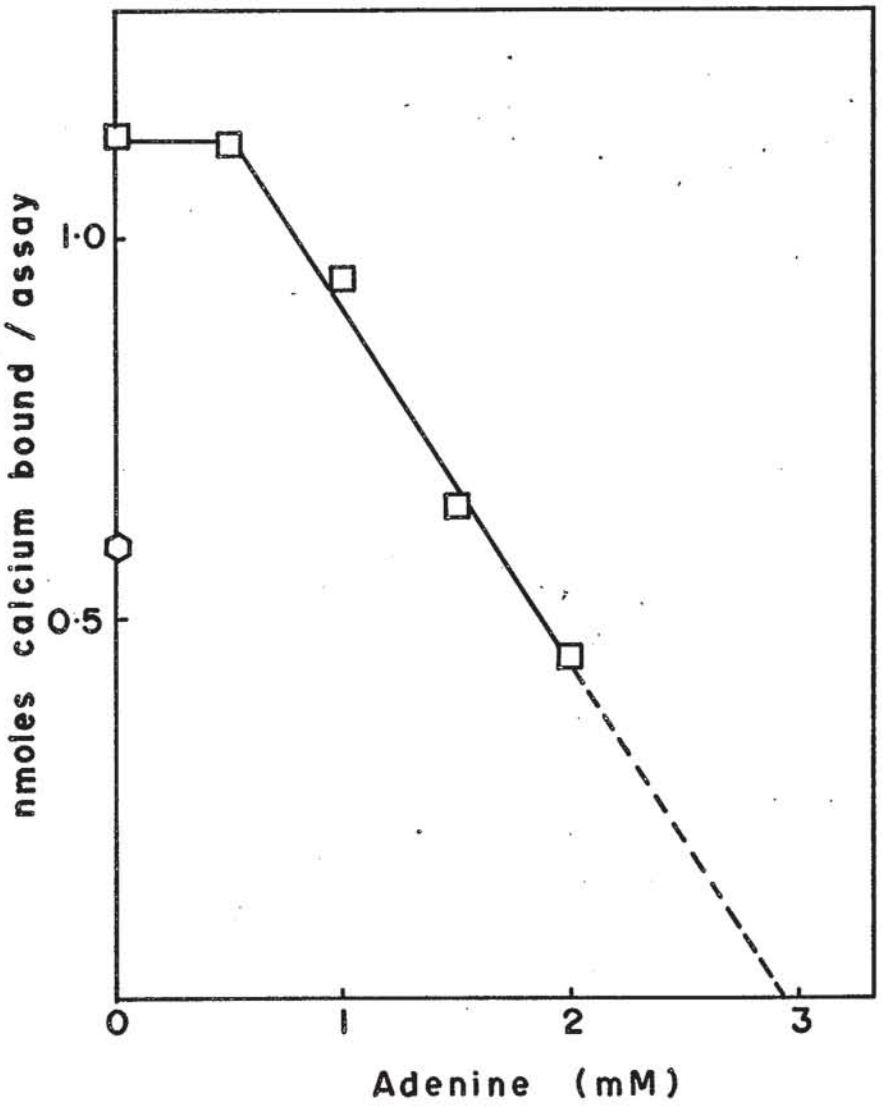
Expt. No.	Agent	Concentration (μM)	Age/Purity of glycoprotein	pH	nmols $^{45}Ca^{2+}$ bound/mg protein
5.	nil	-		9.0	628.6
	Xanthine	500			203
	Hypoxanthine	500			406.6
	Isopentenyladenine	500			91.1
6.	nil	-	Purified CaBP	9	2153.0
	Xanthine	1			1611.0
		10			1561.0
		100			1102.0
		500			575.0

*Crude shock fluid stored on ice at 0° to 4° during the interval from time of extraction.

[a]Triacanthine, 6-amino-3-(3-methyl-2-butenyl)-purine

[b]Zeatin, N^6-(trans-4-hydroxy-3-methylbut-2-enyl)adenine

Figure 49: Effect of adenine on glycopeptide calcium binding activity in the presence of 0.02 mM thimerosal (□), and in the absence (⬡).


nmoles calcium bound / assay
1·0
0·5
0
1
2
3
Adenine (mM)

transport component throughout the growth cycle, but another plant-hormone was found to interact with the glycopeptide complicating an at first seemly straight forward regulation by cytokinin.

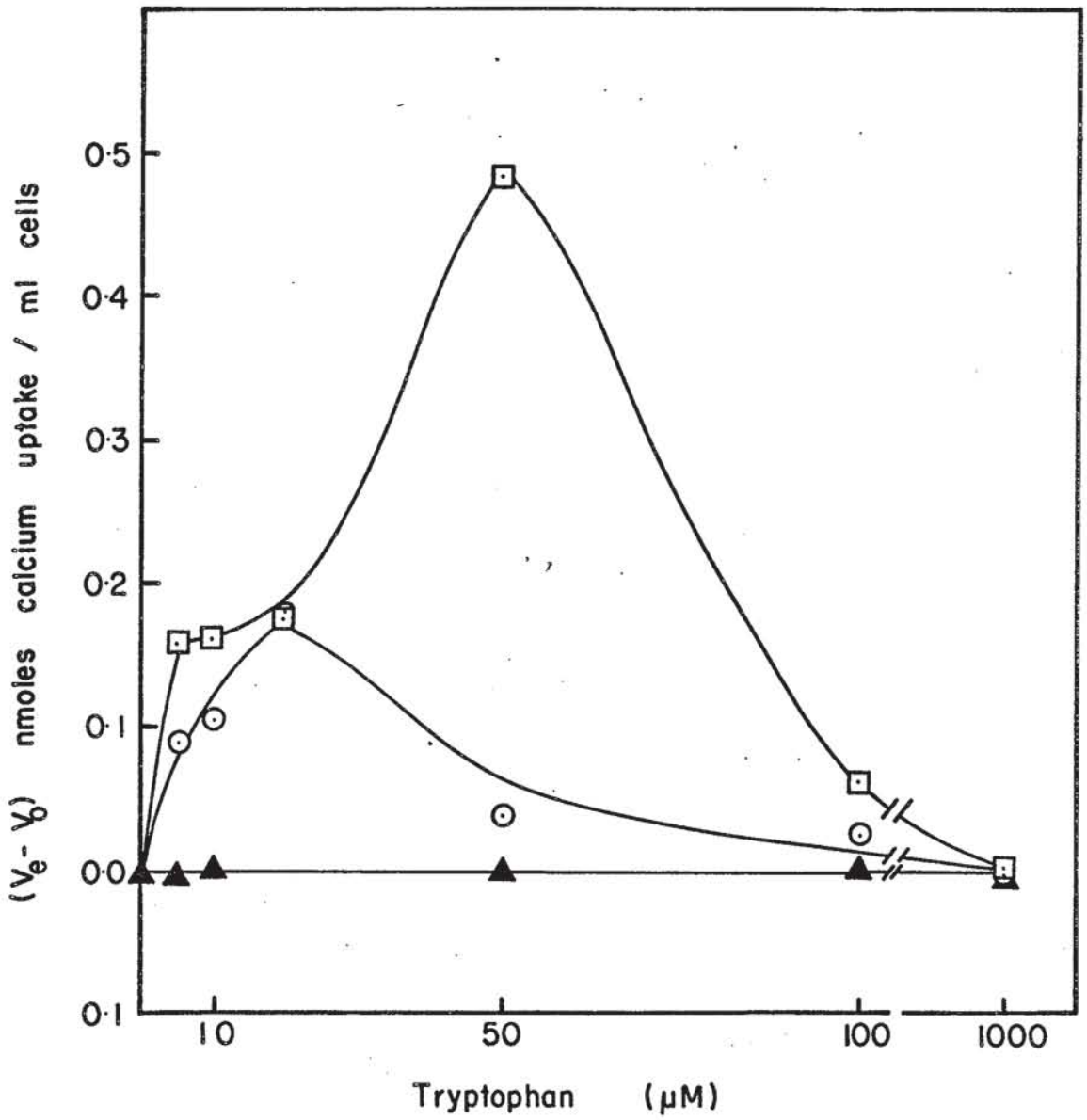
Auxin effects: It was of interest to investigate the importance of another plant hormone, auxin on the calcium transport system. Stevenson (1974) and LéJohn (1975) showed that cytokinins antagonized amino acid uptake in Achlya with the sole exception of tryptophan. Further tests by Stevenson (1974) showed that cytokinin and tryptophan or IAA interacted with the same component on the cell surface and enhanced apparent uptake of tryptophan was due to binding to this component. It was found that in cytokinin apparent uptake-activation of the amino acid, tryptophan was in fact due to its relation to auxin, indole-3-acetic acid. Tryptophan is the precursor for indole-3-acetic acid synthesis. In an extension of these studies, it was found that tryptophan influenced calcium binding by the glycopeptide (Table XXIII). The auxin-effect on calcium binding was strongly concentration dependent. At 10 μM and 100 μM, tryptophan had little effect on calcium binding, but at 50 μM tryptophan, total calcium binding was increased 2.5 fold. The activating effect of tryptophan on calcium transport was affirmed in intact cells, as shown in Figure 50. Activation was maximal at 50 μM tryptophan and in the presence of $HgCl_2$. Upon addition of isopentenyladenine, tryptophan activation was reduced to the normal level whether in the presence or absence of $HgCl_2$. In osmotically shocked cells, tryptophan

TABLE XXIII

Ability of Achlya calcium binding glycopeptide to bind calcium and other compounds. Assay was by equilibrium binding on Sephadex G-50 equilibrated with labelled ligand in 1 mM Tris-acetate, pH 7 buffer. Calcium binding glycopetide was present as a monomer with a molecular weight of 5960 daltons, but an aggregate form did appear in the presence of calcium.

Ligand	Concentration (μM)	Addition	Nmoles bound/mg protein
tryptophan	10	-	60.7
	10	6ipAde, 50 μM	104.8
	100	-	276.4
	100	6ipAde, 10 μM	317.9
6ipAde	10	-	111.1
	10	tryptophan, 100 μM	470.6
	10	calcium, 10 μM	
		monomer	21.8
		aggregate	54.9
calcium	10	-	
		monomer	579.3
		aggregate	447.4
	10	tryptophan, 100 μM	
		monomer	459.5
		aggregate	325.1
	10	tryptophan, 10 μM	
		monomer	553.0
		aggregate	377.0
	10	tryptophan, 10 μM + 6ipAde, 100 μM	
		monomer	436.0
		aggregate	378.6
	10	tryptophan, 50 μM	
		monomer	1719.2
		aggregate	849.2

Figure 50: Influence of tryptophan on calcium upake in intact cells in the presence (⊡) and absence (⊙) of 0.01 mM $HgCl_2$ in addition to 0.50 mM 6ipAde (▲). V_e is the reaction rate in the presence of tryptophan and V_o the rate in its absence.


0·5
0·4
0·3
0·2
0·1
0·0
0·1
(Ve - Vo) nmoles calcium uptake / ml cells
10
50
100
1000
Tryptophan (μM)

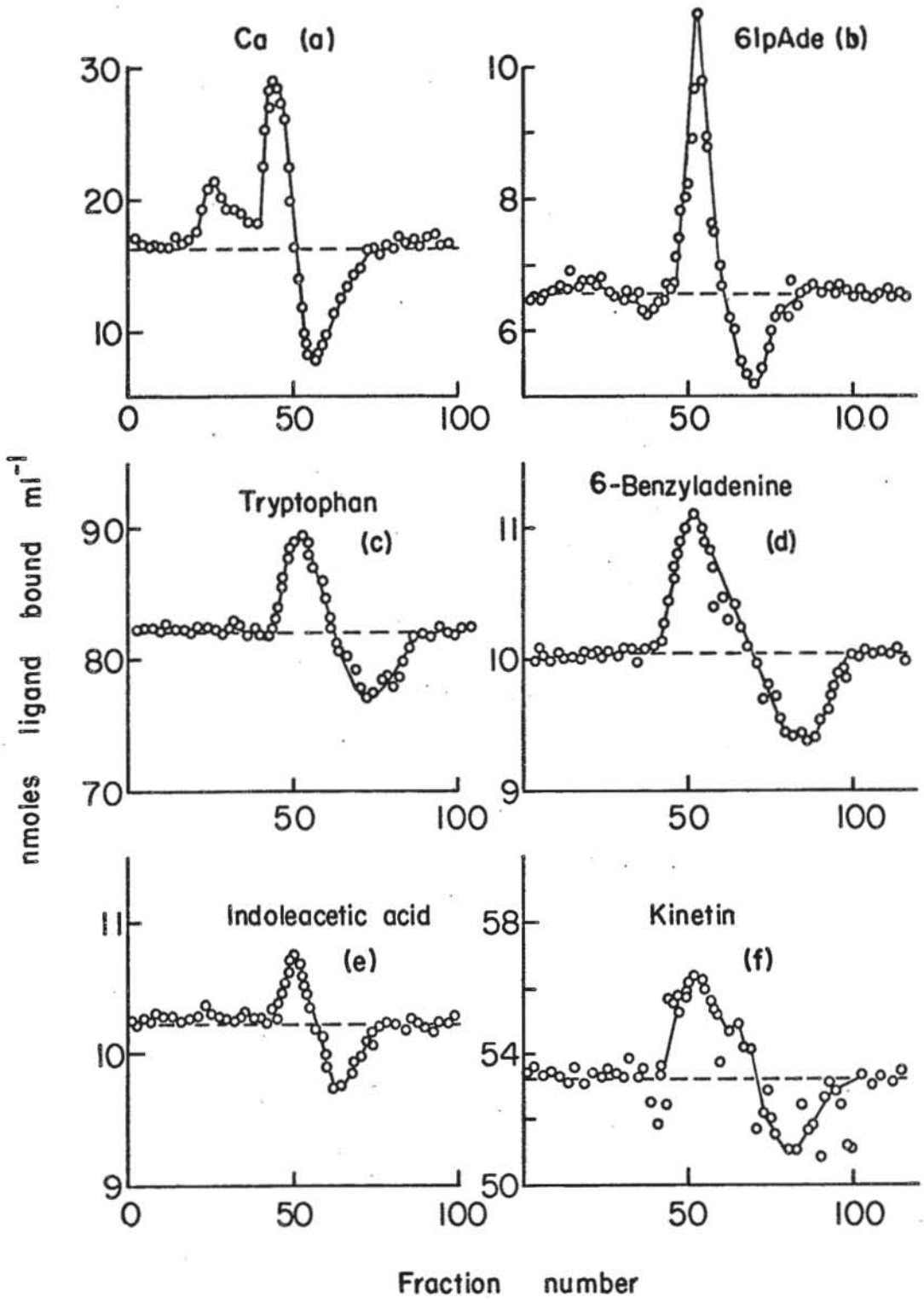
activation of calcium could only be shown in the presence of $HgCl_2$ and cytokinin no longer antagonized the tryptophan effect.

Interaction of glycopeptide ligands: To clarify the apparent interplay of auxin and cytokinin on the calcium transport system, ligand binding by the glycopeptide was studied. Hormonal ability to influence the amount of calcium bound by the glycopeptide was verified by showing that both cytokinin and auxin can bind to the glycopeptide (Fig. 51). These results indicate that calcium was the most effective of all the ligands to bind to the glycopeptide, and that only in the presence of calcium did an aggregate form of the glycopeptide appear (Fig. 51a). The glycopeptide bound isopentenyladenine relatively well, but kinetin binding could only be shown when the concentration was raised to 50 μM. The poor binding of kinetin may be due to the furfuryl group in this cytokinin since all nucleoside forms of cytokinin and purines failed to interact with the molecule (Table XXII). A variety of other compounds were tested for their ability to bind to the glycopeptide, such as histidine, phenylalanine, thymidine, and cyclic AMP but none of these compounds were capable of binding. Of interest was the ability of indole-3-acetic acid to bind to the glycopeptide only at acidic pH. When the pH was raised to pH 5.5 no binding occurred.

To further characterize hormone action, binding of each ligand was studied in the presence of the other ligand. The results are summarized in Table XXIII. Binding of hormonal ligand by the aggre-

Figure 51: Glycopeptide-ligand equilibrium binding on Sephadex column equilibrated with (a) $^{45}Ca^{++}$, (b) ^{3}H* [N^{6}(Δ^{2}-isopentenyl)adenine] , (c) ^{3}H-tryptophan, (d) ^{14}C-8-benzyladenine, (e) ^{14}C-2-indole-3-acetic acid, and (f) ^{14}C-6-furfuryladenine (kinetin).

* Generally-labelled [G]


Ca (a)
6IpAde (b)
Tryptophan (c)
6-Benzyladenine (d)
Indoleacetic acid (e)
Kinetin (f)
nmoles ligand bound ml⁻¹
Fraction number

gate glycopeptide only occurred in the presence of calcium. Of importance are the following interactions on the glycopeptide: (i) cytokinin alone caused the release of calcium and increased auxin binding; (ii) auxin alone enhanced both calcium and cytokinin binding; (iii) calcium antagonized cytokinin binding; and (iv) cytokinin nullified auxin enhancement of calcium binding, but this interaction was concentration dependent. This evidence suggests that both hormones are interacting with the glycopeptide in some complex manner. Moreover, it is apparent that both hormones are important under normal physiological conditions for the regulation of calcium movement through the cell wall and membrane.

DISCUSSION

Characterization of the Calcium Transport Process

Transport of calcium in Achlya seems to be by a carrier-mediated process that is indirectly associated with metabolic energy. The results obtained with metabolic energy antagonists are difficult to analyze, since inhibition of calcium transport by 2,4-DNP may also be due to ionophoric transport reversal by 2,4-DNP (McLaughlin, 1972) facilitating diffusion of calcium out of the cell. Moreover the reason why metabolic antagonists do not appear to directly affect the calcium transport process to the same degree as active transport processes in Achlya could be because 40% of the calcium taken up by the cell is reversibly bound to the cell surface and not actually transported. But this seems unlikely since both azide and 2,4-DNP partially inhibit reversible sulfhydryl-activated calcium binding of the cell (see Fig. 14). The transport process is saturable at low external concentration, but as the concentration of calcium is raised, a gradual uptake of calcium continues even at 1 mM Ca^{++}. Temperature and pH influence on calcium uptake also shows that the calcium transport process is carrier-mediated.

The calcium transport process appears to contain two components as shown by a biphasic uptake curve of Fig. 4, and two pH optima (Fig. 8). Sulfhydryl group reacting compounds signified that there are two components to the calcium system because they differentially affected pH and temperature optima. When the transport system is in

an oxidized state, maximal uptake of calcium occurs. Activation by oxidizing agents appears to be at easily accessible sulfhydryl groups since the oxidizing agent's mode of action is by oxidation of sulfhydryl groups. $HgCl_2$ and organic mercurials employed, normally interact with sulfhydryl groups by mercaptide formation that is easily reversible, except after prolonged periods of incubation or high concentration, these mercurials become inhibitory due to diffusion into the membrane (see Seidel, 1972). Also, $HgCl_2$ at high concentration or prolonged periods of incubation can interact with phosphoryl and carboxyl groups. The secondary inhibition of $HgCl_2$ could be due to (i) binding to sulfhydryl groups of the intrinsic component of the calcium transport system, (ii) removal of calcium bound to carboxy groups and/or (iii) release of sulfhydryl activated component of the calcium transport process as was shown by Cater (1973) for release of loosely associated red blood cell membrane proteins by pCMBS.

Two Component Calcium Transport Process

Intrinsic component: Kinetic analysis of the calcium transport process in intact cells revealed a complex pattern of calcium uptake in Achlya. Calcium uptake was simplified after cold osmotic shock treatment of the cells. In cold osmotic shocked cells the initial rate of calcium transport is by normal Michaelian kinetics. The affinity of the transport system is almost identical to the low affinity transport component in normal cells. Temperature and pH

studies in the presence and absence of sulfhydryl-reacting compounds no longer indicate a two component system. The partial antagonizing influence of metabolic inhibitors may indicate that the intrinsic calcium transport process is associated with cell energy but this is difficult to discern. An attempt to check for ATPase activity was futile, no ATPase activity could by found over a wide range of Ca^{++} and Mg^{++}-ATP concentrations. Moreover, oligomycin had no effect on transport. Another possibility could be that active transport proceeds down a potential gradient generated by uptake or extrusion of another cation. A facilitated means of intrinsic calcium transport seems to be the most attractive method since calcium is one of the few solutes that can accumulate in osmotically shocked cells (experimental details are presented in a later section).

Calcium binding glycopeptide: A calcium binding glycopeptide was released into the surrounding medium of cold osmotic shocked cells. The isolation of this glycopeptide was of interest in understanding the complexities of the calcium transport system of Achlya. The glycopeptide calcium binding was found to be stimulated by mercurials analogous to the reversible mercurial-stimulated component of the calcium transport system of intact cells and have a K_d equal to the low K_m value of whole cells. Indirect evidence, ionic strength, sensitivity, rapid binding, reversibility of binding, and release of glycopeptide by mild treatment indicates that the glycopeptide is located at the cell membrane surface. Apparent association of the glycopeptide with the calcium transport process is indicated by a reduction

in calcium uptake upon osmotic shock treatment. Osmotically shocked cells placed promptly in suitable growth medium, after a lag period of 10 h, were able to re-synthesize the calcium binding glycopeptide (Fig. 28) whose synthesis coincided with resumption of growth. The simultaneous re-synthesis of glycopeptide and start of growth infers that the calcium binding glycopeptide is essential for growth.

Usually, loosely associated transport proteins bind only one or so substrate molecules, but the calcium binding glycopeptide bound 7 - 14 moles of calcium per mole of glycopeptide. Thus, the calcium binding glycopeptide may act as an external storage site for calcium, as calcium was found to be so important for growth, energy-related transport activities and metabolism of Achlya (see later section). Apparently, surface calcium binding glycopeptides are a general phenomenon in water moulds as other Oömycetes were found to have these glycopeptides (Fig. 2b).

As the glycopeptide is associated with both calcium transport and storage, the significance of this glycopeptide in differentiation and growth was of interest. The glycopeptide was found to be produced throughout the ontogenic cycle, but as the mycelial mats aged the affinity of calcium binding decreased with only low affinity calcium binding ability remaining after 6 h (Table XVIII). However, the external concentration of calcium was found to have no effect on the amount of glycopeptide produced and its specific activity. One role that the glycopeptide may play during differentiation is the storage of calcium until the cell signals its uptake; a possible explanation why only low affinity calcium binding is detected in older cell mats

since higher than normal calcium levels are required for sporulation as shown by Griffin (1966). Of interest was the finding that the glycopeptide could not be released by osmotic shock treatment if the cells were first germinated in defined medium of Bardsdale (1963), but the significance of this not yet understood.

Characterization of Calcium Binding Glycopeptide

The calcium binding glycopeptide released into the surrounding medium upon osmotic shock treatment was purified to homogeneity as shown by a single band by electrophoresis. The amount of glycopeptide that could be recovered after purification was small presenting difficulties for its chemical and physical characterization. Only by large scale growth of the germinated sporangiospores was this problem partly overcome.

The nature of the high and low affinity binding sites was investigated by enzymatic degradation, and the results indicate that both intact peptide and carbohydrate moiety are required for proper calcium binding by the glycopeptide. As would be expected for a surface location, the calcium binding activity was found to be extrememly sensitive to ionic strength (Fig. 34). At low ionic strength, calcium binding activity was found to increase with increasing pH having no defined optimum, but at higher ionic strength there was an ill-defined optimum at pH 6 to pH 8.

The glycopeptide was found to contain 65% carbohydrate and 35% protein. The high content of glutamic acid (55%) contributes to the

acidic nature of the glycopeptide. The remaining amino acid residues are glycine, aspartic acid, alanine, lysine, serine, and cystine, and all are D-isomers. The majority of glutamic acid was also found to be D-isomeric but some L-isomer was also present. The carbohydrate component contained 80% glucose, 19% ribose or fucose, and the remaining sugars are unidentified but tentatively classified as uronic acids.

By correlating kinetic data, chemical composition, physical properties of isolation etc., the location of this glycopeptide is speculated to be on the cell membrane. The D-isomeric forms of amino acids indicate that the glycopeptide is apparently synthesized by surface enzymes not the normal ribosome protein synthesizing machinery of the cell, and that the glycopeptide is in some manner associated with the cell wall. The neutral and basic amino acids present may permit membrane attachment by the glycopeptide or hydrophilic amino acids, glutamic and aspartic may form a loose ionic attachment with phospho- groups of phospholipids. Furthermore, calcium, or some other divalent cation may play an important role in attachment of the glycopeptide to the surface membrane. One other possibility is that the glycopeptide may be free in a putative "periplasmic space". Also, the presence of a large quantity of glucose indicates a possible association with the cell wall. The presence of ribose in the cell surface binding glycopeptide was unusual and what the significance of ribose is is unknown. In mammalian cells, acidic surface binding proteins usually contain sialic acid as terminal residues, but an

attempt to find sialic acid in the glycopeptide of water moulds was unsuccessful. Due to the tentative characterization of ribose, the other sugar that ran almost coincident was fucose. If indeed, fucose was present it would be of significance since in the majority of surface binding proteins that do not contain sialic acid, fucose is the terminal sugar.

The binding sites of calcium have been indirectly shown to be both carbohydrate and protein, one type of binding site could be attributed to glutamic acid and aspartic acid carboxyl groups as was shown by Matthews et al (1972) for calcium binding by thermolysin and Nockolds et al (1972) for fish parvalbumin, and an additional calcium binding site by ionic association with carbohydrate oxygen atoms; hydroxyl or if fucose is present, deoxy groups.

The primary structure of the glycopeptide was not investigated due to problems of enzymatic hydrolysis of D-amino acids. Crude pronase was able to reduced calcium binding activity, but the molecular weight remained unchanged after hydrolysis. Speculation of possible structures for the glycopeptide in itself is difficult. The peptide component may form a backbone (single unit due to small number of amino acids) for glyco- attachment but the number of oligosaccharide chains attached and the type of attachment are unknown. The nature of oligocaccharide chains is extremely difficult to analyze due to inherent heterogeneity caused by the nature of synthesis and possible degradation steps involved.

The glycopeptide molecular weight was found to be 5,960 daltons as determined by Sephadex gel filtration. Further tests, SDS electrophoresis and chemical composition, confirmed that the molecular weight is in the region of 6,000 daltons.

Cytokinin and Auxin Regulation of Calcium Transport

Mercurial activation of the calcium transport system can be viewed as (i) a natural activator (ii) mimicking a natural activator, or most probably (iii) desensitizing the system to natural regulators. If the latter action is occurring, this implies that cytokinins in conjunction with auxin and Mg^{++} or possibly Mn^{++} regulate calcium transport.

At first notice, cytokinins appear to inhibit the transport of calcium in intact cells, but this occurred only under mercurial desensitizing conditions. Most important, calcium transport activation by cytokinin could be shown after prolonged periods of pre-incubation. In addition, in osmotically shocked cells, cytokinin enhanced calcium transport (Fig. 47). Auxin, unlike cytokinin, enhanced calcium uptake in normal cells in the absence or presence of $HgCl_2$, but to a much greater extent for the latter condition, and also activated calcium uptake in osmotically shocked cells but this time, $HgCl_2$ had no effect.

Both cytokinin and auxin can bind to the glycopeptide, each enhancing the binding of the other. A chelating effect of cytokinin causing calcium release by either complexing with $HgCl_2$ or $CaCl_2$ was excluded because of the (i) specificity of ^{6}N-(substituted)adenines,

the calcium binding glycopeptide is closely associated with the intrinsic calcium transport system adding specificity to the system, and, in addition, the calcium binding glycopeptide stores calcium whose movement is regulated by auxin-cytokinin concentration ratio.

Implications of Cytokinin and Auxin Effects

The importance of calcium for growth and differentiation throughout the ontogenic cycle suggests that calcium transport regulators, cytokinin and auxin or some natural agents play an essential role in the regulation of morphogenesis of this water mould, Achlya. As other members of this species is characterized by parasitism of higher organisms, the regulation of calcium movement by cytokinin and auxin in these fungi may have a physiological role associated with its parasitic nature. The ability of these fungi to produce auxin and cytokinin is unknown, whether the physiological role of these hormones is by fungal production at the site of parasitism producing a nutrient 'sink' or by using cytokinin and auxin present in the host to the advantage of the fungi during disease production.

PART II

CALCIUM EFFECTS ON GROWTH

RESULTS

A. Growth Requirement

Asexual growth cycle of Achlya: Calcium was found to be required throughout the growth cycle of the coenocytic filamentous water mould Achlya, as shown in Fig. 52a. A schematic diagram of Achlya sp. asexual life cycle is presented in Figure 52b, and substantiated by photographs of major stages of interest for experimental purposes in Fig. 52c. The cycle was repeated every 24 h under normal growth conditions, stationary cultures in GY medium at 22°. The description of the individual stages of the growth cycle has been previously outlined under Organism and Growth. Griffin (1966) first demonstrated that calcium was required for differentiation of sporangia of Achlya. After addition of chelators at hourly intervals to cells growing in GY medium, the results showed that EGTA (0.2 mM), EDTA (0.1 mM) and citrate (10 mM) immediately stopped growth. Cells were not able to proceed beyond their existing stage of the cycle. The specificity of the calcium requirement was shown by growth resumption only upon addition of calcium. To affirm the requirement of calcium for growth throughout the ontogenic cycle, calcium uptake over a 30 h period was studied. Ungerminated spores failed to take up

Figure 52a: Calcium requirement throughout the growth cycle of Achlya.

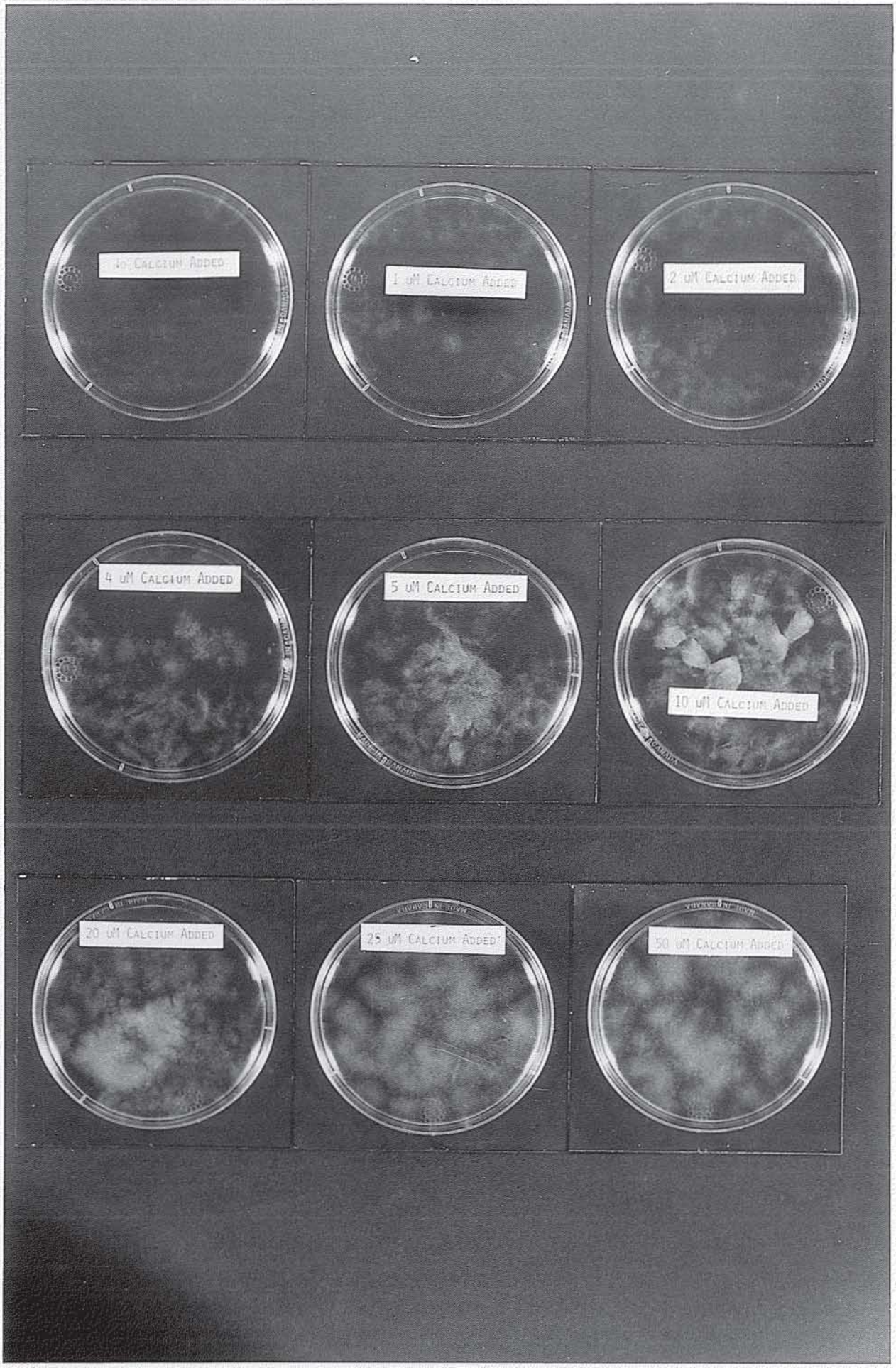

0 uM Calcium Added
1 uM Calcium Added
2 uM Calcium Added
4 uM Calcium Added
5 uM Calcium Added
10 uM Calcium Added
20 uM Calcium Added
25 uM Calcium Added
50 uM Calcium Added

Figure 52b: A schematic summary of Achlya sp. life cycle. The numbered stages have been arbitrarily choosen to categorize the growth cycle. The inner circle correlates stage number with morphological development and time of growth.

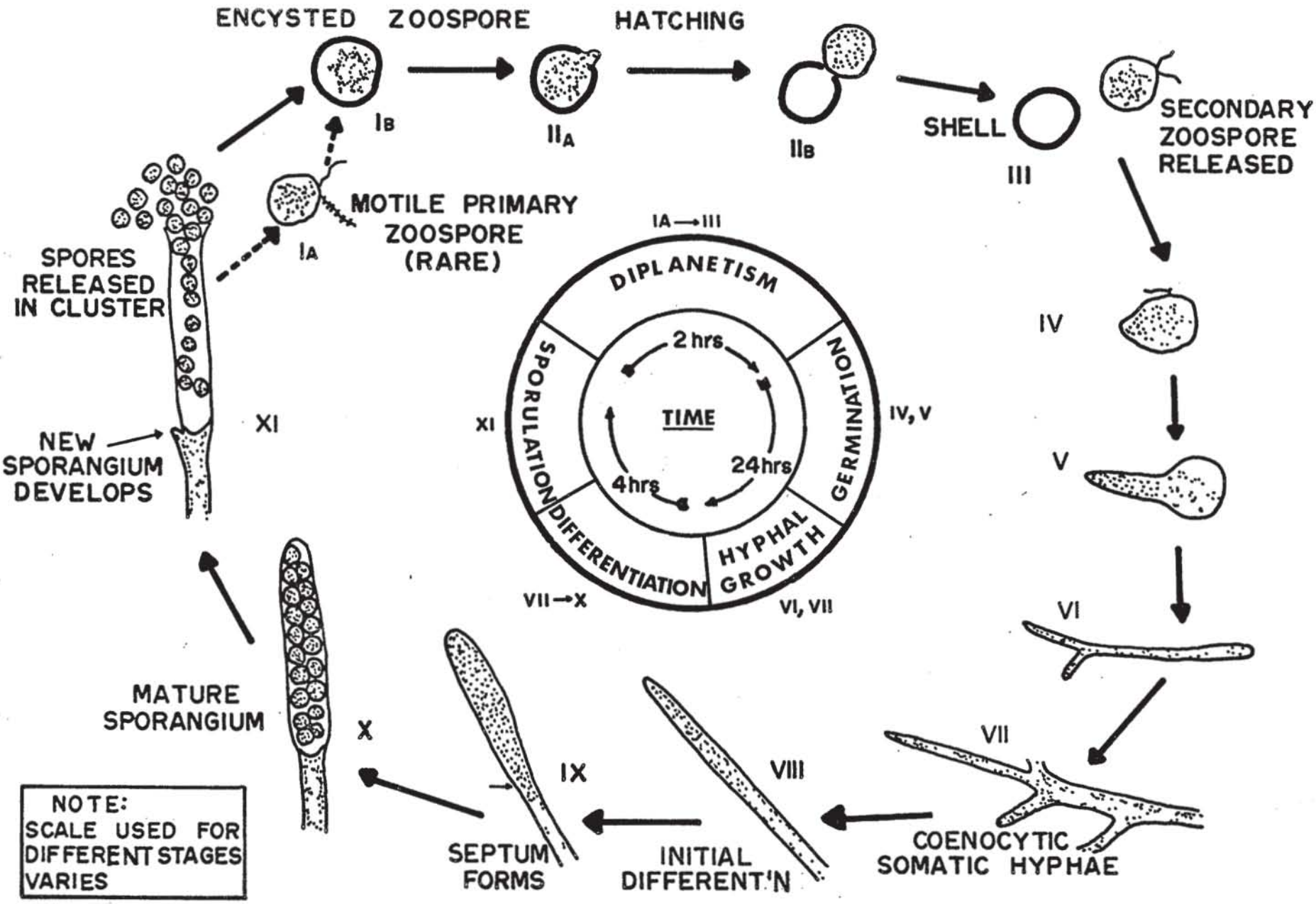

ENCYSTED ZOOSPORE
HATCHING
IB
IIA
IIB
SHELL
III
SECONDARY ZOOSPORE RELEASED
MOTILE PRIMARY ZOOSPORE (RARE)
IA
SPORES RELEASED IN CLUSTER
IA→III
DIPLANETISM
2 hrs
TIME
24hrs
4hrs
SPORULATION
GERMINATION
DIFFERENTIATION
HYPHAL GROWTH
XI
IV, V
VII→X
VI, VII
IV
V
VI
VII
VIII
IX
X
XI
NEW SPORANGIUM DEVELOPS
MATURE SPORANGIUM
SEPTUM FORMS
INITIAL DIFFERENT'N
COENOCYTIC SOMATIC HYPHAE
NOTE:
SCALE USED FOR DIFFERENT STAGES VARIES

Figure 52c: Photographs of Achlya sp. asexual growth cycle at various stages: (a) stage 1_B, encysted zoospore; (b) stage II_A and II_B, zoospore hatching; (c) stage III, motile secondary zoospore (arrow); (d) mid-stage V, small germinated sporangiospores; (e) late-stage V, germinated sporangiospores; (f) stage VI, initiation of hyphal branching; (g) stage VII, hyphal filaments; (h) stages VIII to XI, initial differentiation to the release of sporangiospores; and (i) stage XI, release of sporangiospores from the sporangium.

a
b
c
d
e

f
g
h



calcium but immediately upon germination, calcium uptake commenced (Fig. 53). The logarithmic rate of calcium uptake closely correlated with the rate of growth and macromolecular synthesis.

When cells were grown in GY medium, increasing concentration of calcium up to 0.1 mM caused a linear increase in dry weight (Fig. 54). For a further ten-fold increase in the calcium concentration the dry weight only doubled. Above this calcium concentration, no further effect was produced until at very high concentrations (greater than 50 mM) growth was inhibited.

Citrate inhibition: Of interest was the ability of citrate to stop growth. Usually, citric acid does not have a detrimental effect on cell growth of organisms and a wide variety of organisms have the ability to use citric acid as a carbon source. The germinated sporangiospores of Achlya were unable to take up citric acid. The results obtained for uptake of dicarboxylic and tricarboxylic acids by growing cells are recorded in Table XXIV. Under non-toxic conditions for these organic acids, less than 1% of the exogeneous supply was associated with the cells. Of the organic acids tested, only succinate may have been transported. As a consequence, the cells are unable to overcome the inhibitory action of citric acid. Other citric acid cycle acids, although not transported, were characteristically different from citric acid due to their inability to antagonize growth even at a concentration of 30 mM. Citrate could be acting in a number of ways:

Figure 53: Uptake of calcium (⊙) correlated with percentage of Achlya spores germinated (⊡); RNA synthesis (△), and protein synthesis (⬡) in GY medium. The uptake of calcium and the increase in dry weight in inset are during Stage I to Stage XI of the developmental cycle.

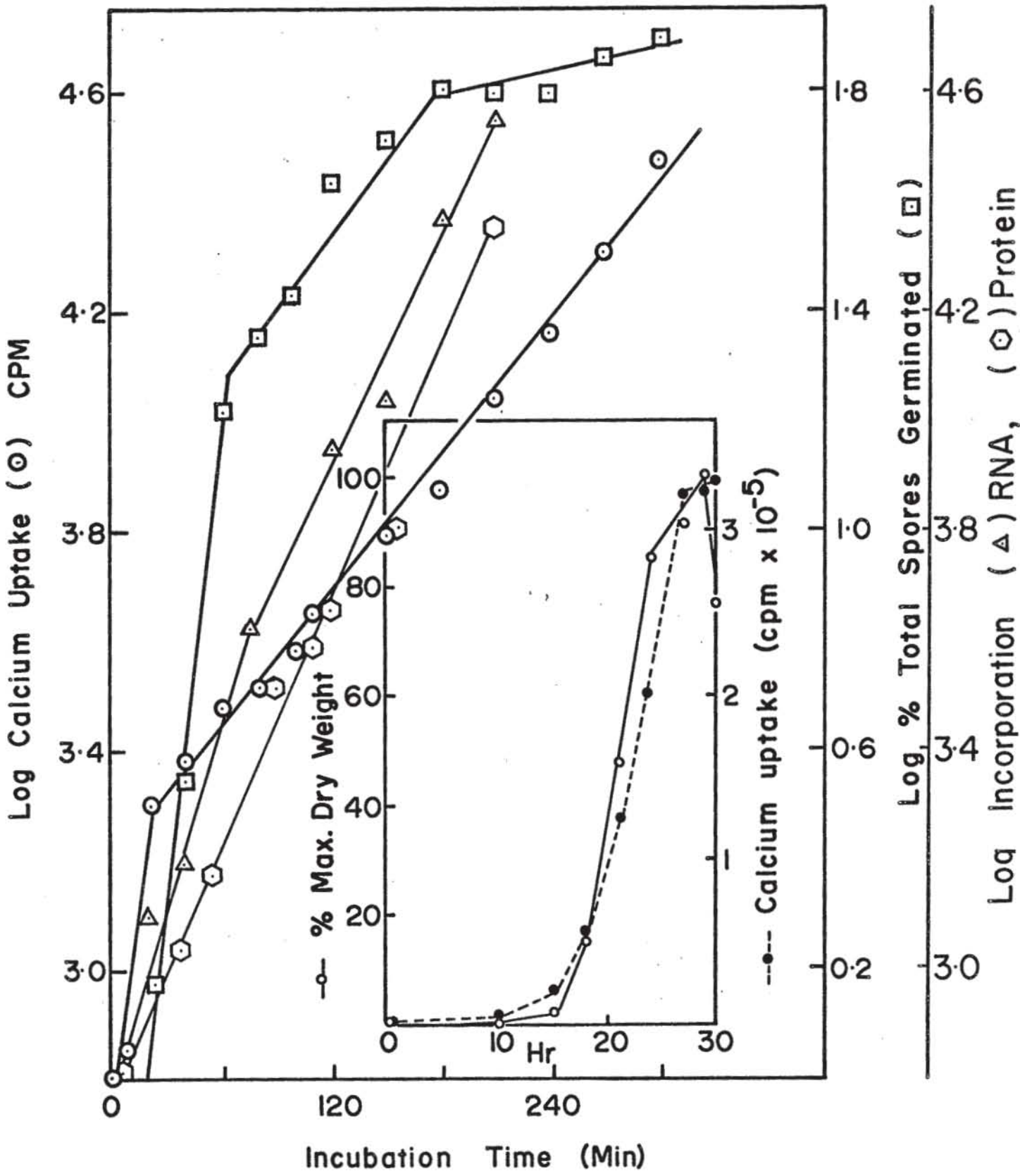

Log Calcium Uptake (⊙) CPM
4·6
4·2
3·8
3·4
3·0
0
120
240
Incubation Time (Min)
1·8
1·4
1·0
0·6
0·2
Log, % Total Spores Germinated (⊡)
4·6
4·2
3·8
3·4
3·0
Log Incorporation (△) RNA, (⊙) Protein
100
80
60
40
20
— % Max. Dry Weight
3
2
1
--•-- Calcium uptake (cpm x 10⁻⁵)
0
10
Hr
20
30

Figure 54: Increase in dry weight as a function of calcium concentration.

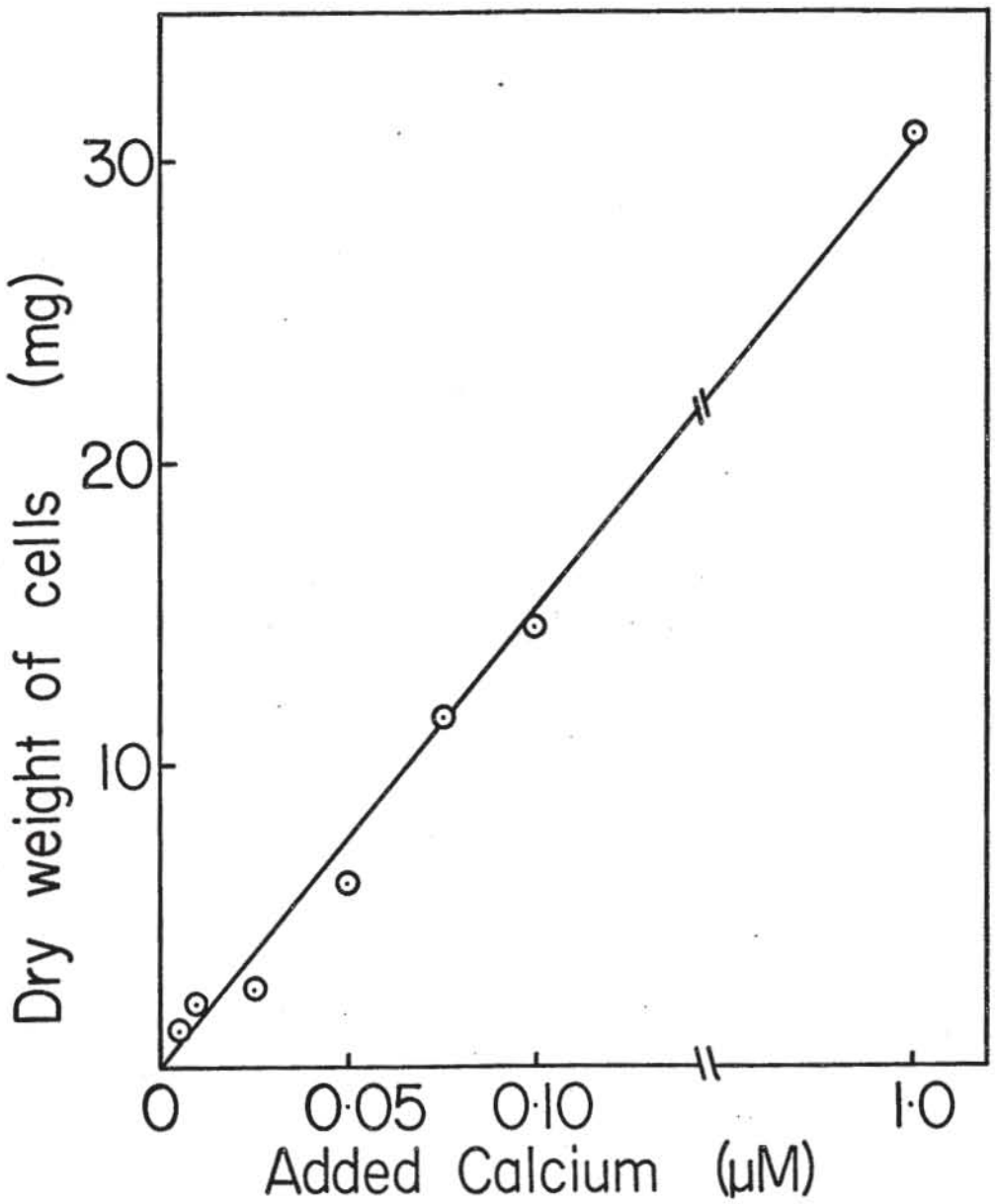

Dry weight of cells (mg)
30
20
10
0
0·05
0·10
1·0
Added Calcium (µM)

TABLE XXIV

Uptake of ^{14}C-labelled organic acids by cells of Achlya growing in GY medium.

Acid	% Total counts associated with cells after				
	1 hr	2 hr	4 hr	6 hr	24 hr
Citric	0.013	0.086	0.015	0.031	0.078
DL-Isocitric	-	-	-	-	0.76
α-Ketoglutaric	-	-	-	0.038	0.10
L-Malic	0.19	0.23	0.66	1.00	-
Fumaric	0.46	0.65	0.39	0.35	-
Succinic	0.86	2.96	4.64	7.14	-
Ethylenediamine tetraacetic	-	-	-	-	0.16

(i) by chelating exogeneous cation, preventing access to the cell; (ii) by binding to cell surface, thereby preventing cation binding and/or transport into the cell, and (iii) by causing an efflux of essential ions. All three possible actions proved to play a part in citrate inhibition of cell growth.

Citrate chelation of calcium was demonstrated by immediate stoppage of calcium uptake (Fig. 55), by competitive inhibition of the calcium transport process (Fig. 56), and by antagonism of glycopeptide calcium binding (Table XV). In addition, that citrate interacted with the cell was suggested by the chelation results whereas EDTA was about 100 times as effective as citrate in inhibiting growth, EGTA, a specific chelator of calcium (over 1000-fold tighter liganding) relative to EDTA was only 20 times as effective as citrate. The third mode of citrate action was inferred from calcium efflux experiments (Fig. 16). After pre-loading Achlya cells with $^{45}Ca^{++}$, citrate caused the release of bound calcium to the same extent as Ba^{++} which is also a strong competitive inhibitor of the calcium transport system. The property of citrate to antagonize cell growth and yet not be transported proved extremely useful when the role of calcium in cell growth and metabolism was examined.

The inhibitory effect of citrate was found to be lethal. When ungerminated spores were suspended in GY medium containing citrate for prolonged periods before calcium was added and citrate removed, a citrate-killing curve for Achlya spores was obtained, as shown in Figure 57. Over 65% of the spores were killed in the first 8 hours

Figure 55: Inhibition of calcium uptake by Achlya germinated sporangiospores in the presence of 10 mM citrate (△) and the absence (⊙).

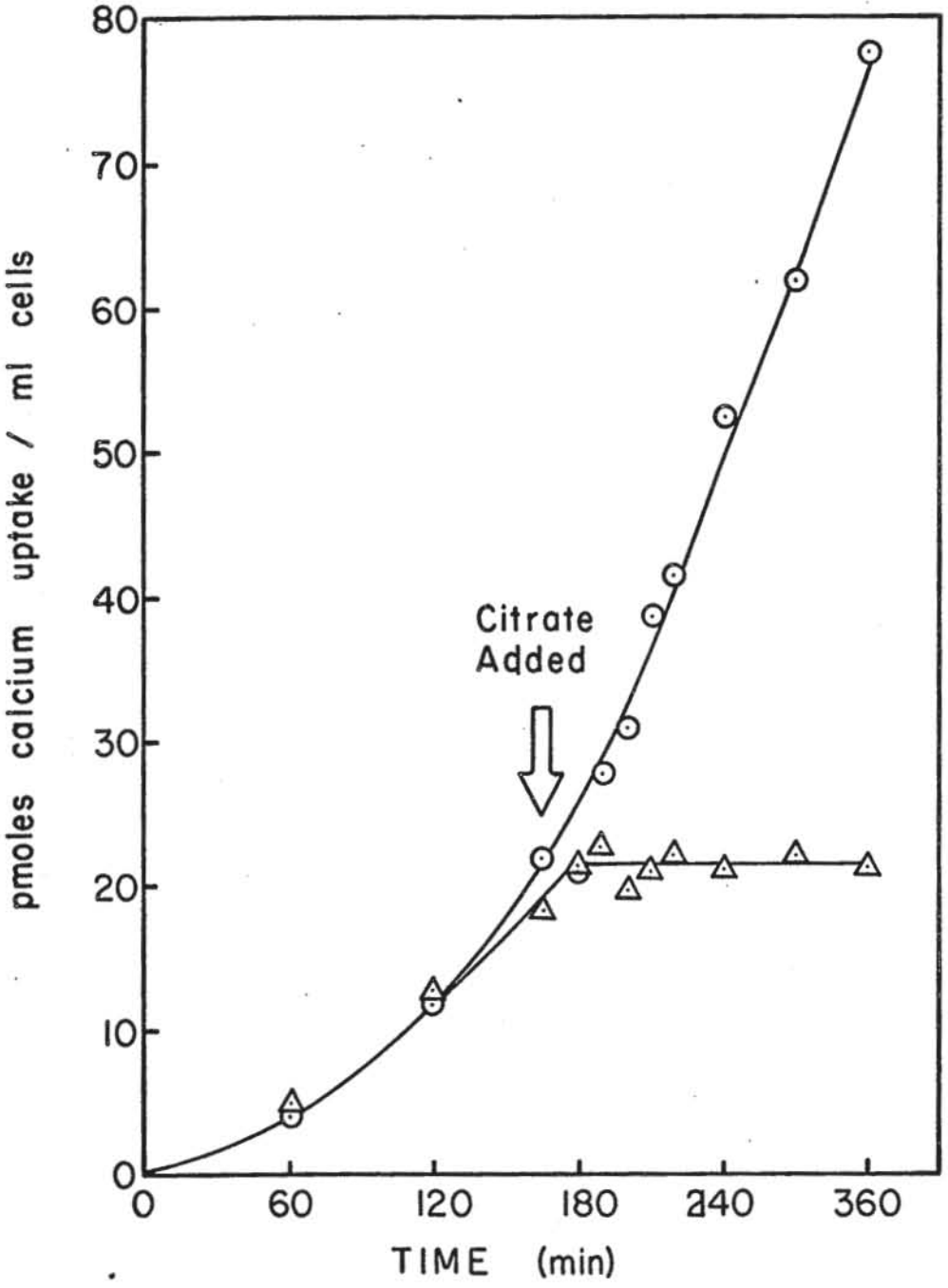

80
70
60
50
40
30
20
10
0
pmoles calcium uptake / ml cells
Citrate
Added
0
60
120
180
240
360
TIME (min)

Figure 56: Competitive inhibition by citrate of calcium uptake by Achlya germinated sporangiospores at the following concentrations: (○), 0 mM; (○), 0.1 mM; (□), 1.0 mM; and (△), 10 mM.

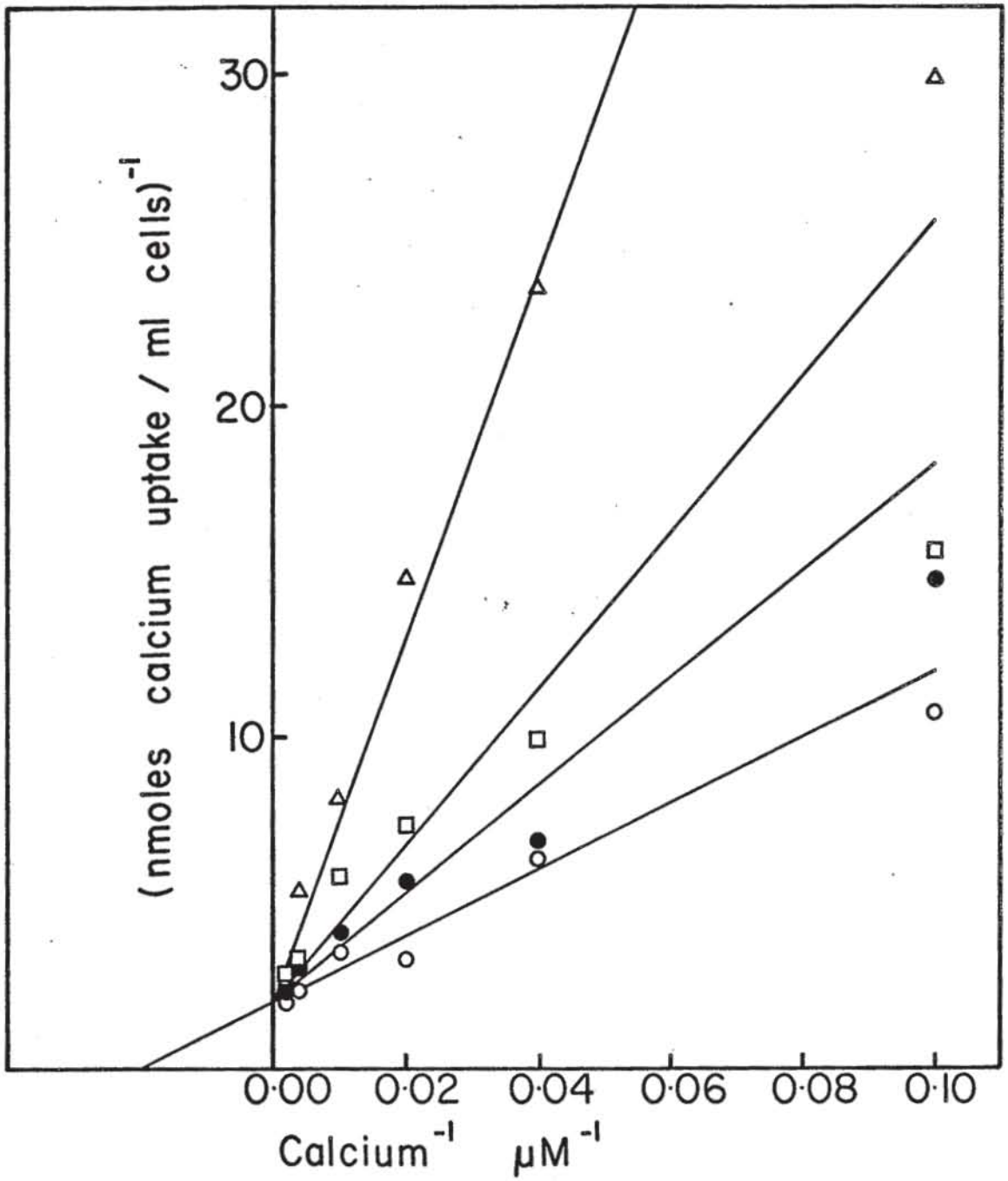
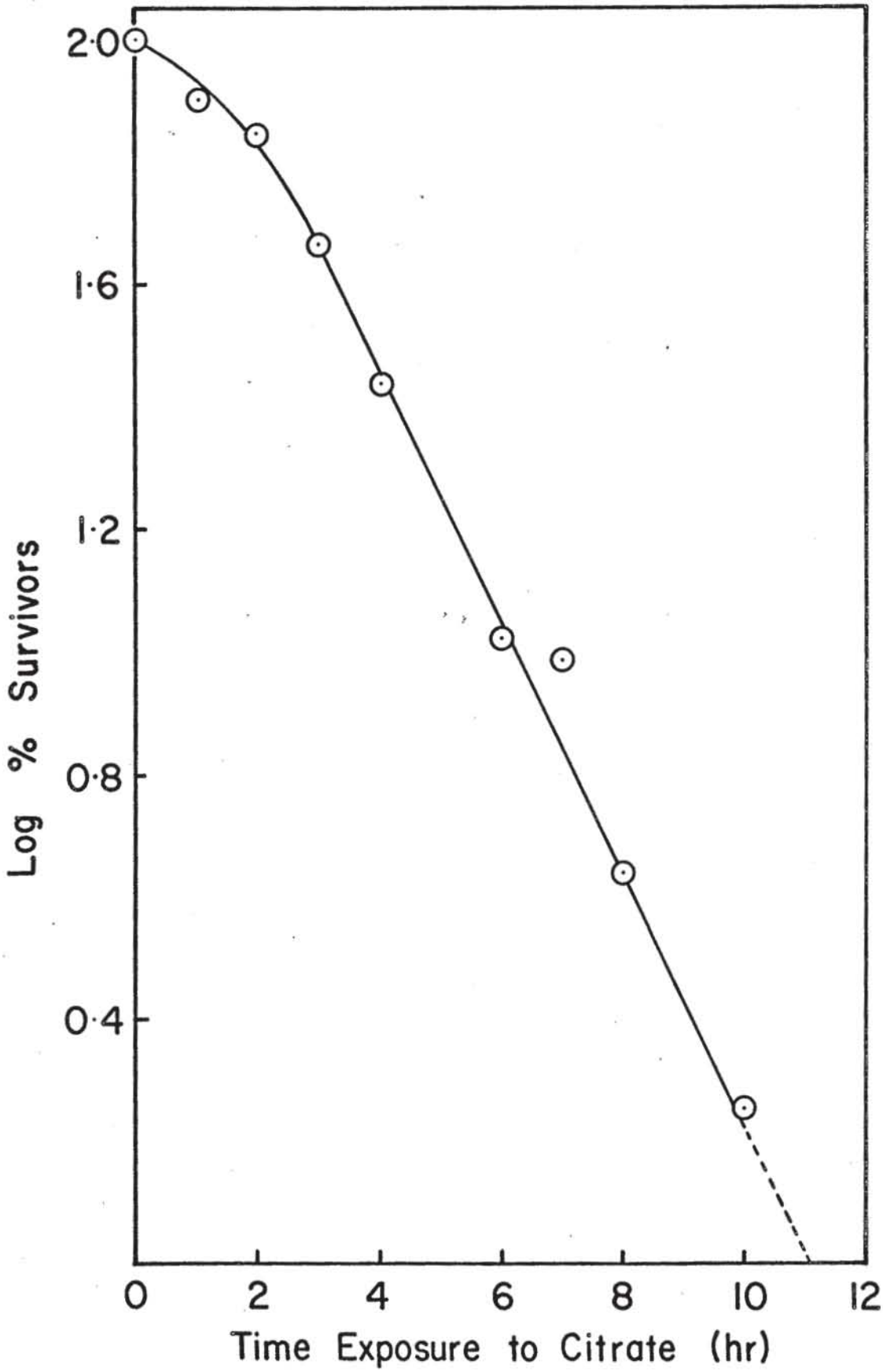

30
20
10
(nmoles calcium uptake / ml cells)$^{-1}$
0·00
0·02
0·04
0·06
0·08
0·10
Calcium^{-1} μM^{-1}

Figure 57: Kinetics of the lethal effect of citrate on ungerminated spores of Achlya.


2·0
1·6
1·2
0·8
0·4
Log % Survivors
0
2
4
6
8
10
12
Time Exposure to Citrate (hr)

and by 24 hours only 1% remained viable. In a further experiment, calcium efflux induced by citrate, indicated that the citrate lethal effect on ungerminated spores was due, in part, to removal of calcium. The kinetics of calcium efflux as a function of time in GY medium are shown in Fig. 58. Citrate induced an efflux of 40% of the bound calcium within 6 hours. A point to note, is that this efflux experiment occurs with time and is not experimentally analogous to the efflux assay described under Methods. An additional difference is that the growth medium used in the normal efflux assay is the defined medium of Barksdale (1963). This would account for the apparent discrepancy in percentage $^{45}Ca^{++}$ released by the mycelial material, 40% Ca^{++} efflux with time for GY medium grown cells and 80% Ca^{++} initial rate efflux for defined medium grown cells. Apparently incubation of spores for a prolonged period in the absence of cations and the resultant efflux of stored ions by citrate causes irreparable damage to the cell.

Calcium relative to other cations: A number of other divalent cations were also chelated by citrate such as, Mg^{++}, Mn^{++}, Ba^{++}, Co^{++}, Sr^{++}, and Fe^{++}. However, the ability of divalent cations other than calcium to counteract the citrate effect was neglible as shown in Figure 59. When the concentration of added cation exceeded 0.1 mM only Ca^{++}, Ba^{++} and Mg^{++} were not toxic, but Ba^{++} and Mg^{++} became toxic at 2 mM. In GY medium containing 2 mM Ba^{++}, growth was arrested but could be reversed if calcium was added even at very low concentration (0.1 mM).

Figure 58: Efflux of calcium from ungerminated spores as a function of time in the presence of 10 mM citrate (○) and in the absence (●); and from fully developed mycelial mats in the presence of 10 mM citrate (▽) and in the absence (▼). Cells grown in GY medium.

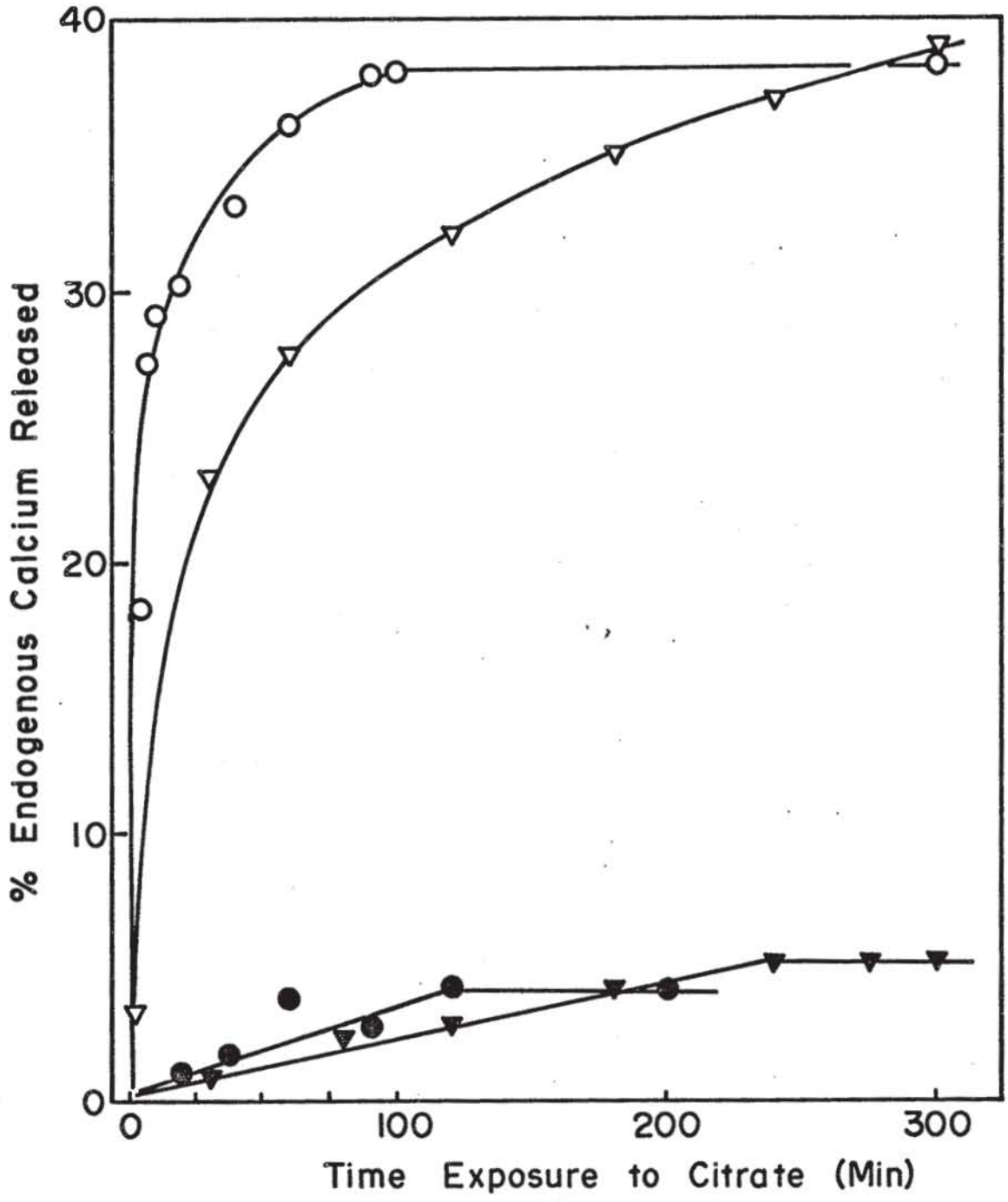
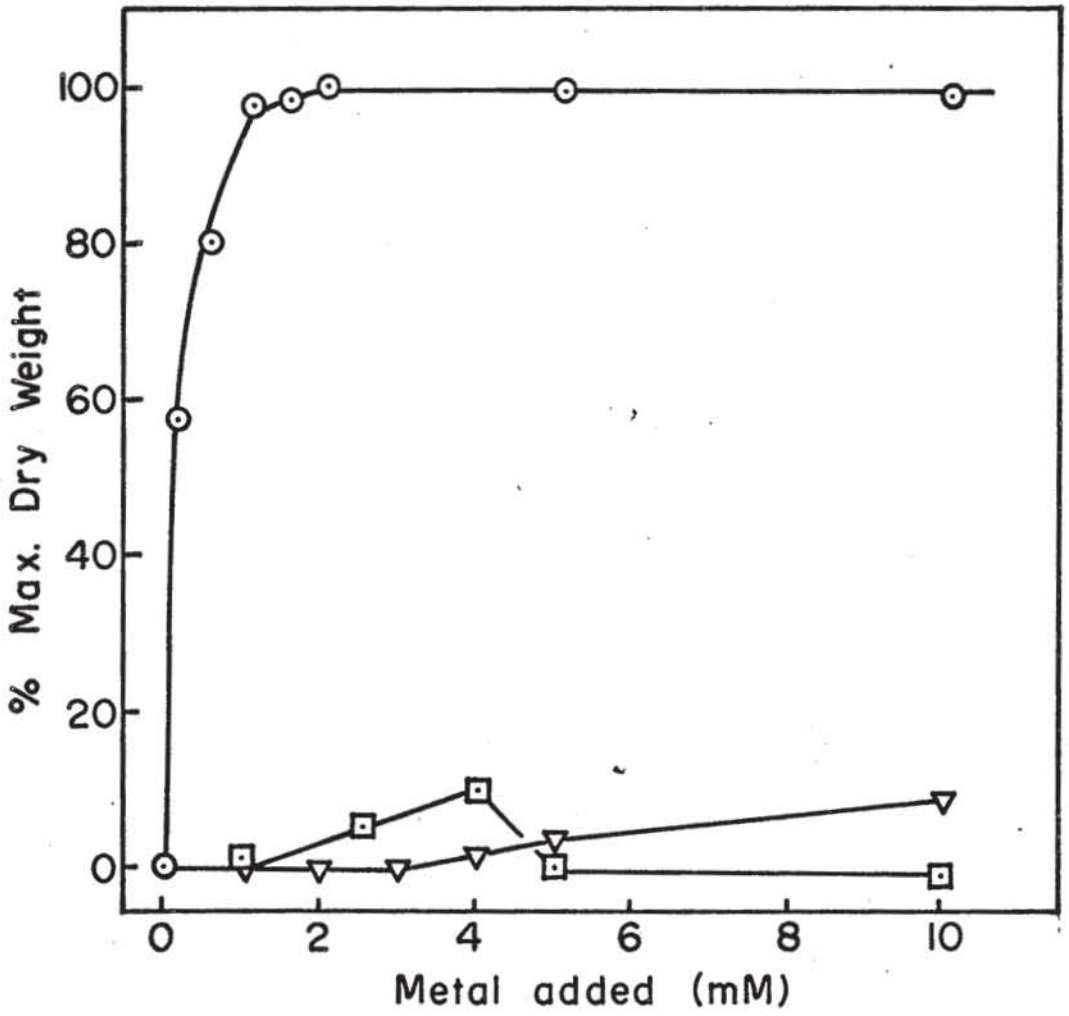

40
30
20
10
0
% Endogenous Calcium Released
0
100
200
300
Time Exposure to Citrate (Min)

Figure 59: Antagonism of the inhibitory effect of citrate (10 mM) by (⊡), Mn^{++}; (▽), Mg^{++}; and (⊙), Ca^{++} on cells growing in GY medium.


100
80
60
40
20
0
% Max. Dry Weight
0
2
4
6
8
10
Metal added (mM)

The unique property of calcium to reverse growth inhibition by citrate does not imply that other divalent cations do not play an essential role in cell growth. As previously described for the calcium transport system, divalent cations interfere with initial rate of calcium transport by direct competition. This was apparent in intact cells for divalent cations, Ba^{++}, Sr^{++}, Pb^{++} and Cd^{++}. Mn^{++} and Mg^{++} at low concentration had little effect on calcium transport, competing only at high concentrations. A study of non-inhibiting concentration of divalent cations on calcium transport during growth affirmed the importance of Mn^{++} and Mg^{++}, as shown in Figure 60. Over a period of 24 hours, both these divalent cations increased calcium uptake, although Ba^{++} inhibited. It appears that divalent cations, produce their toxic effect on growth at high concentration by direct competitive interaction with the calcium transport process.

Cellular distribution of calcium: About 40% of the calcium taken up cannot be released. Presumably this calcium is either taken up by the cell or irreversibly bound in some manner. To compartmentalize the irreversible bound calcium, subcellular fractions of cell material were obtained from $^{45}Ca^{++}$ pre-loaded cells. Individual fractionations steps and $^{45}Ca^{++}$ associated with each fraction are listed in Table XXV. Greater than 90% of the total cellular calcium was detected in the cytoplasmic fraction which had been freed of cell walls, membranes, mitochondria, and nuclei. Of interest was the 2 to 5% associated with ribosomes, but the sifnificance of ribosomal associated calcium is

Figure 60: Influence of divalent cations on calcium uptake. Effect of (○), no addition; (△), 0.1 mM Mn^{++}; (□), 1.0 mM Mg^{++}; (●), 1.0 mM Ba^{++} on calcium uptake.

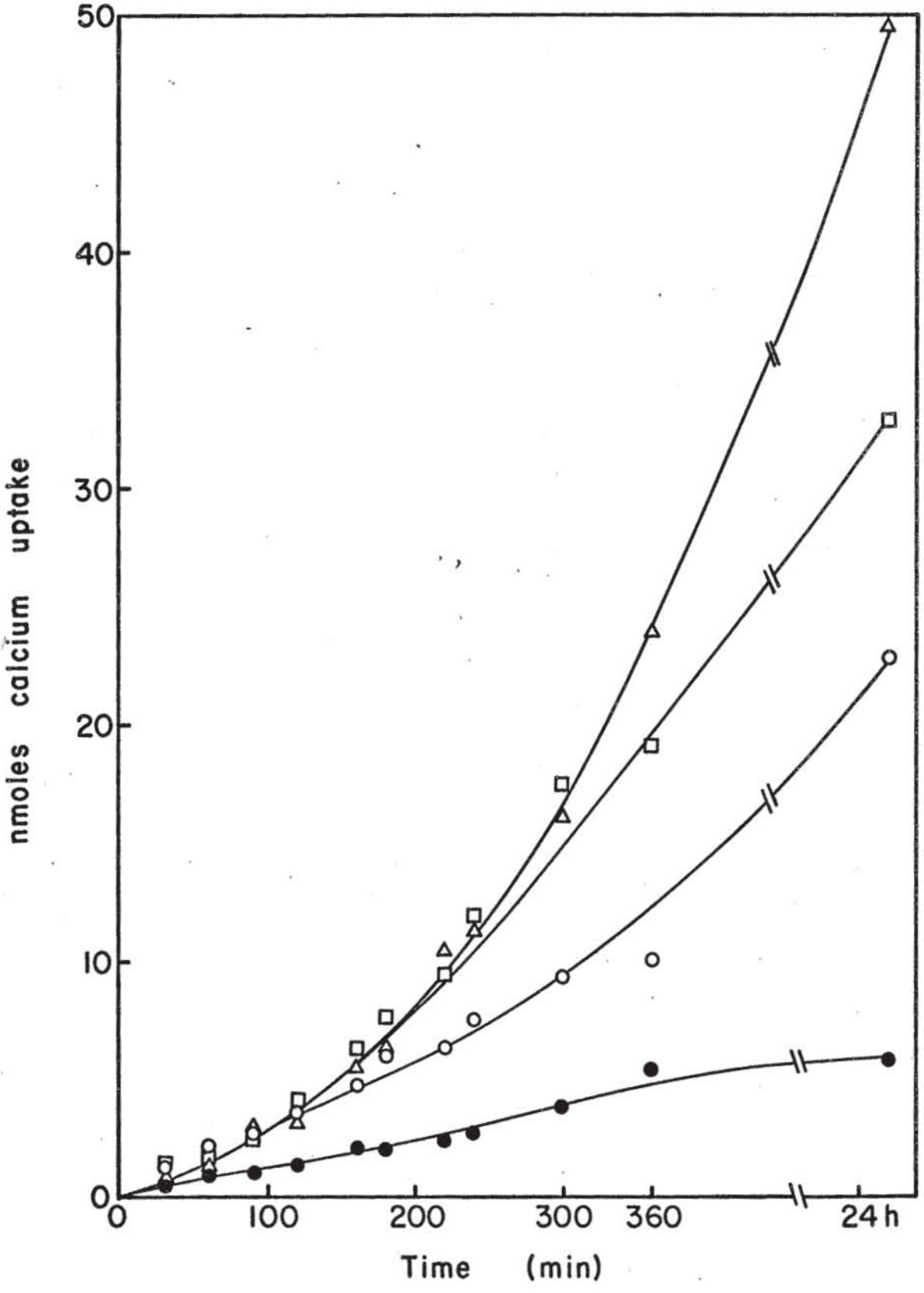

50
40
30
20
10
0
nmoles calcium uptake
0
100
200
300
360
24 h
Time (min)

TABLE XXV

Distribution of $^{45}Ca^{++}$ among cellular components of Achlya.

Ten petri plates of Achlya cultures developed to stage IX of the life cycle in GY medium containing $^{45}Ca^{++}$ (input of 0.5×10^6 dpm per petri plate) were harvested washed exhaustively with 500 ml deionized H_2O and the mycelia mixed with 50 g of unlabelled cells at the same stage of development. The hyphae were then suspended in 150 ml of a buffer composed of 0.44 M sucrose, 5 mM Mg^{++}, 1 mM KCl, and 10 mM tris-acetate, pH 7.2 and homogenized in a Waring blendor. Blending was performed 5 times by applying 2-min bursts and an interval of 5 min chill between bursts in an ice-salt bath. Unbroken cells and cell wall were collected by filtering through cotton gauze, resuspended and the blending repeated. The final debris was washed 6 times by resuspension and recentrifugations at 1,500 x g for 5 min. The cell brei obtained after the two successive homogenizations was then fractionated as described from step 2 to 4 in the Table.

Step	Fraction	% Total Counts
1.	Washed cells	100
2(a).	Cell walls and membranes (washed six times)	0.095
(b).	Wash fractions from Step 2(a)	12.4
3(a).	Mitochondria and nuclei (15,000 x g pellet) (washed three times)	< 0.1
(b).	Wash fractions from Step 3(a)	99.5
4(a).	Ribosome pellet (washed once) (105,000 x g pellet)	4.1
(b).	105,000 x g supernatant from Step 4(a)	80

unresolved. Only trace amounts of $^{45}Ca^{++}$ was found to be associated with microsomal material, however, most of the membrane attached calcium may have been lost since calcium binding would be ionic at best.

B. Macromolecular Synthesis

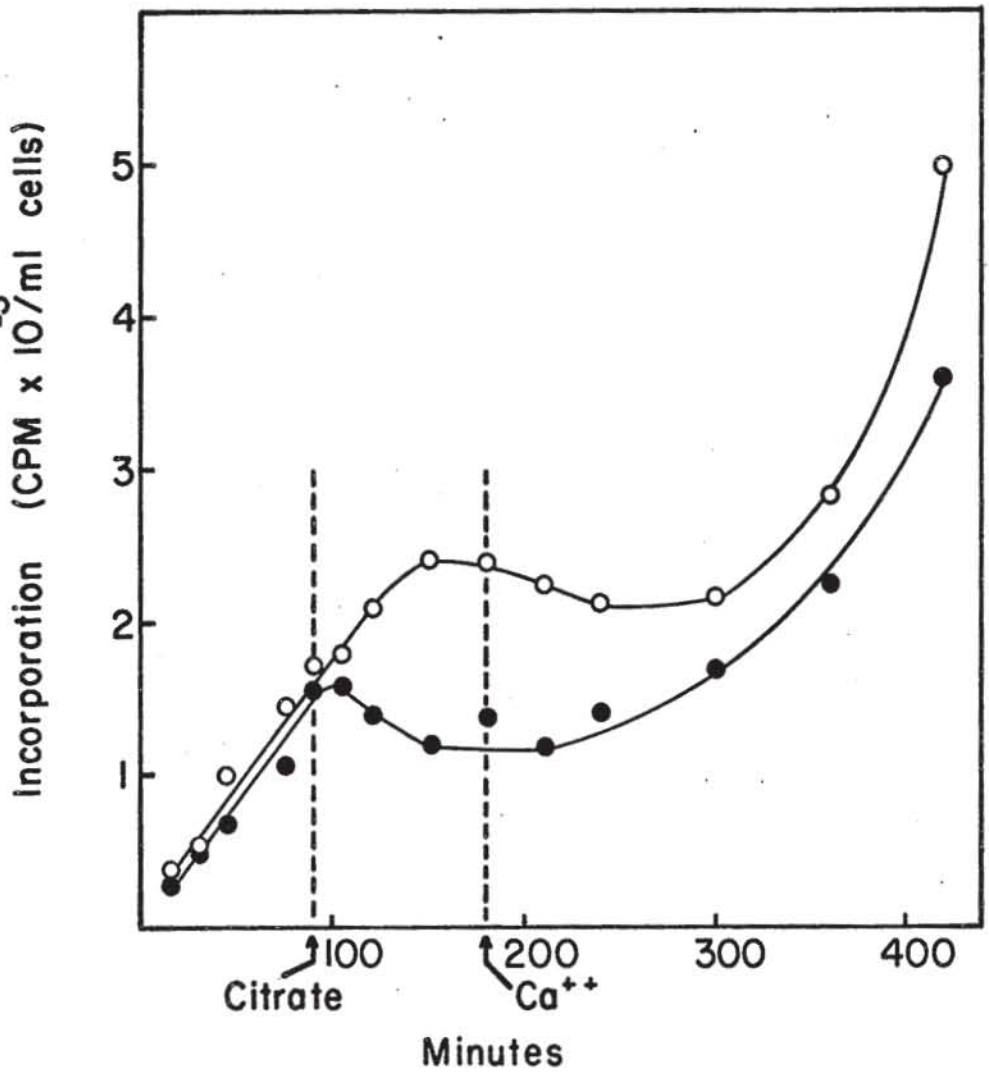
Protein, RNA and DNA synthesis: The specific requirement of calcium throughout the growth cycle was found not to be attributed solely to its incorporation into cellular structure. A concurrent study on macromolecular synthesis showed that protein, RNA, and DNA synthesis were inhibited by citrate (Fig. 61). These studies showed citrate inhibition of protein synthesis to be immediate, while RNA synthesis proceeded for 2 to 3 hours at a slightly reduced rate before plateauing. DNA synthesis also was promptly inhibited. These responses will be correlated to inhibition of amino acid transport below. Apparently, protein synthesis is the target of citrate inhibition and the inhibition of RNA and DNA synthesis is a consequence of this effect. A time dependent reversal study of citrate inhibited macromolecular synthesis by calcium supported the conclusion that protein synthesis was the prime target of citrate. Calcium reversed citrate inhibited protein and RNA synthesis as shown in Figure 62. However, resumption of RNA synthesis lagged 2 hours behind the start of protein synthesis.

When investigating the effect of calcium on protein synthesis, the problem of associated amino acid transport had to be considered.

Figure 61: Influence of citrate on protein (a), RNA (b), and DNA (c) synthesis in germinated spores of Achlya in GY medium. Incorporation in the absence (O) and the presence of 10 mM citrate added at 120 min (●) as shown by the vertical broken line.

Incorporation (CPM x 10^{-4}/ ml cells)
Protein
(a)
RNA
(b)
DNA
(c)
Minutes
0
200
400
2
4
6
1
3

Figure 62: Differential effects of citrate on RNA (O) and protein (●) synthesis and reversal by Ca^{++} in germinated sporangiospores growing in GY medium. 10 mM citrate was added at 90 min and 10 mM Ca^{++} was added at 180 min.

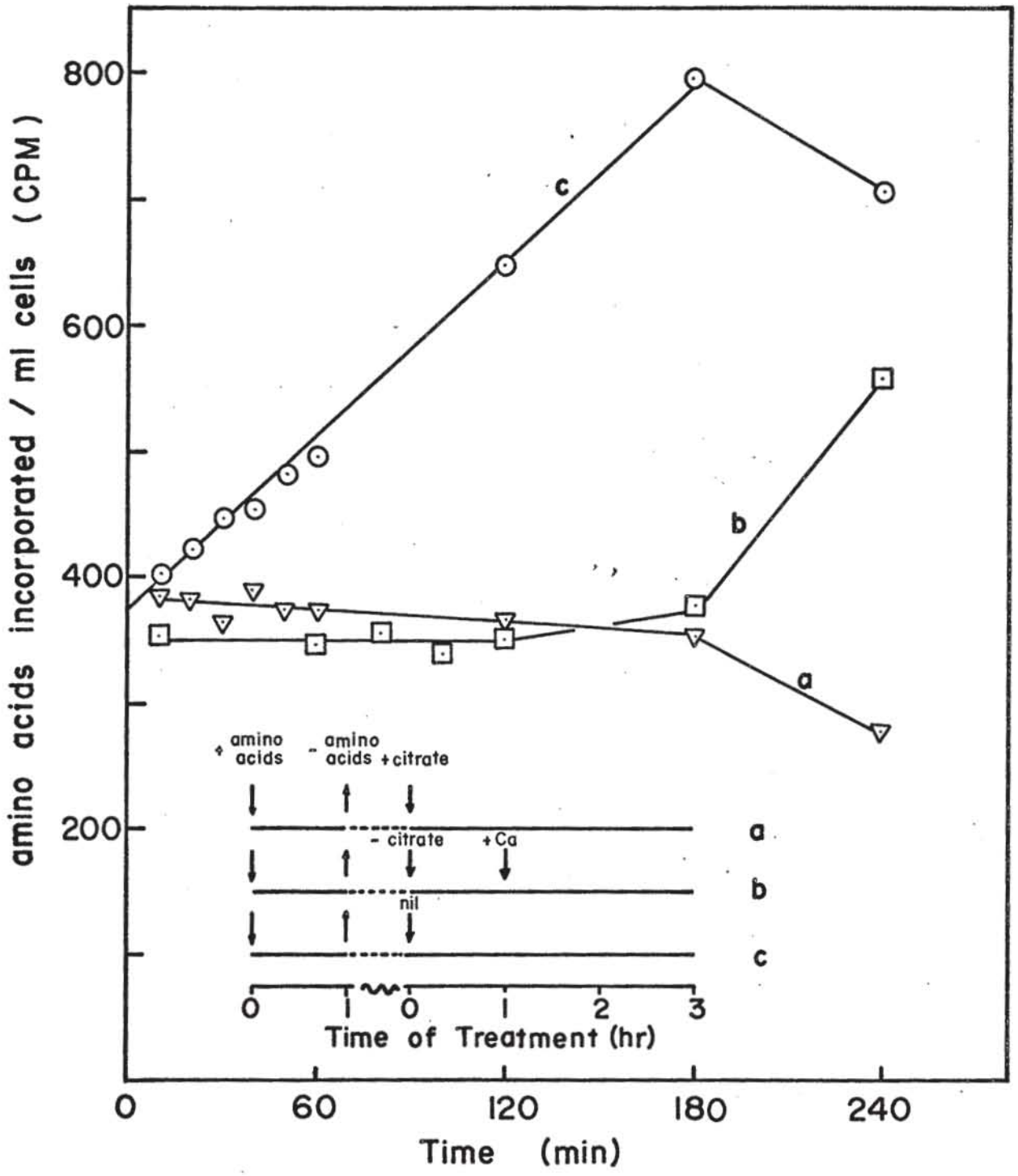

Incorporation (CPM x 10^{-3}/ml cells)
5
4
3
2
1
100
200
300
400
Citrate
Ca^{++}
Minutes

A pulse experiment for protein synthesis was devised to alleviate this difficulty. After pulse labelling the cells for 60 min with ^{14}C-amino acids, all exogenous radioactive label was removed by washing and the incorporation of endogenous amino acid into protein was followed. The results (Fig. 63) indicate that citrate still exerted some inhibitory effect on protein synthesis and this was antagonized by Ca^{++}. As a precaution, the loss of amino acid during the pulse experiment was monitored, but no loss was apparent. Although calcium reversed protein synthesis there was a considerable lag period. Here again, the effect of calcium on growth could not be attributed to protein synthesis effect alone. As protein synthesis and transport of amino acids are closely coupled in a number of organisms the inhibitory effect on protein synthesis may be in part due to inhibition of amino acid transport, as demonstrated in a later section.

Induction of glutamate dehydrogenase: A second method to alleviate the problem of associated amino acid transport was to study the effect of citrate and calcium on specific enzyme activity. A study of calcium and citrate on individual enzymes was also undertaken to characterize endogenous calcium effect as to whether it is a general activator of overall protein synthesis or an activator of specific enzyme synthesis.

In germinated sporangiospores of Achlya, glutamate dehydrogenase activity was increased five-fold when cells were grown in the presence of 0.5% glutamate. In contrast, substituting glucose for glutamate repressed glutamate dehydrogenase synthesis permitting only a 0.5

Figure 63: Influence of citrate on the ultilization of endogenous amino acid for protein synthesis by germinated sporangiospores incubated in Buffer A. Effect of no citrate (⊙), 10 mM citrate (▽), and 10 mM citrate followed by 10 mM Ca^{++} after 60 min (⊡).


amino acids incorporated / ml cells (CPM)
800
600
400
200
0
60
120
180
240
Time (min)
c
b
a
+ amino acids
- amino acids
+ citrate
- citrate
+ Ca
nil
a
b
c
0
1
0
1
2
3
Time of Treatment (hr)

fold increase in specific activity (Fig. 64). As previously shown by LéJohn and associates (LéJohn & Stevenson, 1970; LéJohn et al, 1970; LéJohn, 1971), glutamate dehydrogenase is an enzyme that is multivalently controlled being activated by high energy compounds (PEP, GTP, ATP, and acetyl CoA) and inhibited by AMP, citrate, Ca^{++}, and Mn^{++}. In addition to allosteric regulation of enzyme activity by these modulators, glutamate dehydrogenase synthesis is sensitive to glucose repression.

Citrate and calcium relation was found to be similar under glutamate dehydrogenase inductive and non-inductive conditions. Citrate essentially blocked the induction of glutamate dehydrogenase (Fig. 65, inset). If calcium was added to glutamate-induced citrate-inhibited cells, glutamate dehydrogenase was induced (Fig. 65).

RNA patterns: An examination of the types of RNA synthesized and the effect of citrate on the synthesis of any one species was next undertaken. The measurement of RNA as described under Methods was of total RNA. In bacteria, under down-shift or amino acid starvation conditions ribosomal RNA synthesis was shown to be preferentially inhibited (Neidhardt, 1964). An attempt to demonstrate an analogous effect in fungi was unsuccessful, as shown in Figure 66. The RNA profiles of citrate treated and untreated cells were similar, the only difference being the quantity of RNA synthesized. There was a 90% reduction in the amount of RNA made in citrate-inhibited cells (Fig. 66, inset).

Figure 64: Induction of glutamate dehydrogenase (a), and concomitant amino acid incorporation into protein (b). Enzyme synthesis with glucose as major carbon source (●) and with glutamate as major carbon source (O). (△) represents the specific enzyme activity of isocitrate dehydrogenase in glucose-grown cells.

Minutes
0
100
200
300
(a)
(b)
% Increase Specific Activity Enzyme
0
100
200
300
400
500
600
CPM / µg protein
100
200
300

Figure 65: Effect of citrate on glutamate dehydrogenase activity and amino acid incorporation into protein. Effect of calcium (10 mM) added 60 min after 10 mM citrate. Symbols are for glutamate dehydrogenase specific activity (⊙) and amino acid incorporation (⊡). For the inset plot 10 mM citrate was present throughout the duration of the experiment.

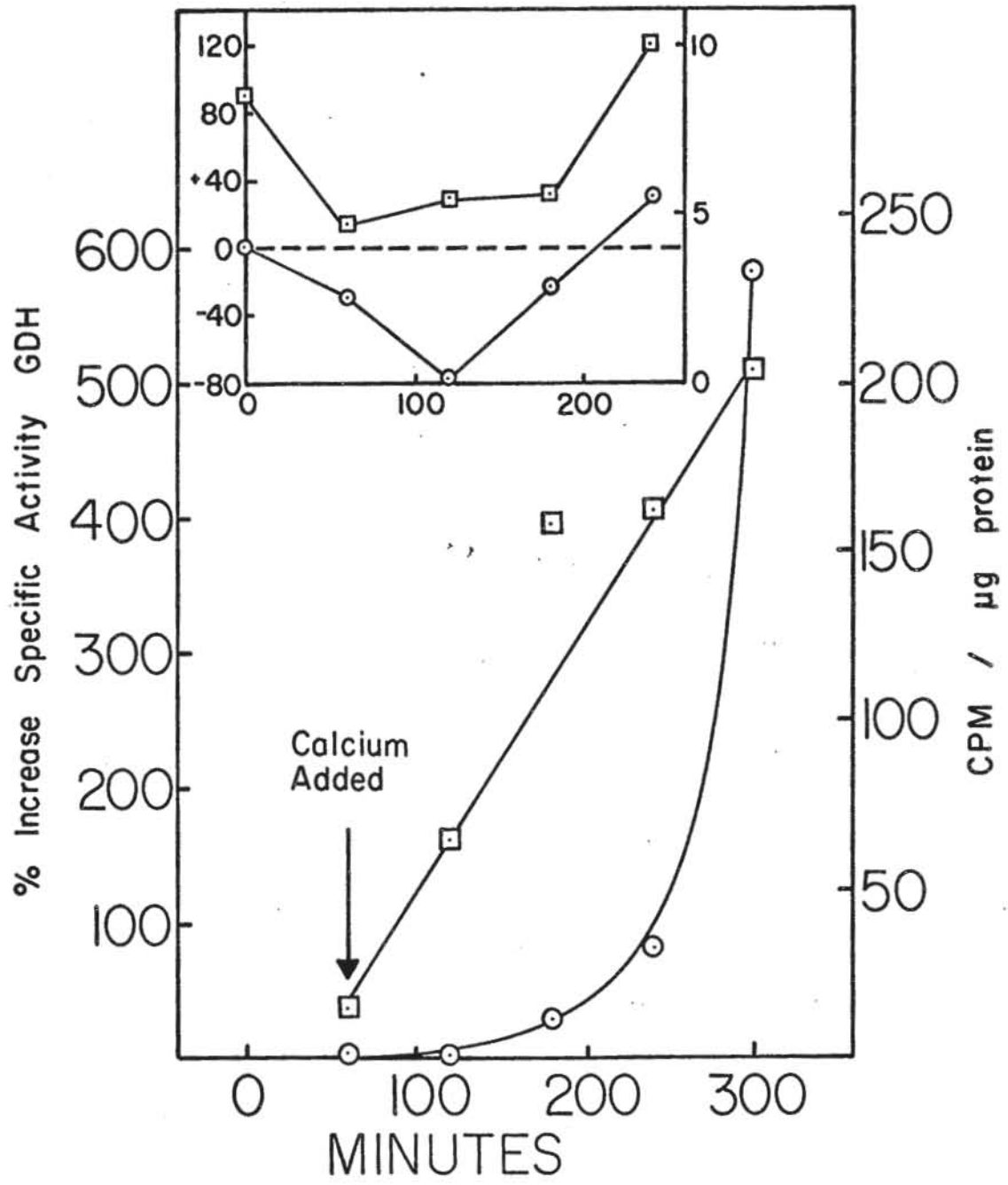
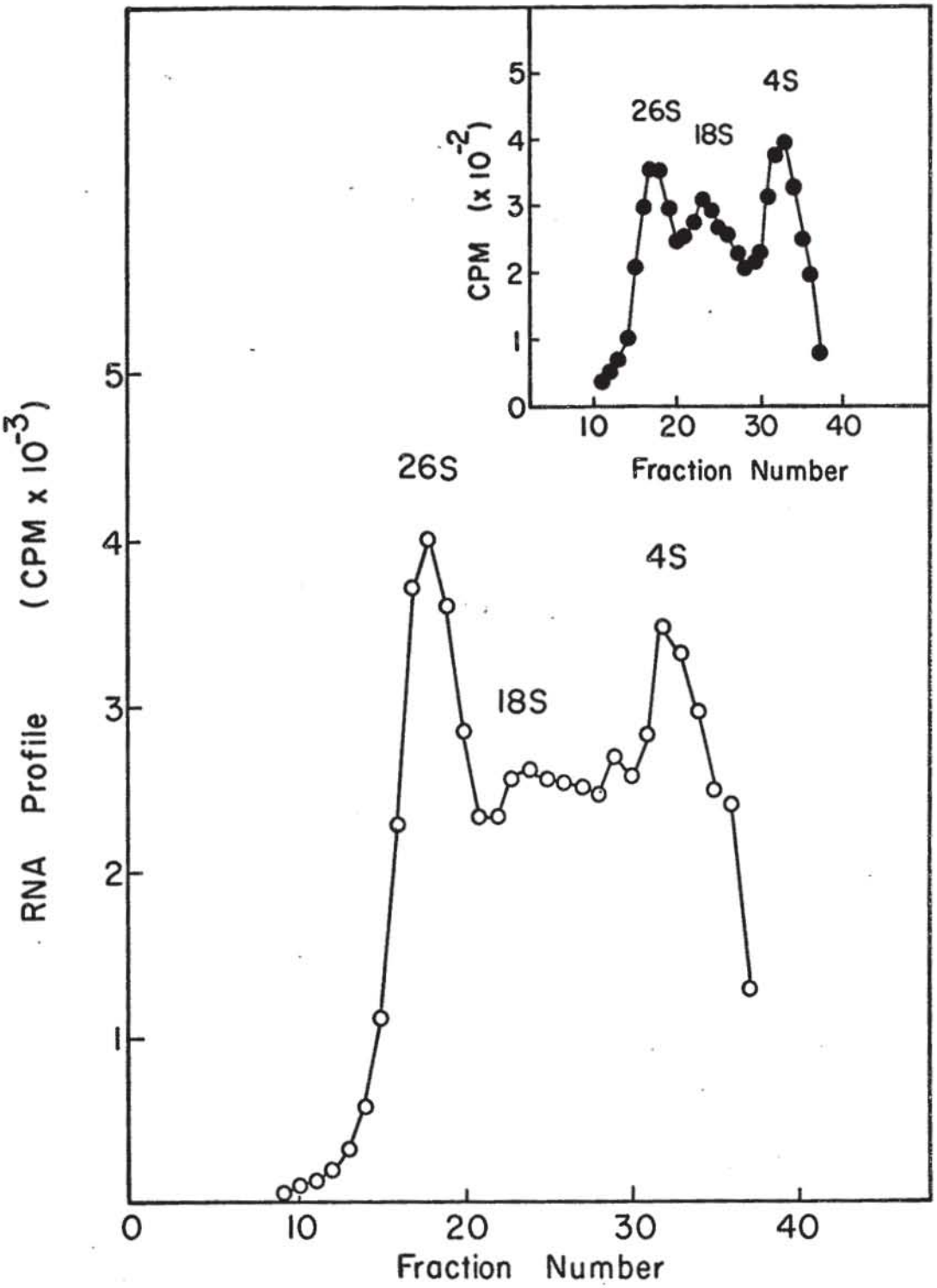

% Increase Specific Activity GDH
CPM / µg protein
MINUTES
Calcium Added
0
100
200
300
600
500
400
300
200
100
250
200
150
100
50
120
80
40
0
-40
-80
10
5
0
0
100
200

Figure 66: Sdeimentation analysis in linear sucrose density gradients of labelled RNA extracted from germinated sporangiospores after 4 hour incubation in GY medium containing ^{14}C-uracil in the presence (●) and absence (O) of 10 mM citrate.


RNA Profile (CPM x 10^{-3})
26S
18S
4S
Fraction Number
CPM (x 10^{-2})
26S
18S
4S
Fraction Number

Coupled RNA and protein synthesis: Citrate reduction of RNA synthesis was of interest to investigate. Cycloheximide proved a useful experimental tool for examination of this problem. Normally, cycloheximide is a specific inhibitor of protein synthesis in eucaryotic cells, but in Achlya, cycloheximide inhibited protein synthesis and RNA synthesis by 90% in both cases (Fig. 67). This result suggested that RNA and protein synthesis in Achlya are tightly coupled.

Cycloheximide effect on RNA synthesis: The nature of cycloheximide interference with RNA synthesis was studied to determine if cycloheximide was specifically inhibiting a particular species of RNA. Cycloheximide appeared to preferentially decrease the amount of ribosomal RNA synthesized, although tRNA was also affected (Fig. 68). As shown in Figure 68a, if cycloheximide was added at the start of measurement of RNA synthesis, no stable RNA species were synthesized, except a small amount of rapid sedimentating material that was estimated to have a sedimentation value of 4 S similar to tRNA. Ribosomal RNA, represented by 26 S and 18 S on the plot, was completely inhibited. When ^{14}C-uracil was removed after 100 min, and cells incubated for a further 100 min, an analogous RNA profile was obtained by cells that had not been incubated a further 100 min which indicates there was no degradation or endogenous supply of labelled uracil to permit further incorporation (Fig. 68b). As cycloheximide had a partial inhibitory effect when added 100 min after the addition of ^{14}C-uracil which was removed at 100 min, cycloheximide may enhance

Figure 67: Inhibition of RNA (▽) and protein (⊙) synthesis by cycloheximide. Plotted as a percentage of incorporation in the absence of cycloheximide.

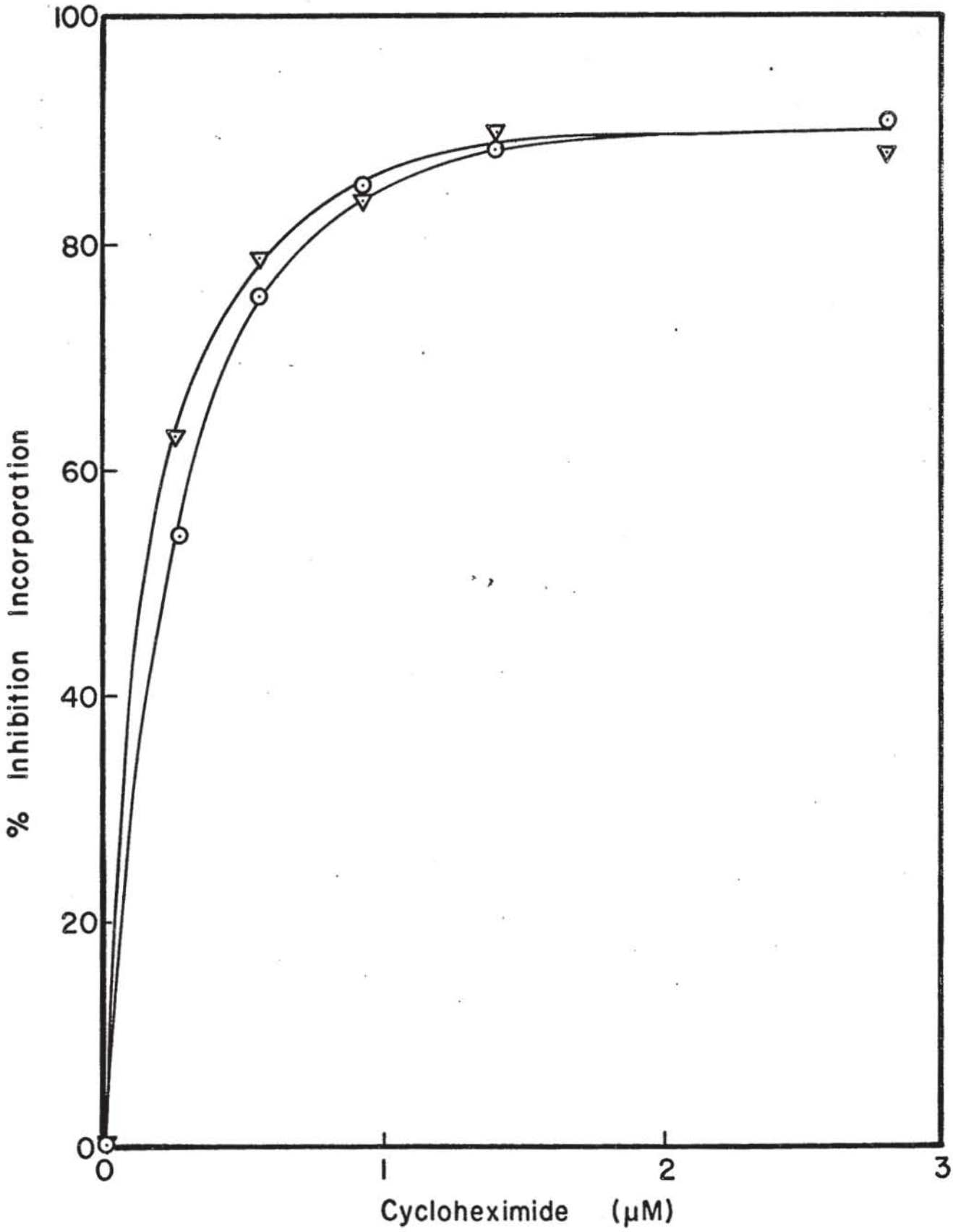
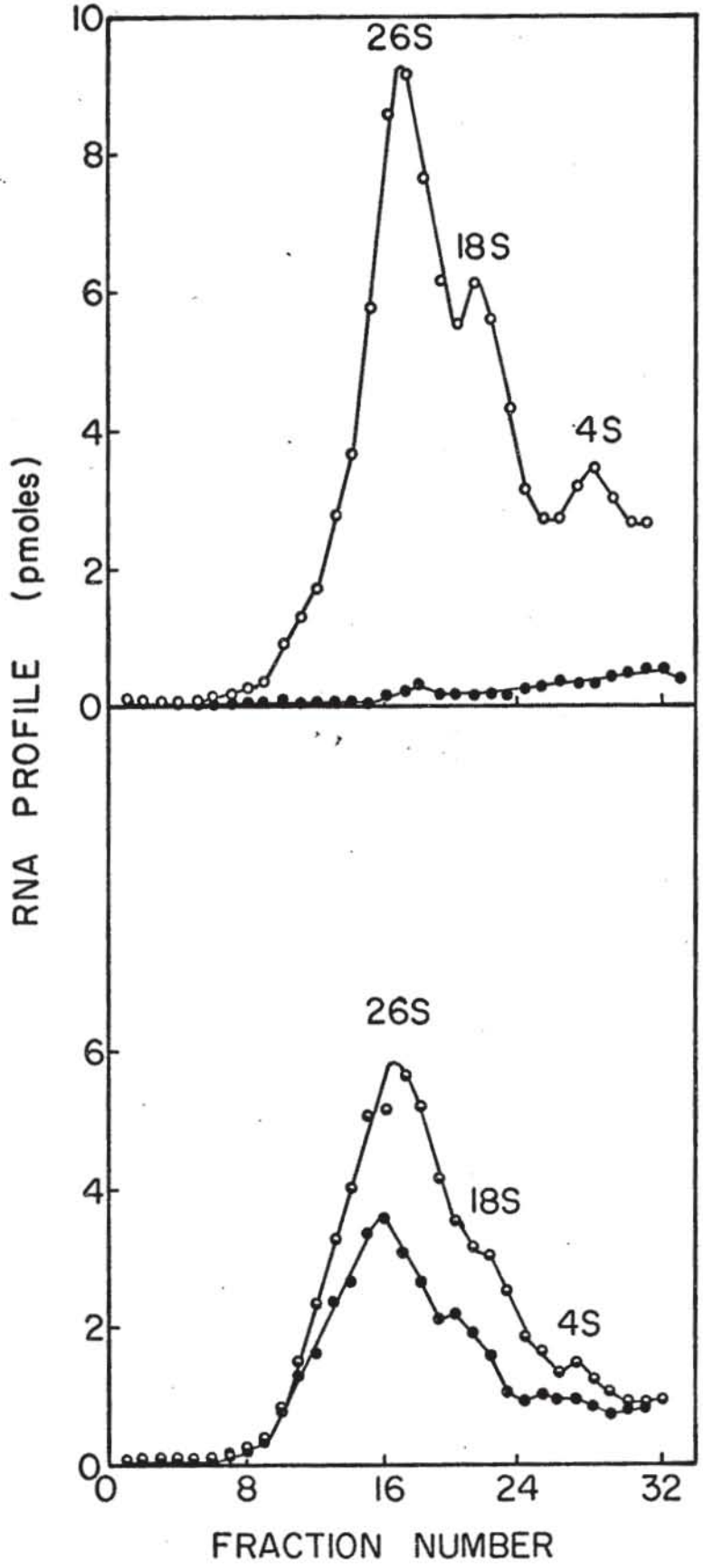

100
80
60
40
20
0
% Inhibition Incorporation
0
1
2
3
Cycloheximide (μM)

Figure 68: Sedimentation analysis of ^{14}C-labelled stable RNA extracted from germinated sporangiospores by linear sucrose density gradients. ^{14}C-labelled RNA profile (a) after 200 min in the presence (●) and absence (○) of 2.8 μM cycloheximide and (b) after 100 min exogeneous ^{14}C-uracil was removed and cells treated as follows: (◒) cells collected or incubated a further 100 min before collecting and (●) citrate added at 100 min and cells collected at 200 min.


26S
18S
4S
RNA PROFILE (pmoles)
26S
18S
4S
FRACTION NUMBER

degradation of RNA, particularly ribosomal RNA. What effect cycloheximide has on messenger RNA was not determined, making it difficult to assess the overall picture of the nature of cycloheximide effect on RNA synthesis.

To further characterize the relation between protein synthesis and RNA synthesis, RNA metabolism was studied when cells were grown under inductive conditions, that is, glucose was replaced by glutamate in the medium. Of interest was the finding that both protein and RNA synthesis was blocked shortly after the addition of citrate as shown in Figure 69. Although subsequent addition of calcium produced an immediate enhancement of protein synthesis while resumption of RNA synthesis lagged about 2 to 3 hours which is analogous to the results obtained for the same study in glucose-treated cells, see Figure 62. This indication that there was even tighter coupling of RNA-protein synthesis in glutamate induced cells, was affirmed by isolation of RNA species made under induced and non-induced conditions (Fig. 70). In glucose-treated cells all RNA species, shown on the plot as 26 S and 18 S (stable RNA) and 12 S to 4 S (presumptive unstable messenger RNA and stable tRNA) were being made in abundance. In contrast, in glutamate-treated cells fractional amounts of stable RNA species were being made, while a wealth of 12 S to 4 S RNA, some presumably continuing in RNA, was being synthesized. In a further study, citrate and cycloheximide inhibition effects were found to be distinct under non-induced conditions. Subsequent addition of cycloheximide after citrate, terminated the lag period of citrate inhibition, in agreement

Figure 69: Differential effects of citrate on protein (●) and RNA (○) synthesis and reversal by Ca^{++} in sporangiospores growing in glutamate medium. Citrate (10 mM) was added at 90 min and 10 mM Ca^{++} was added at 180 min.

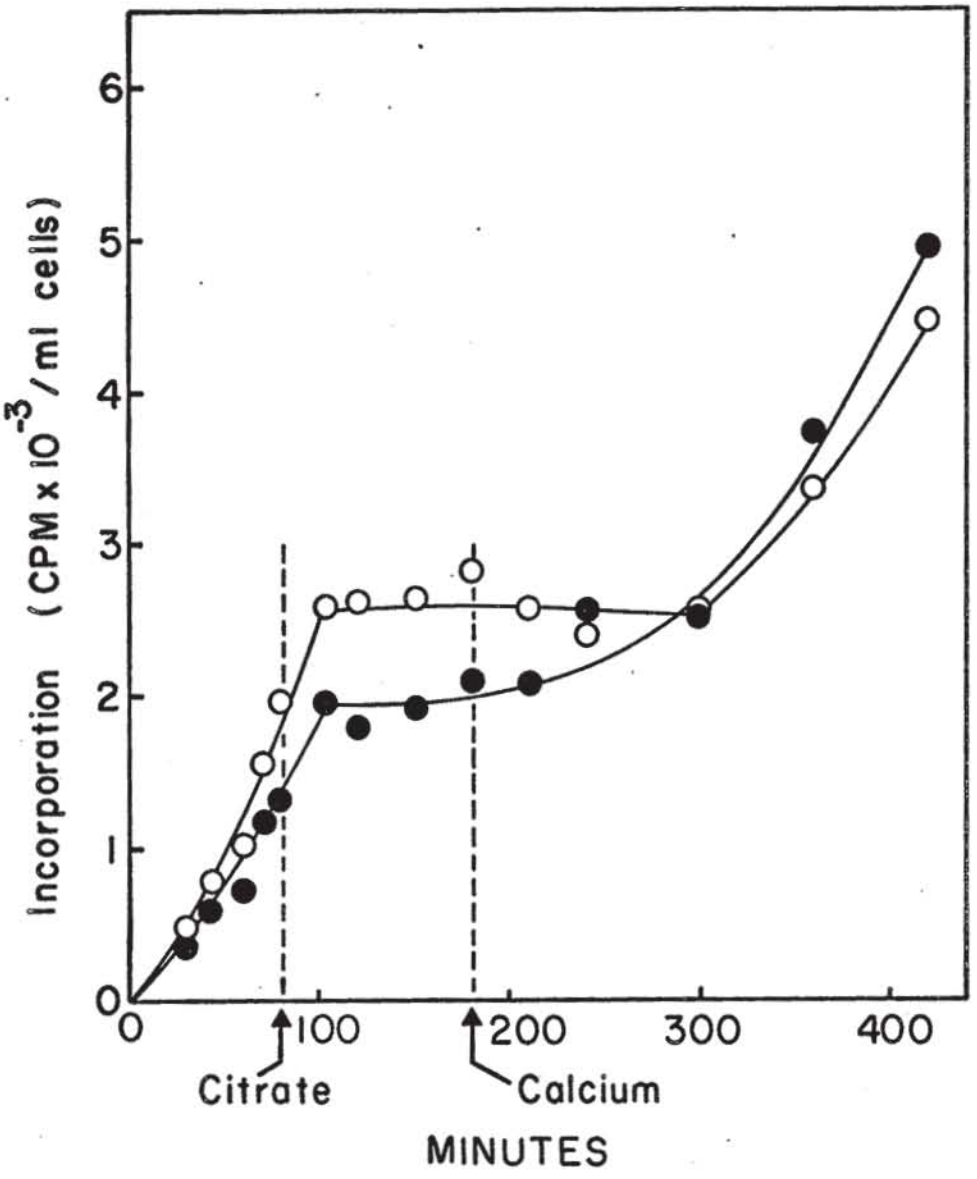
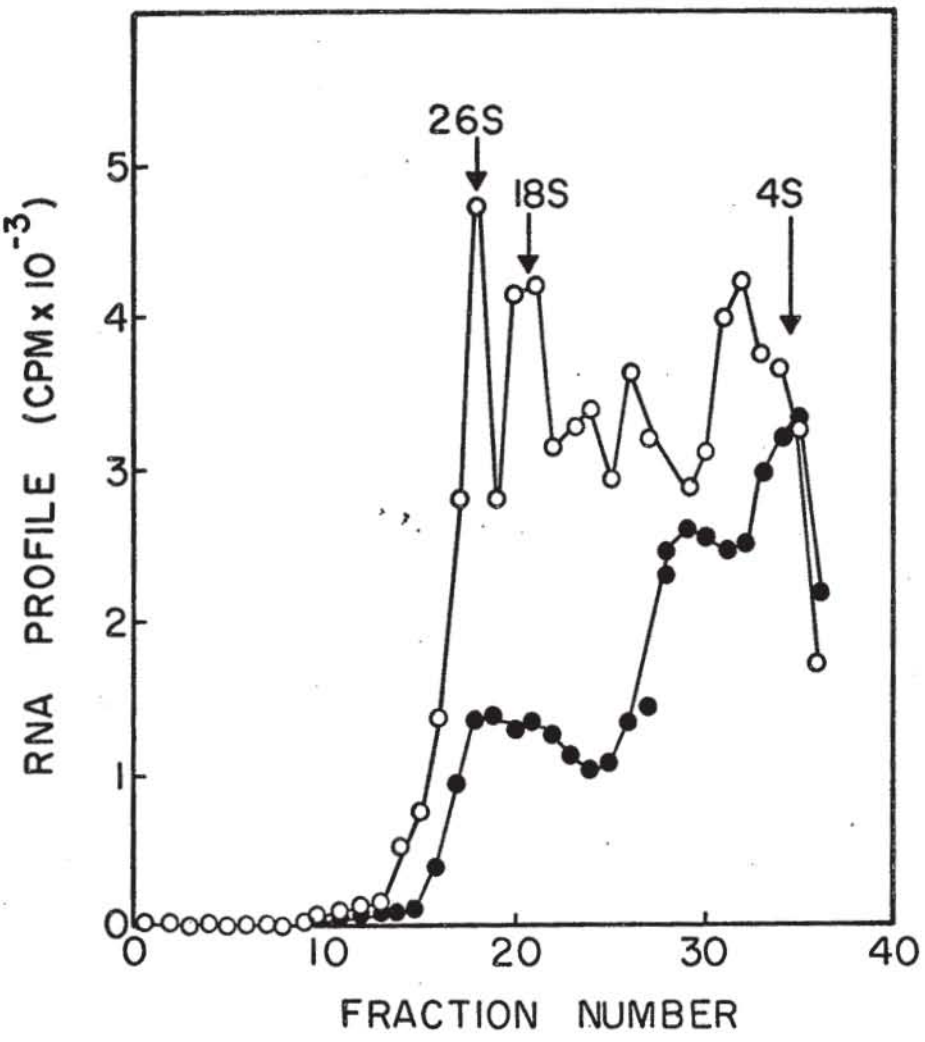
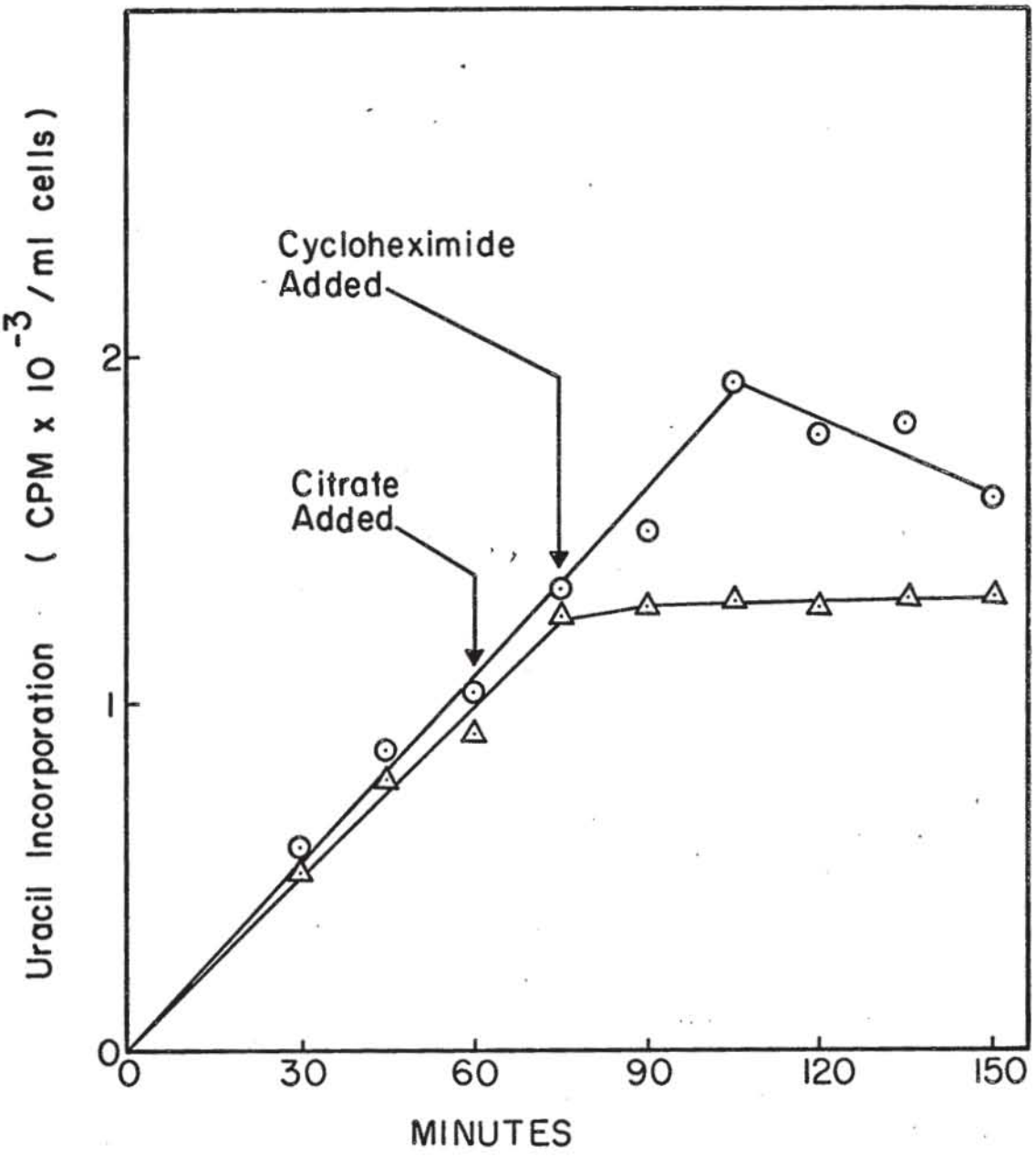

Incorporation (CPM x 10^{-3}/ml cells)
0
1
2
3
4
5
6
0
100
200
300
400
Citrate
Calcium
MINUTES

Figure 70: Sedimentation analysis in linear sucrose density gradients of labelled RNA extracted from germinated sporangiospores after 6 hours incubation in glucose (O) and glutamate (●) medium containing yeast extract.


26S
18S
4S
RNA PROFILE (CPM x 10^{-3})
0
1
2
3
4
5
0
10
20
30
40
FRACTION NUMBER

with earlier results which indicated that cycloheximide preferentially inhibited ribosomal RNA (Fig. 71). A feasible conclusion is that the deprivation of cellular calcium immediately inhibits protein synthesis and messenger RNA synthesis due to tight metabolic coupling causing a probable feedback inhibition of stable RNA species synthesis in some yet to be determined regulatory manner. In depth studies in this area of research are vigorously being pursued.

Figure 71: Combined inhibition effects of citrate and cycloheximide on RNA synthesis in germinated sporangiospores growing in GY medium: effect of citrate (⊙) and citrate plus cycloheximide (△). 10 mM citrate was added at 60 min and 2.8 μM cycloheximide was added at 75 min.


Uracil Incorporation (CPM x 10^{-3} / ml cells)
Cycloheximide Added
Citrate Added
0
1
2
0
30
60
90
120
150
MINUTES

DISCUSSION

Calcium Requirement for Growth

Calcium was found to be required for growth throughout the ontogenic cycle (Fig. 52a). Other divalent cations (Mg^{++}, Mn^{++}, and Ba^{++}) could not substitute for the calcium requirement, as only Ca^{++} could reverse the inhibition effect of citrate. While other divalent cations at high concentrations relative to calcium antagonized growth, calcium had little detrimental effect on growth even at a concentration of 50 mM. Calcium growth tests showed concentration dependent increase in dry weight up to 1 mM Ca^{++}. A coincident logarithmic increase in uptake of calcium and weight of cells, RNA, and protein synthesis indicated the importance of calcium for growth processes only. This was indirectly affirmed by the inability of ungerminated spores to take up calcium. Furthermore, the assimilation of calcium only during growth indicates that calcium is essential for growth. A study of cellular distribution of calcium added little information about the location of intracellular calcium since it was found in the cytoplasmic milieu. However, the harshness of the experimental procedure may have caused the release of membrane associated calcium.

Citrate Inhibition

The inhibition of growth by citrate and the specificity of reversing this reaction by calcium suggests more than a ion chelation effect on growth. Achlya cells are unable to overcome citrate inhibition by assimilation of this tricarboxylic acid. In subsequent studies, citrate proved to be of unique experimental value due to immediate inhibition of growth while only acting at the cell surface, and the specificity of complete reversibility by calcium. Moreover, citrate causes a considerable efflux of reversibly bound calcium. When spores were incubated in the presence of citrate for prolonged periods of time prior to removal, citrate caused the release of calcium resulting in lasting detrimental effects that bring about the death of the spores. These results indicate that cations, particularly calcium, are important for the maintenance of spore integrity even though cations are not accumulated by the spores.

Calcium Relative to Other Divalent Cations

Although calcium was found to be the only divalent cation that could reverse the inhibition effect of citrate, other divalent cations at appropriate concentrations are also important for optimal growth of Achlya. One major difference that can be discerned between calcium and other divalent cations such as Ba^{++}, Mg^{++}, and Mn^{++}, is their ability to inhibit growth at concentrations relative to calcium that

actually stimulate growth. Studies of the effect of Mg^{++} and Mn^{++}, at optimal growth concentration, on calcium transport show activation of calcium uptake. Of interest was the results previously shown that Mg^{++} and possibly Mn^{++} may be regulators of the calcium transport system, and when present at high concentration, competitively inhibit calcium transport. The probable conclusion is that Mg^{++} regulation of calcium transport is reflected in growth.

Macromolecule Synthesis

Protein, RNA, and DNA synthesis are all inhibited by citrate, but in each case, citrate effect was found to be characteristic (Fig. 61). RNA synthesis was not stopped until 2 to 3 hours after the addition of citrate, and DNA synthesis inhibition-lag varied depending on the stage of DNA replication. It appears that once a replication stage had begun, citrate had no immediate effect, however, a new replication cycle could not proceed (Fig. 61c). This is reminiscent of amino acid deprivation for bacteria, once a DNA replication cycle has begun removal of amino acid has no effect, but a new replication cycle cannot be initiated. If a correlation can be drawn, this result gave the first hint of the importance of calcium for amino acid transport. At the macromolecular level, it was protein synthesis that was specifically affected by citrate, producing an immediate blockage of protein synthesis. Citrate inhibition of protein and RNA synthesis, like growth could only be reversed by the addition of calcium, other divalent cations being ineffective.

Calcium antagonism of citrate inhibition was s hown to be almost immediate for protein synthesis but for RNA synthesis there was a lag period of 2 to 3 hours after the addition of calcium. The question of direct calcium involvement in protein synthesis was studied by following the incorporation of endogenous amino acids into protein (Fig. 63). Calcium did stimulate protein synthesis after citrate treatment even though the enhancement was smaller than expected. All attempts to investigate calcium effect on in vitro protein synthesis for Achlya were unsuccessful. Unlike bacterial systems, as in vitro protein synthesizing system was extremely difficult to set up, possibly due to RNases and other degradative enzymes in the S100 fraction and special problems that may be encountered when dealing with an eucaryotic system. Controls varied widely making interpretation of the results for in vitro protein synthesis in the presence and the absence of calcium impossible.

The effect of calcium and citrate on protein synthesis was studied at the enzyme level to eliminate the problem of coupled amino acid transport. These studies confirmed calcium direct involvement in protein synthesis. The results obtained for calcium activation of specific enzyme synthesis or of general protein synthesis were inconclusive.

Initially it was thought that citrate may differentially affect the different RNA species, preferentially inhibiting ribosomal RNA, but this was shown to be unfounded as all species, rRNA, tRNA, and messenger RNA were inhibited to the same extend. Why inhibition of RNA synthesis occurred when citrate action appeared to be at the level of transport was an interesting problem. This was resolved by the use

of cycloheximide which showed that transcription and translation are closely coupled processes in Achlya. Cycloheximide is usually considered as an inhibitor of protein synthesis in eucaryotic cells. In Neurospora, cycloheximide at a concentration less than 4 μM reduced protein synthesis by 90% but RNA synthesis was unaffected (Horowitz et al, 1970). In Achlya, cycloheximide was an effective antagonist (90% inhibition) of both RNA synthesis and protein synthesis at the low concentration of 1.5 μM (Fig. 67). The nature of RNA inhibition by cycloheximide seems to be preferential inhibition of ribosomal RNA, although only initial investigatory studies were done in this area.

Enzyme Induction

Under normal growing conditions used for this study (GY medium), the finely controlled enzyme glutamate dehydrogenase was partially repressed by glucose. Replacement of glucose with glutamate produced a five-fold increase in the specific enzyme activity without a corresponding increase in the rate of overall protein synthesis (Fig. 64) indicating a coarse control as well.

The effects of calcium reversal of citrate inhibition of macromolecule synthesis were not identical in cells growing in glutamate and glucose media. The notable difference was the effect on RNA synthesized under the two conditions. Both RNA and protein synthesis were immediately stopped shortly after the addition of citrate to glutamate grown cells, while RNA synthesis continued for at least 2 hours

in glucose grown cells. The reversal of inhibition by calcium was similar in both cases.

Further studies by sedimentation analysis in sucrose gradients of ^{14}C-uracil labelled extracted RNA showed that the majority of RNA synthesized under induction conditions may be presumptive messenger RNA. This would be expected if there is an abrupt change in cellular metabolism to synthesize inducible enzymes. Moreover, cycloheximide inhibition was additive with respect to citrate when added within 30 min of each other to the culture indicating the inhibitory effects are different.

PART III

CALCIUM EFFECTS ON TRANSPORT

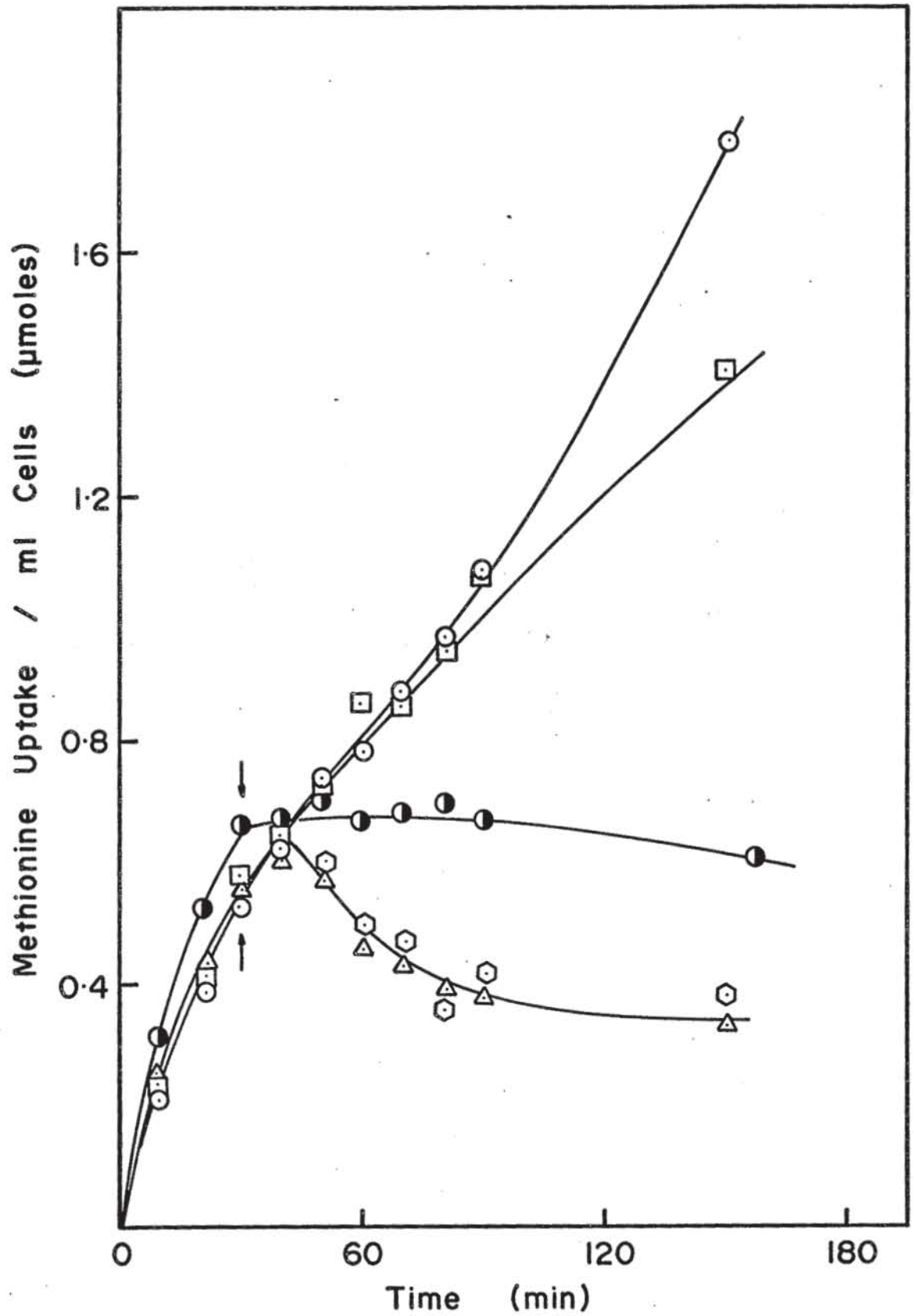
RESULTS

A. Amino Acid Transport

The previous section shows the importance of calcium for growth and macromolecular synthesis. After an initial appraisal of the calcium effect, it seemed that calcium acted on the protein synthesizing machinery of the cell. But upon further investigation, the activation of endogenous protein synthesis by calcium did not correlate with the antagonistic effect of calcium on protein synthesis *in vitro*. Amino acid transport studies helped to clarify at what level calcium was probably controlling metabolism.

Cycloheximide action: In *Achlya*, amino acids are transported by specific energy-dependent amino acid transport systems as characterized by Singh (1974). Addition of metabolic inhibitors such as azide, CCCP and 2,4-DNP inhibited further uptake and caused an efflux of some amino acids (Fig. 72). Apparently, metabolic antagonists disturb the transport system in such a way as to cause the reversal or destruction of a concentration gradient, thereby allowing accumulated amino acid to return to the surrounding medium. Before a study of calcium effect

Figure 72: Uptake of methionine in germinated sporangiospores under non-growth conditions (Buffer A). (⊙), No addition; influence of (⊡), 2.8 μM cycloheximide; (◑), 10 mM citrate; (⬡), 1 mM azide; and (△), 10 μM 2,4-DNP added at 30 min as indicated by the arrow.


Methionine Uptake / ml Cells (µmoles)
1·6
1·2
0·8
0·4
0
60
120
180
Time (min)

on amino acid transport could be considered, two events, the uptake of amino acids and their subsequent incorporation into protein had to be separated. Cycloheximide strongly inhibited RNA and protein synthesis (Fig. 67) but had no effect on calcium and amino acid transport under the assay conditions used for this study. Figure 72 shows the negligible effect that cycloheximide had on amino acid uptake. The amino acid used in this particular case was methionine, but uptake of all the amino acids showed a similar effect (see Singh, 1974). The effect of cycloheximide on calcium transport is shown in Figure 73. Shortly after the addition of cycloheximide, calcium uptake during growth was slightly enhanced, but by 3 hours calcium uptake was reduced. The latter inhibition may reflect on inhibition of macromolecular synthesis. In Figure 73b, cycloheximide had no effect on calcium transport under non-growth conditions (Buffer A). Moreover, from a study of amino acid uptake, the kinetic pattern indicated amino acids first entered a cellular pool before they are incorporated into protein (Fig. 74). Trichloroacetic acid insoluble fraction represents macromolecular material and trichloroacetic acid represents endogenous amino acids. Attention should be drawn to the fact that uptake was followed under growth conditions and a normal saturation curve was not obtained because of this.

The possibility that an intermediate amino acid pool existed between amino acid uptake and incorporation indicated that protein synthesis and transport may be separated without the use of cycloheximide. In further tests, initial transport rates for amino acids were found to

Figure 73: Influence of cycloheximide (1 µg/ml) on calcium uptake by cells growing in GY medium (a) and Buffer A (non-growth) assay conditions (b). Effect of cycloheximide added at 60 min after $^{45}Ca^{++}$ (Δ) and no addition (O).

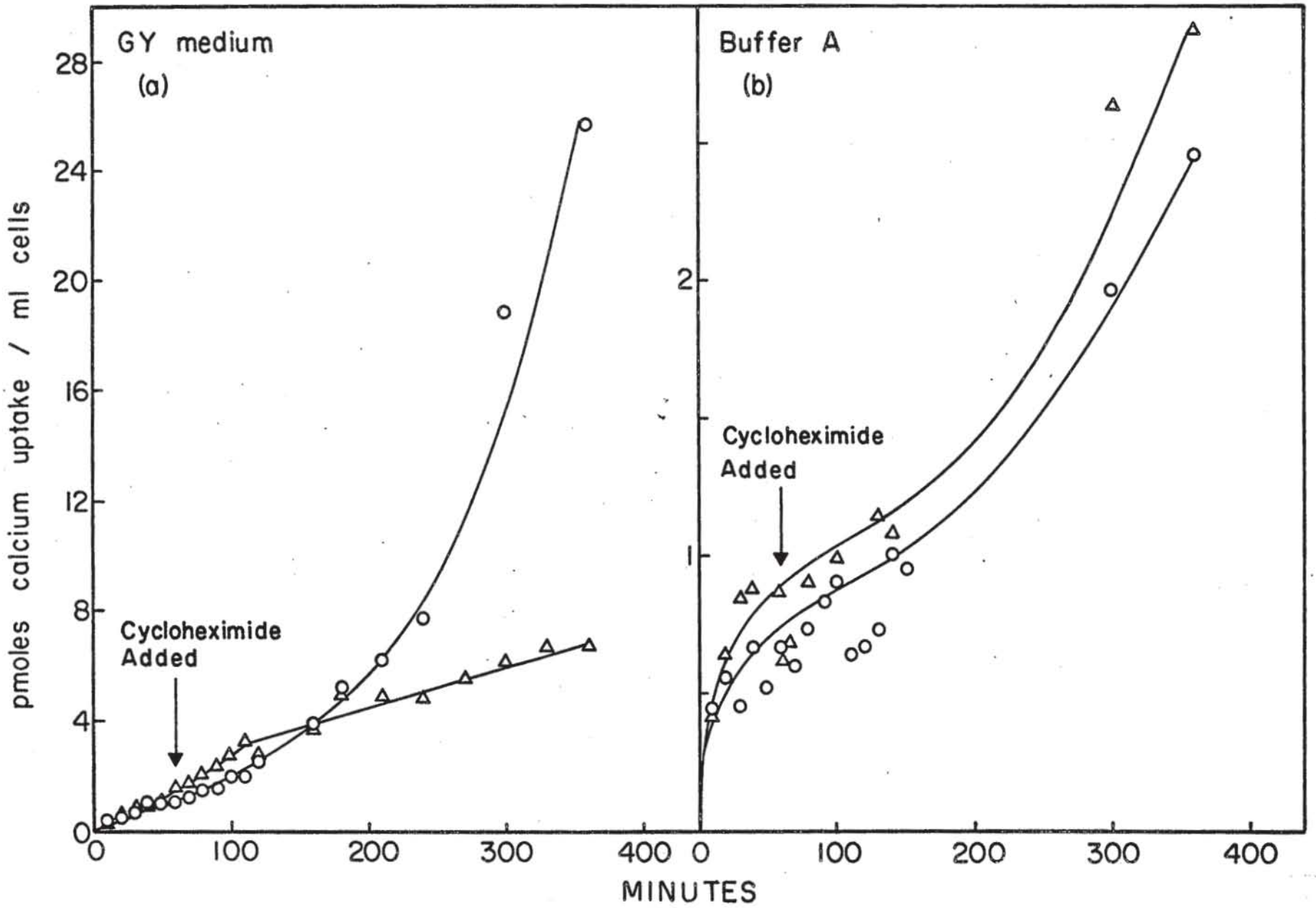
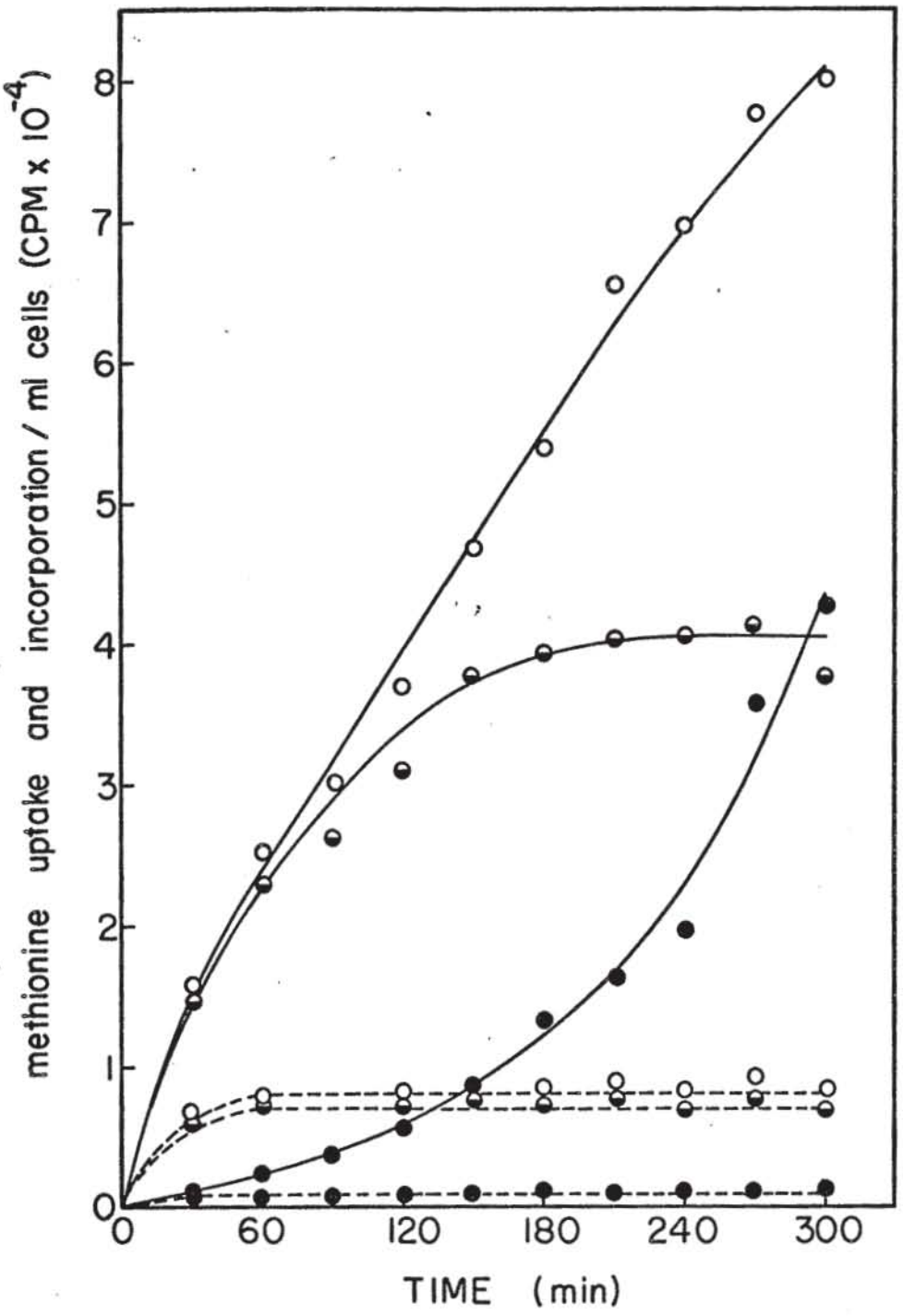

GY medium
(a)
Buffer A
(b)
Cycloheximide Added
Cycloheximide Added
pmoles calcium uptake / ml cells
MINUTES
0
4
8
12
16
20
24
28
1
2
0
100
200
300
400

Figure 74: Effect of citrate on methionine uptake and incorporation into germinated sporangiospores growing in GY medium: whole cell uptake (○), trichloroacetic acid soluble material (◒), and trichloroacetic acid insoluble material (●). All solid lines refer to studies in the absence of 10 mM citrate and all broken lines refer to studies in the presence of 10 mM citrate.


methionine uptake and incorporation / ml cells (CPM x 10^{-4})
0
1
2
3
4
5
6
7
8
0
60
120
180
240
300
TIME (min)

be identical in the presence and absence of cycloheximide, eliminating the necessity of including cycloheximide in transport experiments.

Citrate inhibition: Whether citrate was added to cells growing in GY medium with labelled amino acid or at a pre-determined time thereafter, inhibition was immediate. Citrate inhibition of methionine uptake is shown in Figure 72, and in addition, the corresponding citrate-effects on amino acid pool size and incorporation into protein (see Figure 74). After addition of citrate, the amino acid pool size remained constant, that is, no efflux of endogenous amino acid. A detailed study of citrate effect on amino acid transport is shown in Figure 75. Regardless of the time of addition of citrate, transport of an amino acid mixture containing 15 of the common amino acids was immediately halted.

The next question that was considered was whether citrate was directly interfered with amino acid transport as such or was exerting its effect via citrate-inhibition of the calcium transport system (see Fig. 55 and 56). Metabolic energy inhibitors caused an efflux of amino acid while citrate did not (Fig. 75). These results suggested that citrate was not interferring directly with the amino acid transport system. Moreover, citrate affected transport of all amino acids in a similar way (confirmed by Singh (1974)).

Competition studies added support to the indirect inhibitory effect of citrate on amino acid transport (Fig. 76). The Lineweaver-Burk plot indicates citrate interferes with methionine transport non-competitively by reducing the maximum velocity, while not changing

Figure 75: Amino acid uptake by germinated sporangiospores growing in GY medium. Effect of citrate (●), no addition; and (⊙) added at 0, 30, 60, and 90 min. A mixture of 15 amino acids was supplied at 0.1 μCi/ml and citrate at 10 mM.

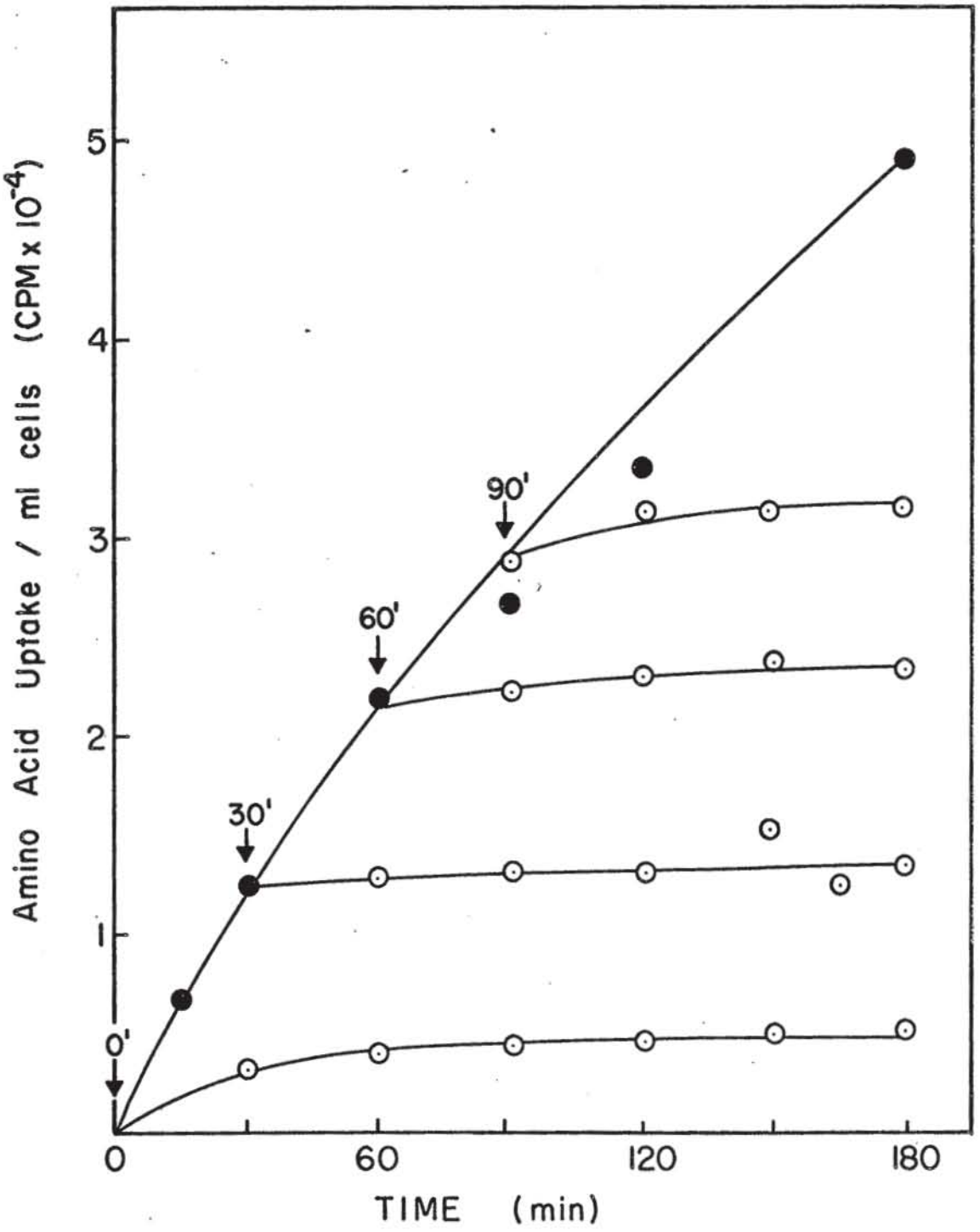
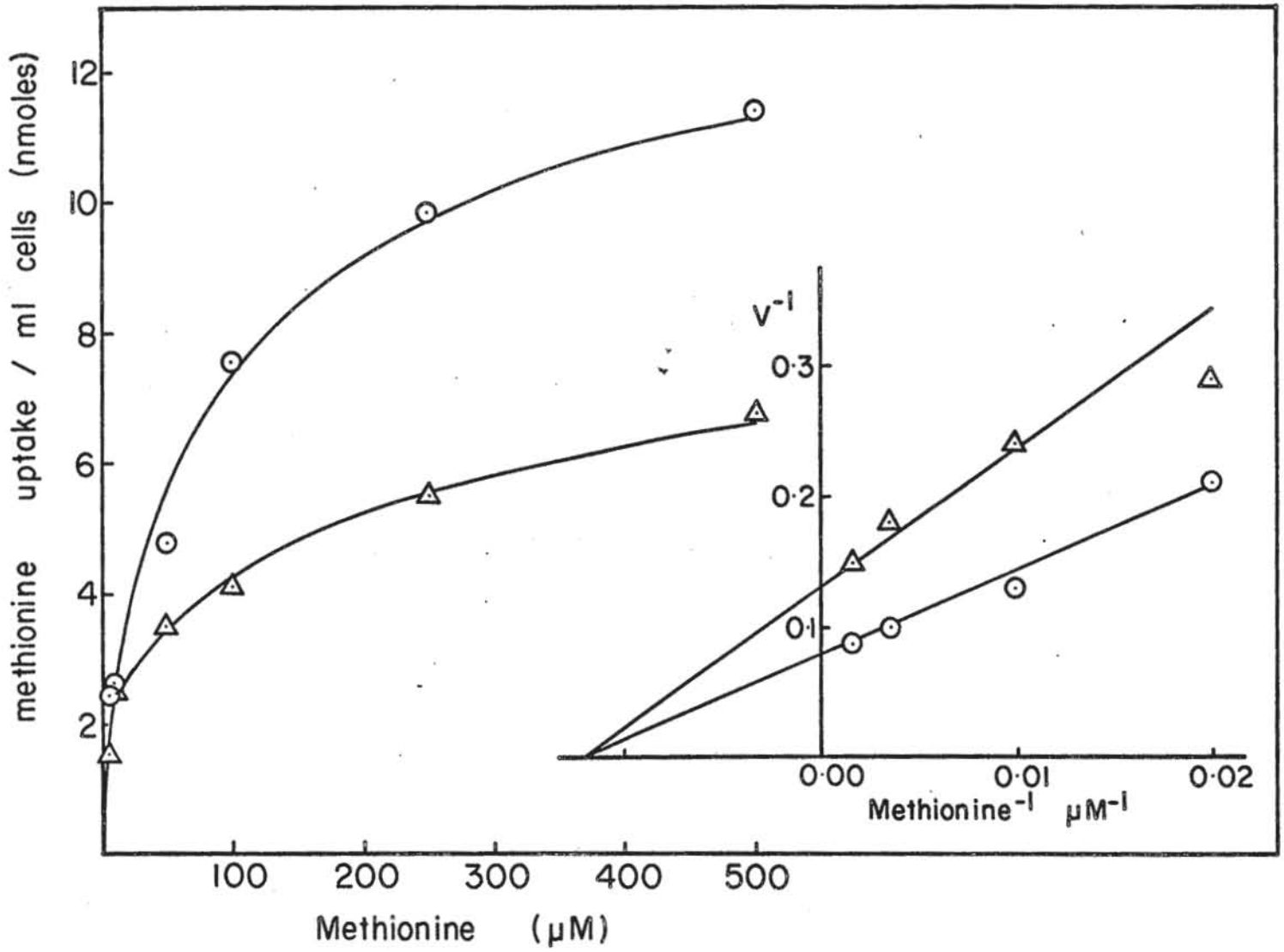

Amino Acid Uptake / ml cells (CPM x 10^{-4})
5
4
3
2
1
0
0'
30'
60'
90'
0
60
120
180
TIME (min)

Figure 76: Rate-concentration plot and Lineweaver-Burk form for methionine transport. Effect of citrate, (⊙), 0 mM; and (△), 10 mM.

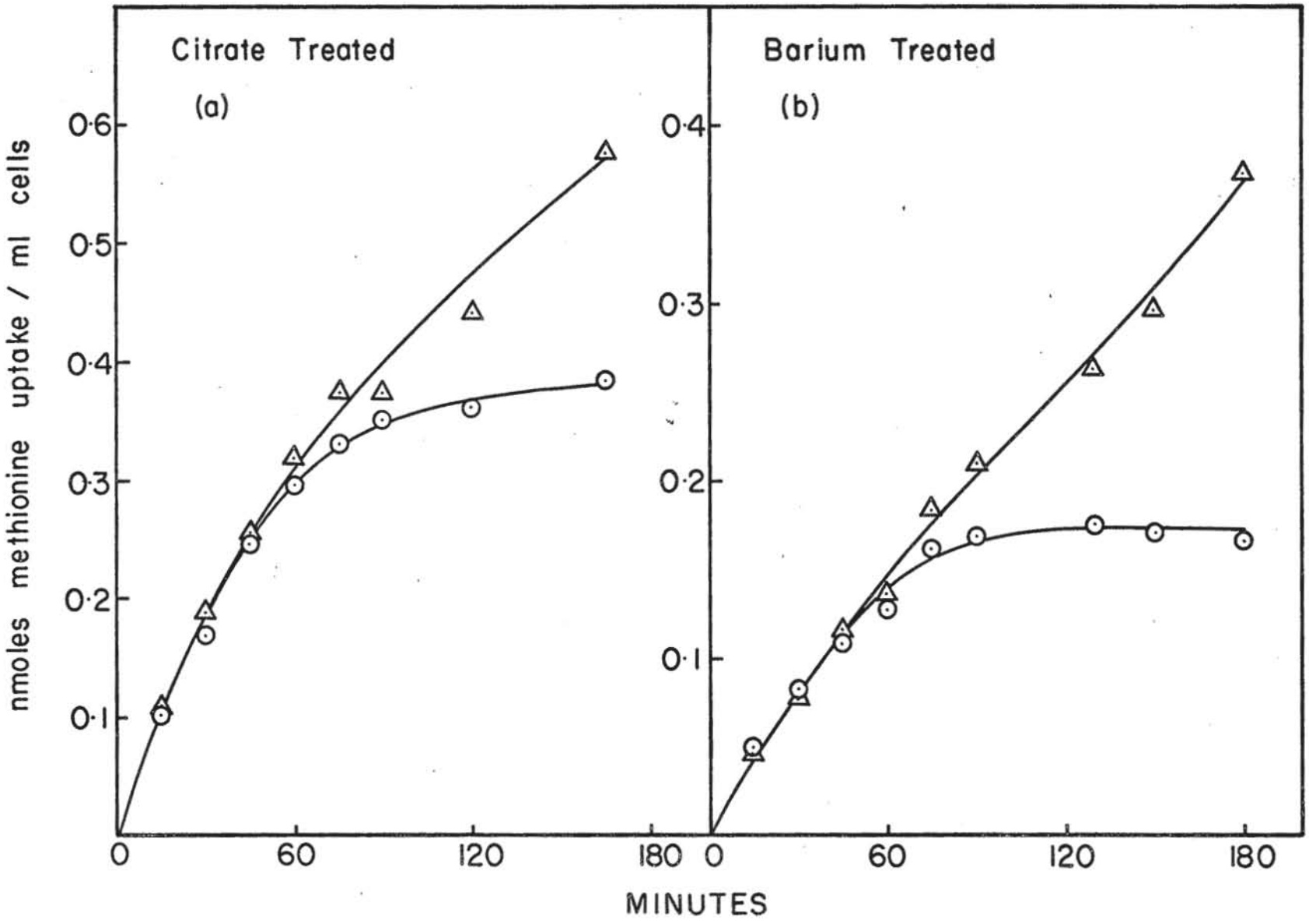

methionine uptake / ml cells (nmoles)
12
10
8
6
4
2
100
200
300
400
500
Methionine (μM)
V⁻¹
0·3
0·2
0·1
0·00
0·01
0·02
Methionine⁻¹ μM⁻¹

the affinity of the methionine transport system. Consequently, citrate and methionine do not bind to the same site. Citrate appears to exert its effect indirectly by inhibiting the calcium transport system and by causing an efflux of reversibly bound calcium.

Of particular interest were the results communicated by LéJohn and Singh (1975) pertaining to activation of amino acid uptake by low concentrations of citrate (50 to 100 μM). Addition of calcium (0.1 to 10 mM) increasingly reduced activated calcium uptake by low citrate even below the basal uptake level of amino acids. This result of low citrate concentration activation of amino acid uptake was interpreted as action by one of the following possibilities: (i) free citrate, (ii) removal of an inhibitor by citrate and (iii) citrate-calcium complex (LéJohn & Singh, 1975). In compliance with this study, the latter possibility seems the most likely choice due to a possible fixed citrate-Ca^{++} chelation complex formed on the glycopeptide producing maximum uptake of energy-linked solutes.

Amino acid activation: Calcium effect on the initial rate of amino acid transport was not evident under normal assay conditions. Neither could the calcium activation effect on protein synthesis be demonstrated without the use of citrate. However, when cells were first washed for a period (60 min) with either citrate, Ba^{++}, or 2,4-DNP, then calcium enhanced amino acid uptake, and also had a slight activating effect on the incorporation into proteins. Calcium uptake in cells pre-treated (1 h) with either citrate or Ba^{++} is shown in Figure 77. Under

Figure 77: Enhancement of methionine uptake by 0.1 mM calcium (△), and methionine uptake without added calcium (⊙). Germinated sporangiospores pre-washed with (a) 10 mM citrate and (b) 2.5 mM Ba^{++} for 60 min, removed by filtration, resuspended in medium lacking inhibitors and methionine uptake studied.


Citrate Treated
(a)
Barium Treated
(b)
nmoles methionine uptake / ml cells
0·6
0·5
0·4
0·3
0·2
0·1
0·4
0·3
0·2
0·1
0
60
120
180
0
60
120
180
MINUTES

these calcium-depleted conditions, added calcium apparently was required to return amino acid uptake to normal level as compared to untreated cells (see Fig. 72). It appears that under normal physiological conditions an intact calcium transport system must be maintained and in some unknown way mediate amino acid transport. Calcium enhancement of methionine uptake in energy depleted cells is shown in Figure 78.

Calcium activation of amino acid transport in cells following pretreatment with 2,4-DNP seemed paradoxical. 2,4-DNP only partially inactivated the calcium transport system, and caused a negligible efflux of calcium, but 2,-DNP does have an immediate effect on amino acid transport. Transport of amino acid was halted and an efflux of amino acids ensued. Since calcium enhancement of protein synthesis could be demonstrated by this experimental procedure, it seemed possible that calcium was aiding the repair of membrane functions (Fig. 78b). Calcium did enhance protein synthesis but the increase was only slight.

Ruthenium red inhibition: The mucopolysaccharide stain, ruthenium red ($Ru(NH_3)_4(OH)Cl_2$), an inhibitor of calcium transport, inhibited the uptake of calcium as shown in Figure 79. Cells were exposed to $^{45}Ca^{++}$ 60 minutes prior to the addition of 50 μM ruthenium red. Inhibition of calcium uptake by ruthenium red was accompanied by an immediate efflux of calcium which suggests that ruthenium red removes reversible bound calcium. Ruthenium red inhibited calcium uptake maximally at 100 μM, reducing transport by 95%. As shown in Figure 80, the inhibition constant ($I_{0.5}$) for ruthenium red was 1.0 x

Figure 78: Enhancement of methionine uptake (a) and incorporation into proteins (b) by calcium in cells pretreated with 0.01 mM 2,4-DNP. Control (untreated cells) open circles and experimental (treated cells), open triangles. 2,4-DNP treatment was for 4 h then removed by filtration and washing. Calcium added at 1 mM.

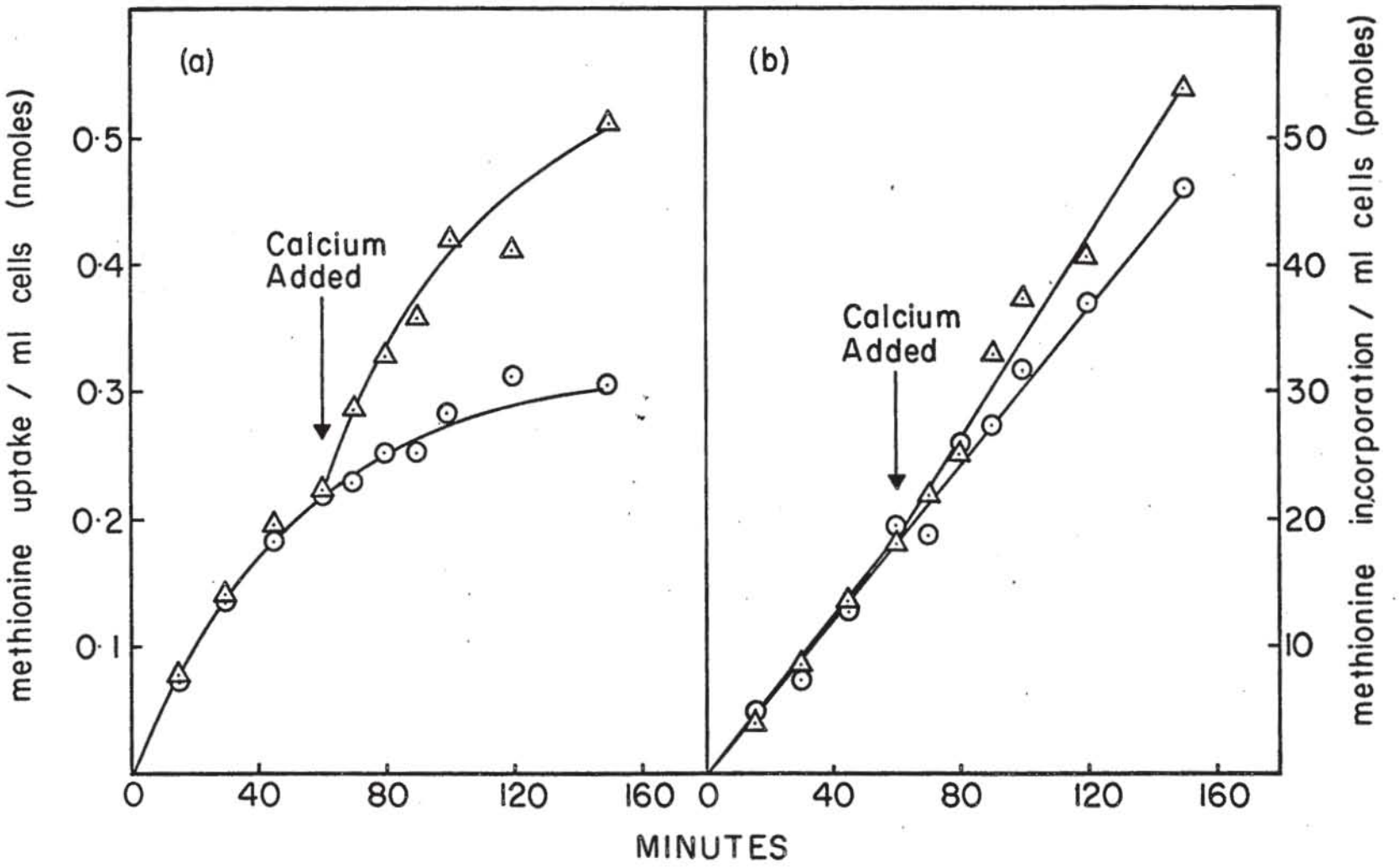

(a)
(b)
Calcium Added
Calcium Added
methionine uptake / ml cells (nmoles)
methionine incorporation / ml cells (pmoles)
0·1
0·2
0·3
0·4
0·5
10
20
30
40
50
0
40
80
120
160
MINUTES

Figure 79: Inhibition of calcium uptake by germinated sporangiospores suspended in Buffer A by ruthenium red added at 60 min (●), point of arrow, and not added (O).

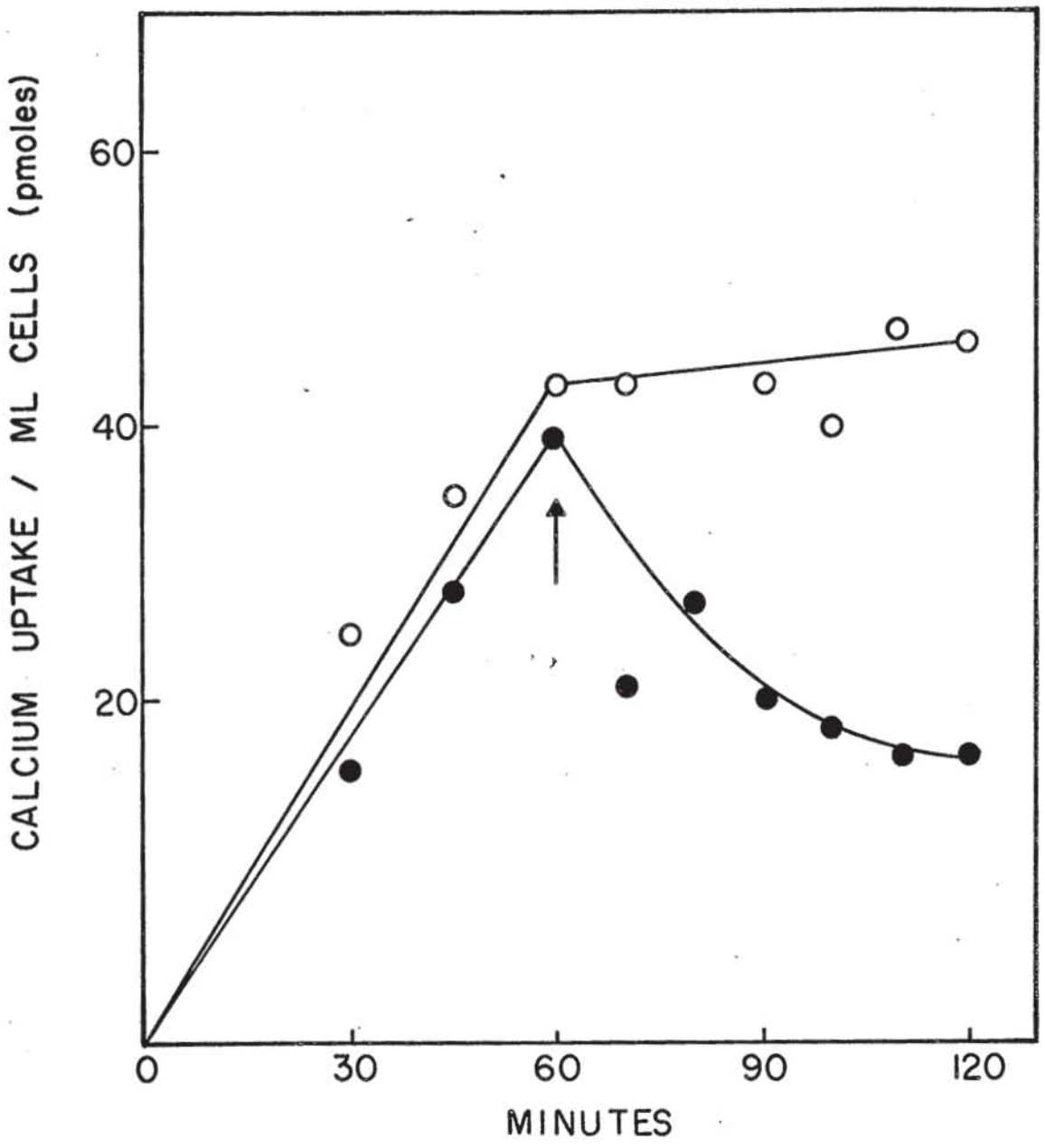
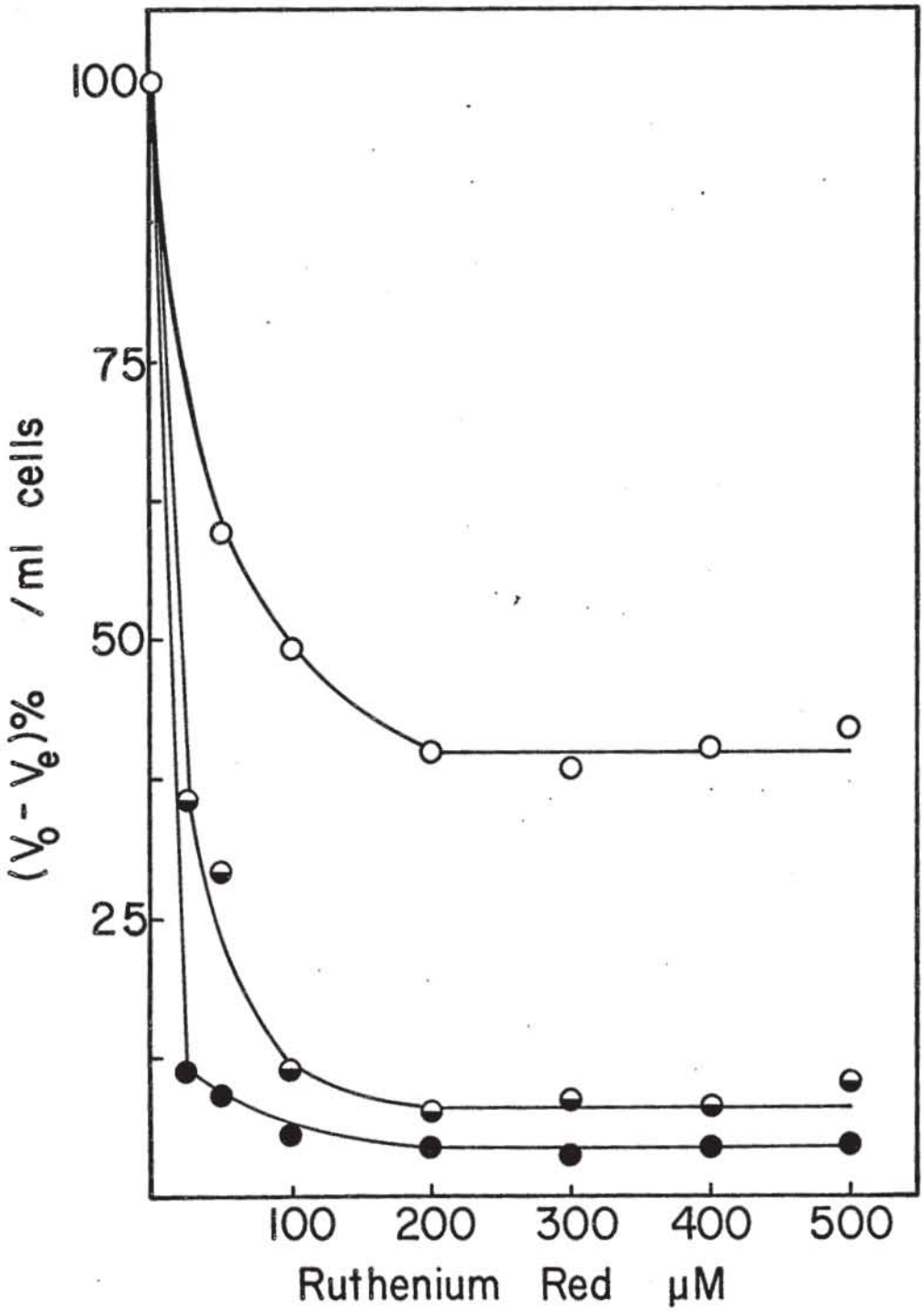

CALCIUM UPTAKE / ML CELLS (pmoles)
60
40
20
0
30
60
90
120
MINUTES

Figure 80: Inhibition of calcium uptake by ruthenium red with 10 mM Ca^{++}(○); 1 mM Mg^{++} (◒) and no addition (●). V_o is calcium uptake in the absence and V_e in the presence of ruthenium red.


100
75
50
25
$(V_o - V_e)$% /ml cells
100
200
300
400
500
Ruthenium Red μM

10^{-5} M. In the presence of 1 mM Mg^{++}, the antagonistic effect of ruthenium red was slightly reduced to an $I_{0.5}$ value of 2.0×10^{-5} M. However, in the presence of 10 mM calcium, the inhibition constant was reduced 10 fold ($I_{0.5} = 1.0 \times 10^{-4}$ M), and maximally inhibited only 55% of calcium uptake at 200 μM ruthenium red.

Kinetic studies of the effect of ruthenium red on calcium transport in Achlya cells indicated that ruthenium red competitively inhibited calcium uptake (Fig. 81). Furthermore, as ruthenium red is known to interact with mucopolysaccharides, the competitive inhibition studies suggest that there may be a carbohydrate moiety associated with the active site(s) of the calcium transport system.

After separation of the calcium transport system into two components, a calcium binding glycopeptide and an intrinsic calcium transport system of osmotically shocked cells, the effect of ruthenium red on each was studied. As shown in Table XVII, ruthenium red reduced calcium binding by the glycopeptide 80% at a concentration of 2.5×10^{-4} M. In osmotically shocked cells, 250 μM ruthenium red non-competitively inhibited calcium transport (see Fig. 20).

As it was clear that ruthenium red specifically interferred with the calcium transport system of Achlya, it was of particular interest to investigate the effect of ruthenium red on amino acid transport. Ruthenium red blocked the uptake of all the amino acids, as shown in Figure 82 for methionine, but unlike effect on calcium, ruthenium red caused no efflux of methionine. Since ruthenium red inhibited the uptake of all the amino acids and as methionine has

Figure 81: Rate-concentration plots and Lineweaver-Burk form of the competition kinetics of calcium transport caused by ruthenium red at (○), 0 mM; (●), 0.05 mM; and (▲), 0.2 mM.

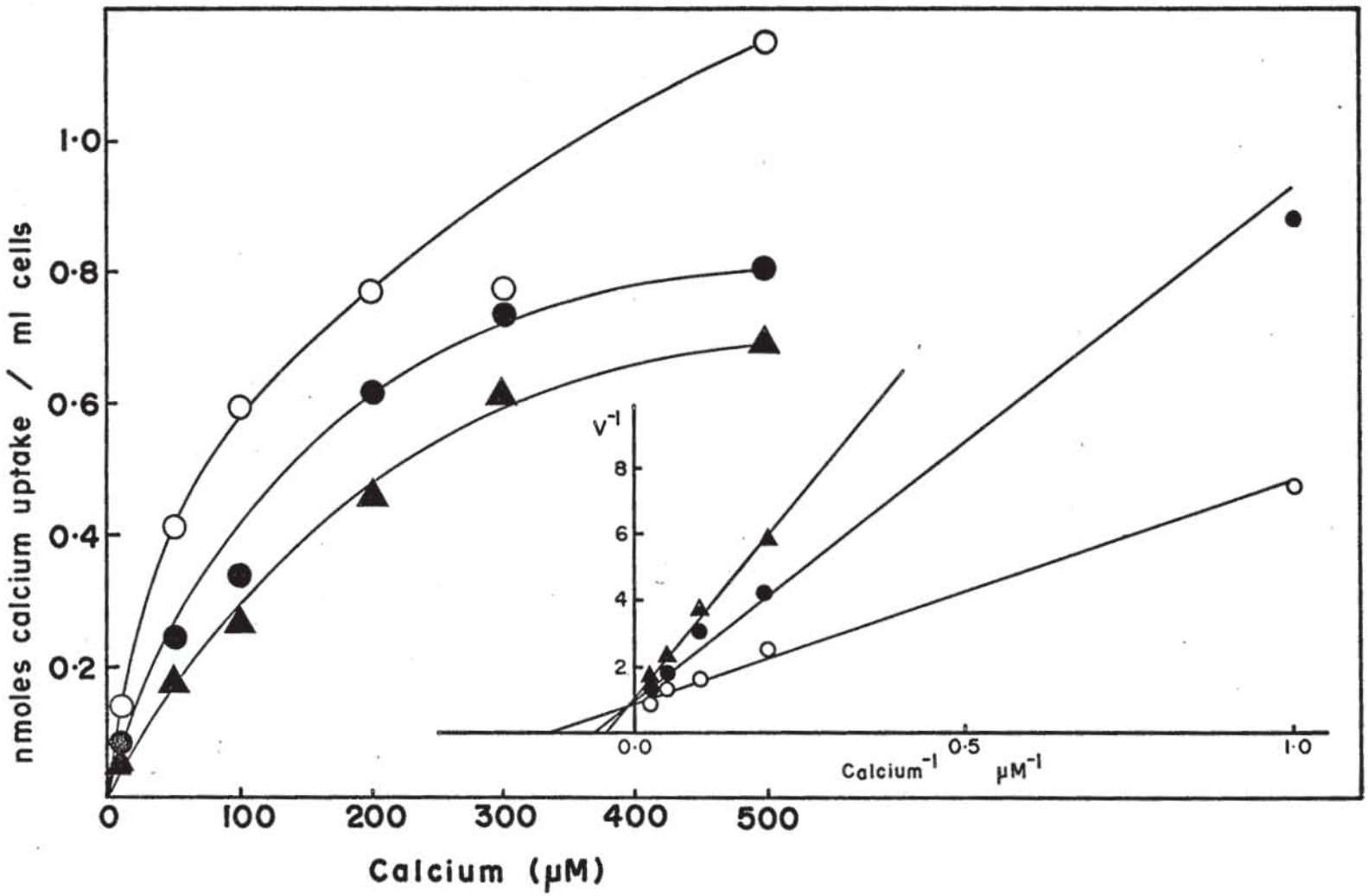
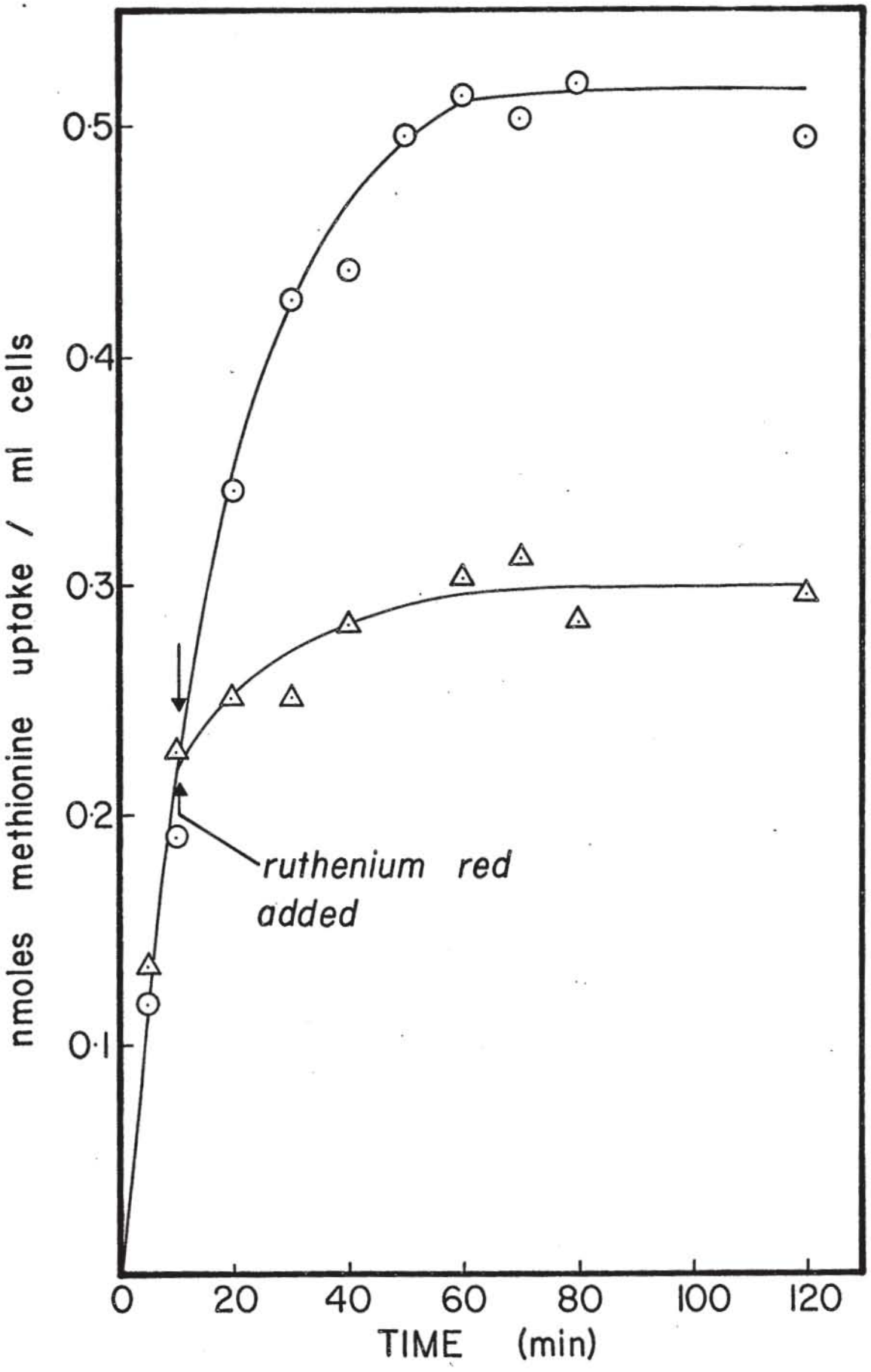

nmoles calcium uptake / ml cells
1·0
0·8
0·6
0·4
0·2
0
100
200
300
400
500
Calcium (μM)
V⁻¹
8
6
4
2
0·0
0·5
1·0
Calcium⁻¹ μM⁻¹

Figure 82: Ruthenium red inhibition of methionine uptake. Methionine uptake in the presence of 12.5 μM ruthenium red (△) and in its absence (⊙).


0·5
0·4
0·3
0·2
0·1
nmoles methionine uptake / ml cells
ruthenium red added
0
20
40
60
80
100
120
TIME (min)

a complex biphasic uptake kinetic pattern, results obtained for phenylalanine-ruthenium red interaction will be discussed. The rate-concentration uptake of phenylalanine was inhibited by ruthenium red and the Lineweaver-Burk plot showed ruthenium red inhibited uptake non-competitively (Fig. 83). Inset replot of slopes as a function of ruthenium red concentration gave an estimated K_i value of 4.0×10^{-5} M for ruthenium red. As determined from the Lineweaver-Burk plot the apparent K_m value was 5.56×10^{-5} M. In further studies, calcium reversal of ruthenium red inhibition of amino acid was shown. As the concentration of calcium was increased from 0 μM to 100 μM, calcium almost completely antagonized ruthenium red inhibition (Fig. 84). To affirm that calcium specifically reversed ruthenium red effect, amino acid uptake was followed in the presence of either Ca^{++}, Mn^{++}, or Mg^{++} (all at 100 μM). As shown in Figure 85a, only calcium was an effective antagonist of ruthenium red, while both Mg^{++} and Mn^{++} in the presence of ruthenium red were slightly inhibitory relative to ruthenium red effect alone. Subsequent protein synthesis was also inhibited by ruthenium red that only could be reversed by Ca^{++}, not Mn^{++} or Mg^{++} (Fig. 85b). These ruthenium red inhibition results verify earlier work with citrate that calcium transport and/or an intact calcium transport system is necessary for the optimum uptake of amino acids.

Osmotically shocked cells: Calcium uptake in osmotically shocked cells was shown to still proceed but at a much reduced rate, and a calcium binding glycopeptide was released into the surrounding milieu.

Figure 83: Inhibition of phenylalanine uptake by rutheniun red supplied at (O), 0 mM; (□), 0.05 mM; (△), 0.1 mM; and (●), 0.2 mM. Inset plot: Replot of slopes against inhibitor concentration to estimate K_i value for ruthenium red.

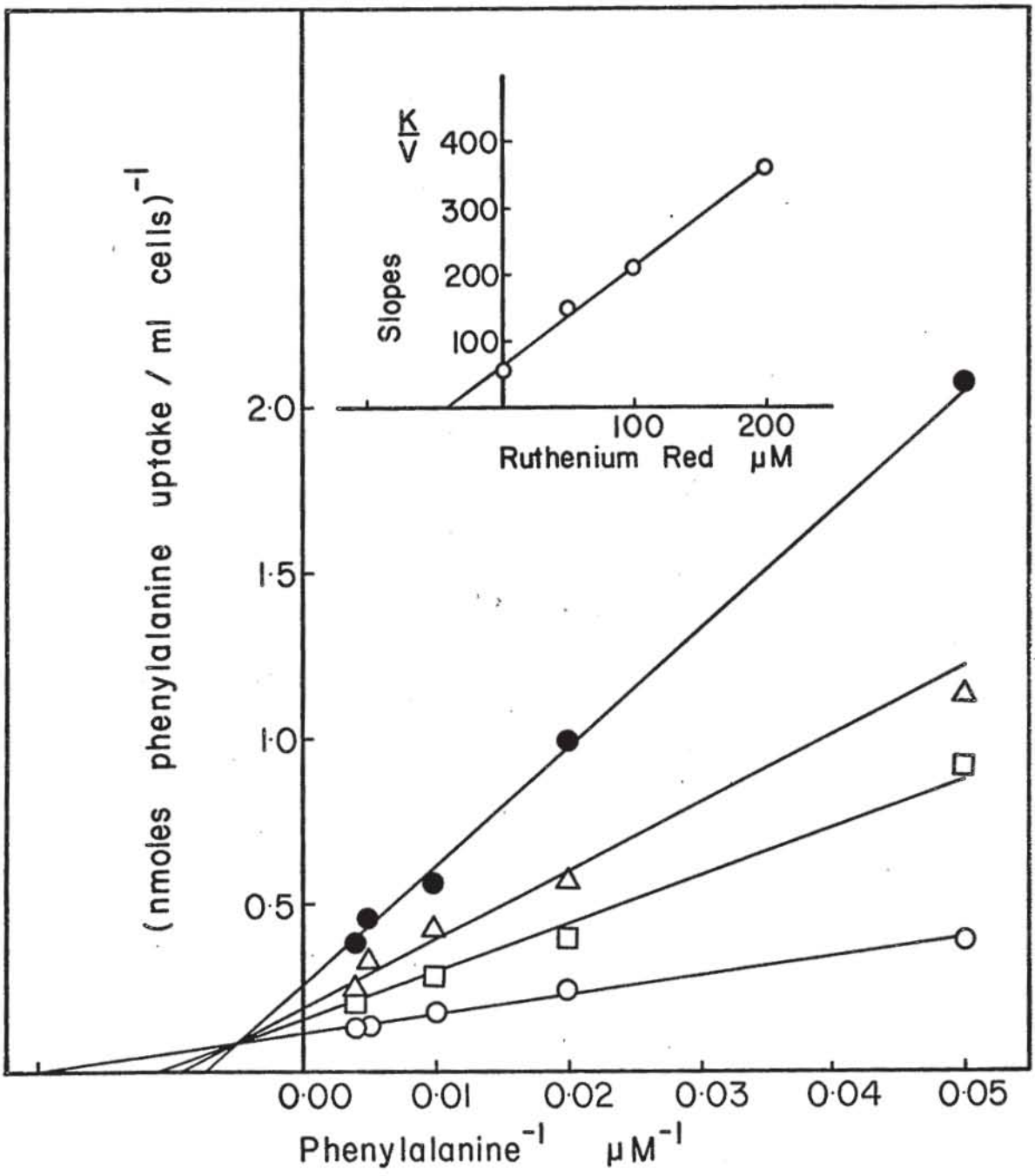

(nmoles phenylalanine uptake / ml cells)$^{-1}$
2·0
1·5
1·0
0·5
0·00
0·01
0·02
0·03
0·04
0·05
Phenylalanine^{-1} μM^{-1}
Slopes $\frac{K}{V}$
400
300
200
100
100
200
Ruthenium Red μM

Figure 84: Lineweaver-Burk plot of the rate of phenylalanine uptake in the presence and absence of ruthenium red (RR) and calcium. Unless specified, all reaction systems contained 0.05 mM ruthenium red.

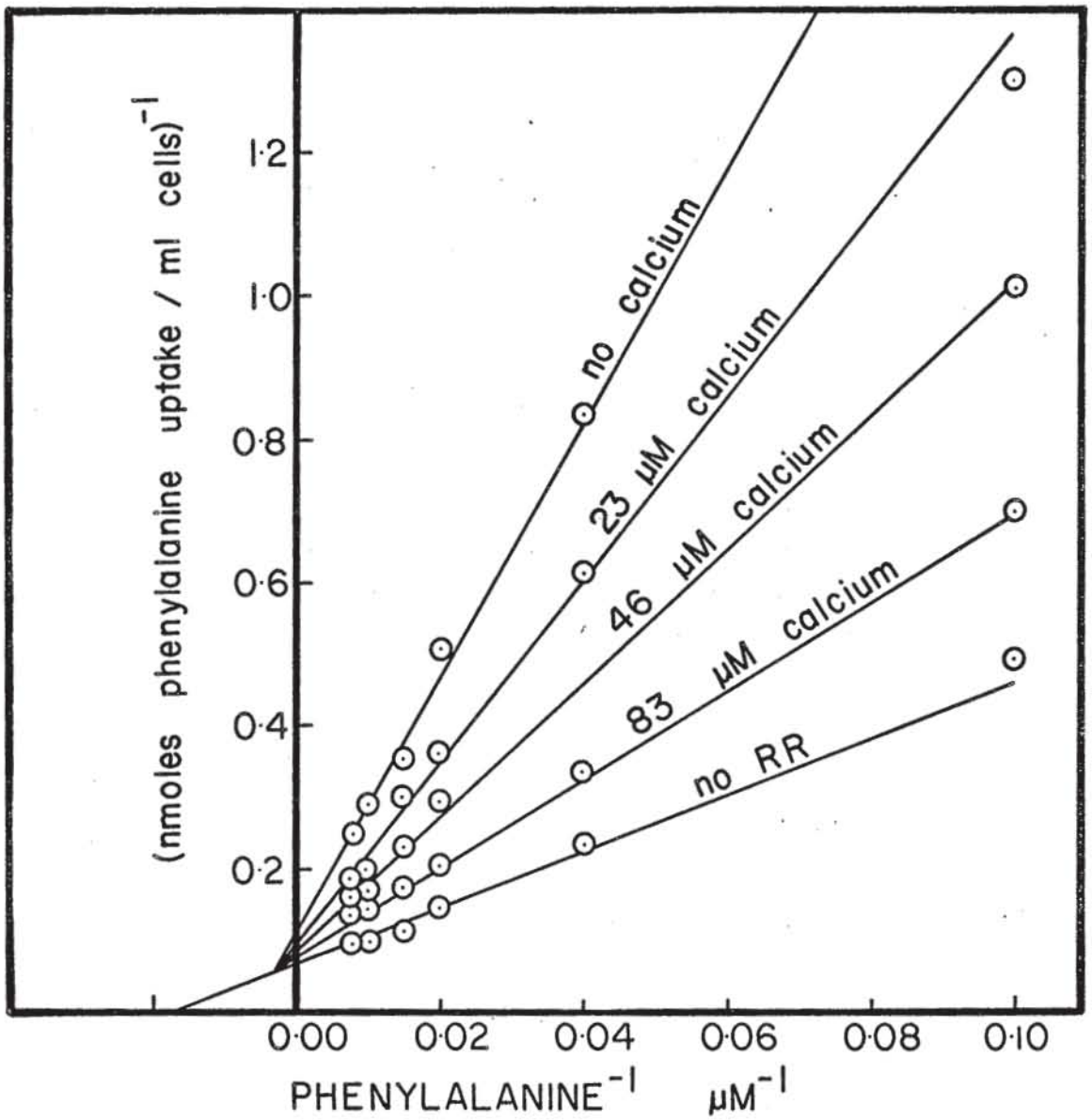
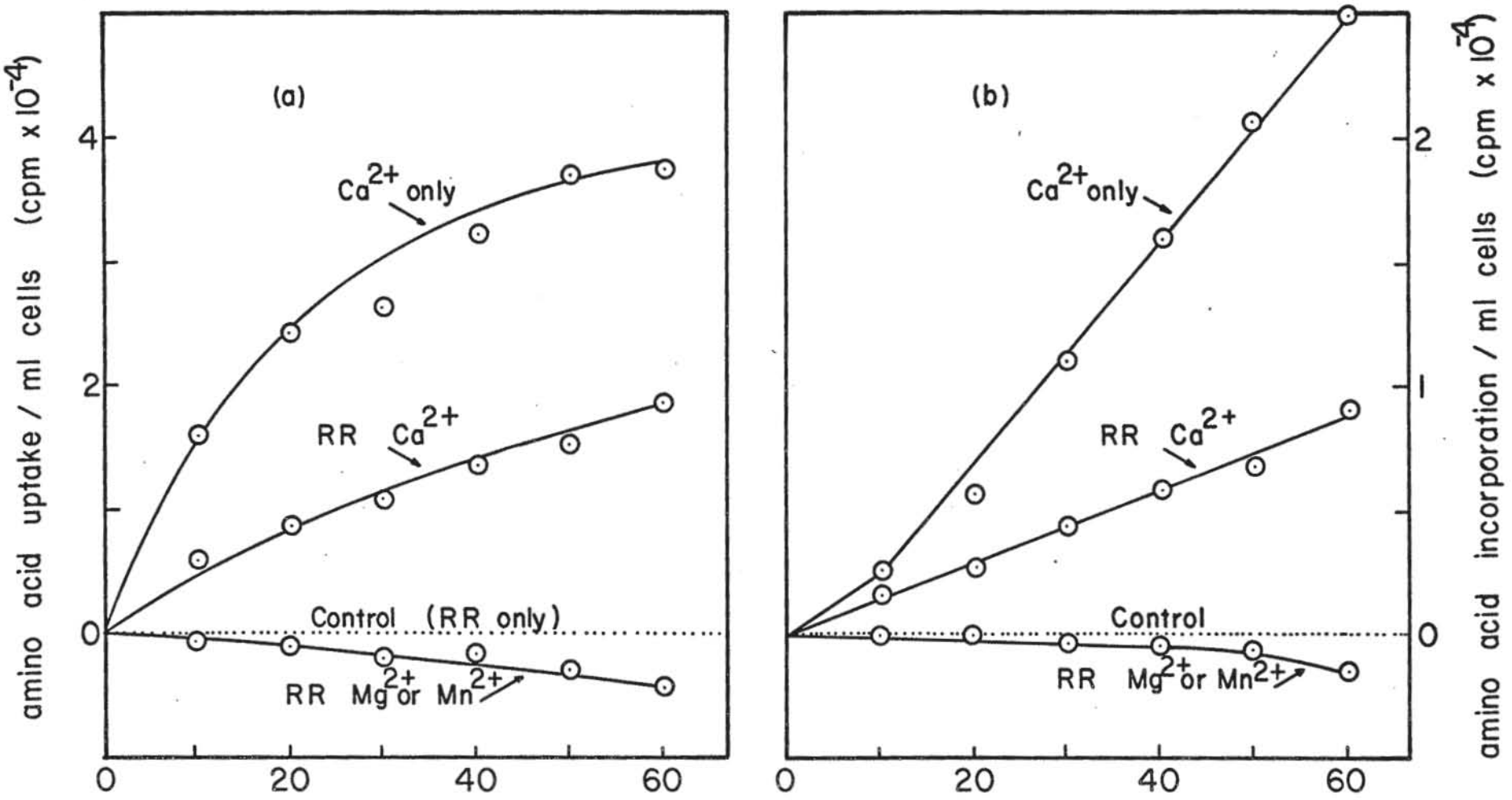

(nmoles phenylalanine uptake / ml cells)$^{-1}$
1·2
1·0
0·8
0·6
0·4
0·2
no calcium
23 μM calcium
46 μM calcium
83 μM calcium
no RR
0·00
0·02
0·04
0·06
0·08
0·10
PHENYLALANINE^{-1} μM^{-1}

Figure 85: Calcium antagonism of ruthenium red inhibition of amino (a) uptake and (b) incorporation. Divalent cations used at a concentration of 0.1 mM and ruthenium red (RR) at 0.01 mM.


(a)
(b)
Ca2+ only
RR Ca2+
Control (RR only)
RR Mg2+ or Mn2+
Control
amino acid uptake / ml cells (cpm x 10-4)
amino acid incorporation / ml cells (cpm x 10-4)
0
20
40
60
MINUTES

Numerous attempts to add calcium binding glycopeptide back to the osmotic shocked cells were unsuccessful. A corresponding study of amino acid uptake in osmotically shocked cells was done, but the cells were unable to accumulate any of the common amino acids. In fact, all energy dependent transport processes in Achlya were halted. Addition of a wide concentration and varied orders of addition of energy metabolites (ATP, PEP, GTP, and phosphocreatine) divalent cations (Ca^{++}, Mg^{++}, and Mn^{++}) and shock fluid failed to reactivate amino acid transport in osmotically shocked cells. A search for amino acid binding proteins in the shock fluid was unproductive. These results indirectly support the thesis that calcium may be involved in amino acid transport. Evidently, calcium is important for membrane integrity, and it is possibly indirectly involved in maintaining a proton gradient across that membrane.

B. Nucleoside and Nucleobase Uptake

Corresponding studies by Stevenson (1974) on Achlya showed nucleobase and nucleoside uptake were carrier-mediated processes but only nucleoside uptake was energy-linked. Nucleobases were transported by facilitative diffusion. As discussed previously, purine, notably N^6-(substituted) adenine compounds regulated calcium binding and transport but sites for purine binding to the calcium transport process and purine uptake were shown to be separate (Stevenson, 1974). This work also indicated that osmotically shocked germlings were unable to accumulate nucleoside.

Citrate and ruthenium red effects: Neither citrate nor ruthenium red had an inhibitory effect on the facilitated transport of uracil (Fig. 86c), while thymidine uptake was immediately blocked and efflux ensued (Fig. 86d). Kinetic analysis of thymidine uptake, indicated non-competitive inhibition by both citrate and ruthenium red. The Lineweaver-Burk plot for citrate antagonism of thymidine uptake is shown in Figure 87. Citrate noncompetitively inhibited the transport of thymidine. Similar results were obtained by Stevenson (1974) for adenosine, cytidine, and uridine. Unlike citrate inhibition of amino acid transport, both the maximum velocity and apparent K_m were reduced for nucleoside transport. A valid conclusion that we can draw from these observations is that the calcium effect on energy-linked nucleoside transport is indirect, while facilitated nucleobase transport is not affected. This is consistent with the result that both ruthenium red and citrate interact with the calcium transport process, reducing energy-related solute transport and subsequent synthesis of macromolecules.

C. Ion Transport

A methodical study of ion transport in Achlya, has not yet been carried out. However, initial studies on the transport of Na^+ have indicated that it is carrier-mediated and energy-linked. The uptake of Na^+ was found to be a saturable process although rate-concentration analysis of Na^+ transport revealed biphasic uptake

Figure 86: Influence of ruthenium red and citrate on the uptake of (a) calcium, (b) methionine, (c) uracil and (d) thymidine. At times indicated by arrows either 0.025 mM ruthenium red or 10 mM citrate was added to the cell suspensions. Broken lines represent uptake patterns for cultures treated with citrate, (⊙) control, and (△) ruthenium red-treated cultures.

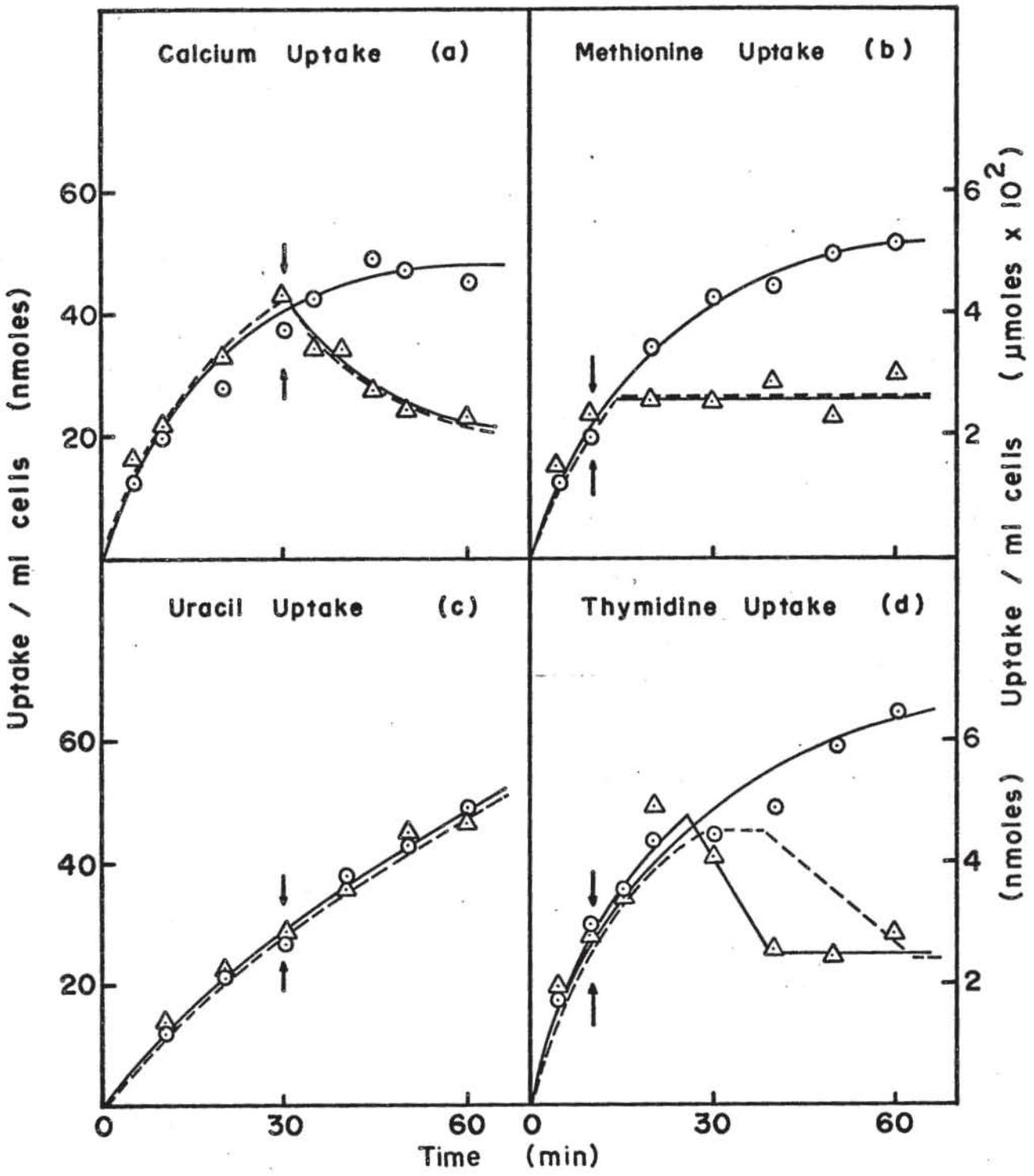
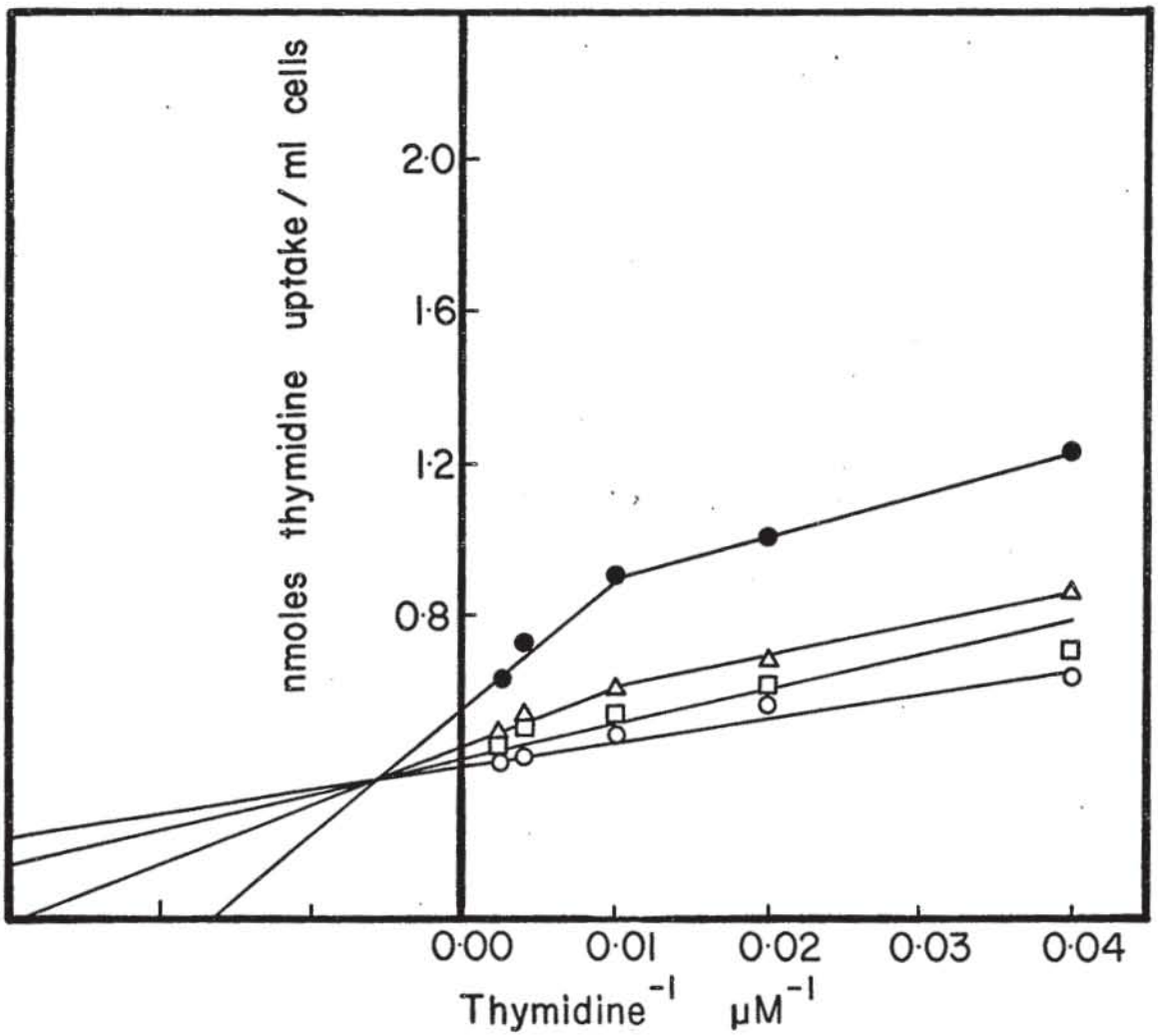

Calcium Uptake (a)
Methionine Uptake (b)
Uracil Uptake (c)
Thymidine Uptake (d)
Uptake / ml cells (nmoles)
Uptake / ml cells (μmoles x 10²)
Uptake / ml cells (nmoles)
Time (min)
0
30
60
20
40
2
4
6

Figure 87: Lineweaver-Burk plot of thymidine uptake. Inhibitory effect of citrate: (O), 0 mM; (□), 2.5 mM; (△), 5.0 mM; and (●), 10 mM on thymidine uptake.


nmoles thymidine uptake / ml cells
2·0
1·6
1·2
0·8
0·00
0·01
0·02
0·03
0·04
Thymidine^{-1} μM^{-1}

(Fig. 88) with a high affinity site (apparent K_m = 4.8 x 10^{-6} M) and a low affinity site (apparent K_m = 6.0 x 10^{-4} M). Na^+ uptake was dependent upon pH and metabolic inhibitors affected the rate of uptake. Na^+ was maximally transported at a well defined pH optimum of 6. Electron transport inhibitors, azide and cyanide and oxidative phosphorylation uncouplers, 2,4-DNP and CCCP antagonized Na^+ transport (Fig. 89).

Like other energy-dependent transport processes, Na^+ transport was inhibited by citrate and ruthenium red, as shown in Figure 90. Citrate maximally inhibited Na^+ transport by 80% with an estimated $I_{0.5}$ value of 6.0 x 10^{-4} M, while ruthenium red reduced transport by 70% with an estimated $I_{0.5}$ value of 3.2 x 10^{-5} M. These results again affirmed that calcium is involved in all energy-related transport processes of this organism. No enhancement of Na^+ uptake could be produced by calcium in the absence of inhibitors but this was not unexpected as 'normal' cells in assay have calcium already bound to the wall and membrane. Another expected result was the reduction of Na^+ uptake in osmotically shocked cells. Only at high external Na^+ concentration did uptake become apparent (Fig. 88). This observation closely correlated with the observed optimal uptake of amino acid with an high external amino acid concentration, eliminating the requirement of an intact calcium transport system.

Figure 88: Rate-concentration plot and Lineweaver-Burk form of Na^+ uptake. Uptake of Na^+ by intact (⊙) and osmotically shocked (△) germinated sporangiospores.

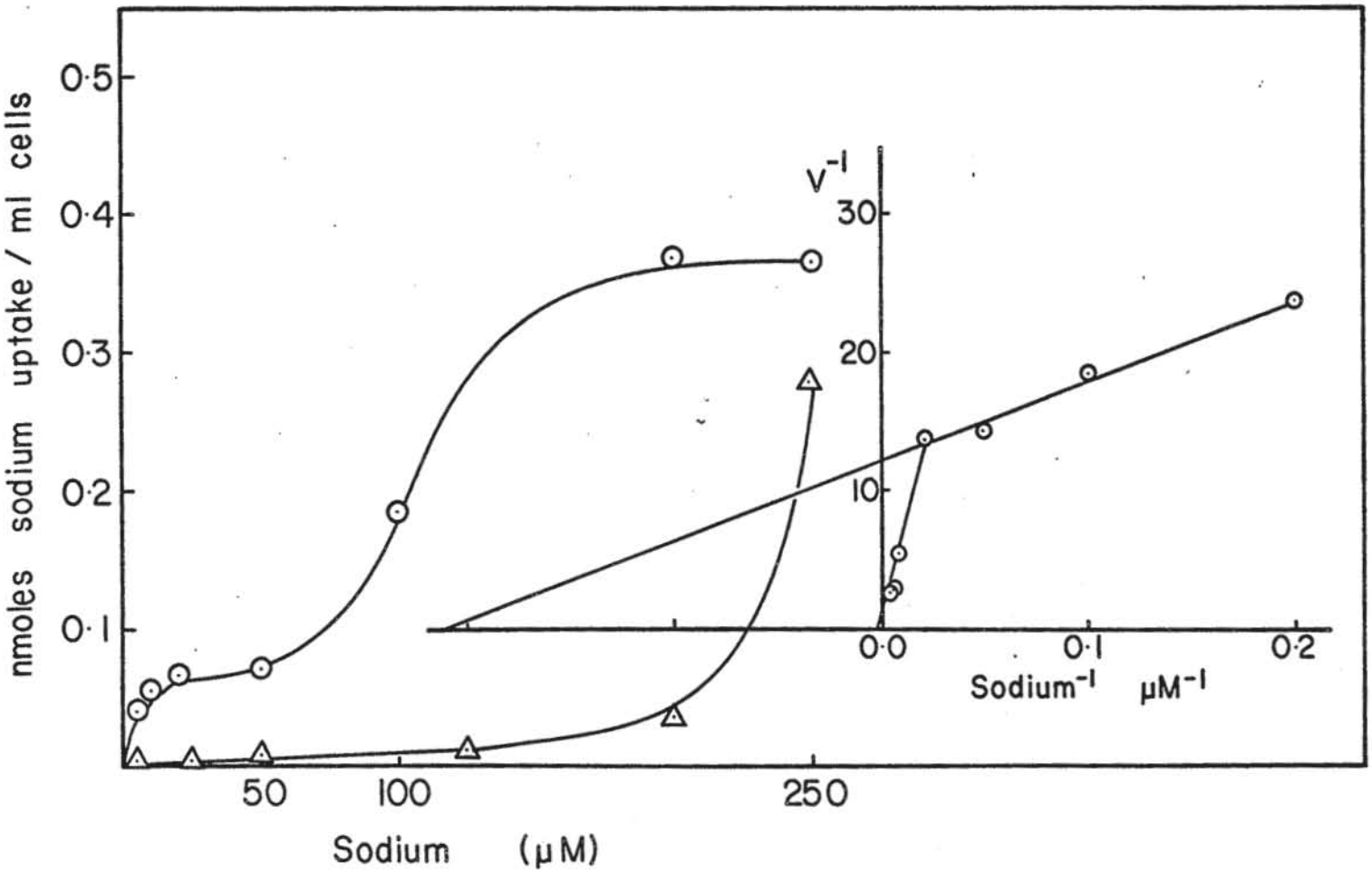

nmoles sodium uptake / ml cells
0·5
0·4
0·3
0·2
0·1
50
100
250
Sodium (μM)
V⁻¹
30
20
10
0·0
0·1
0·2
Sodium⁻¹ μM⁻¹

Figure 89: Inhibition of Na^+ uptake by metabolic antagonists. Effect of (O), azide; (●), cyanide; (Δ), CCCP; and (▲), 2,4-DNP on transport.

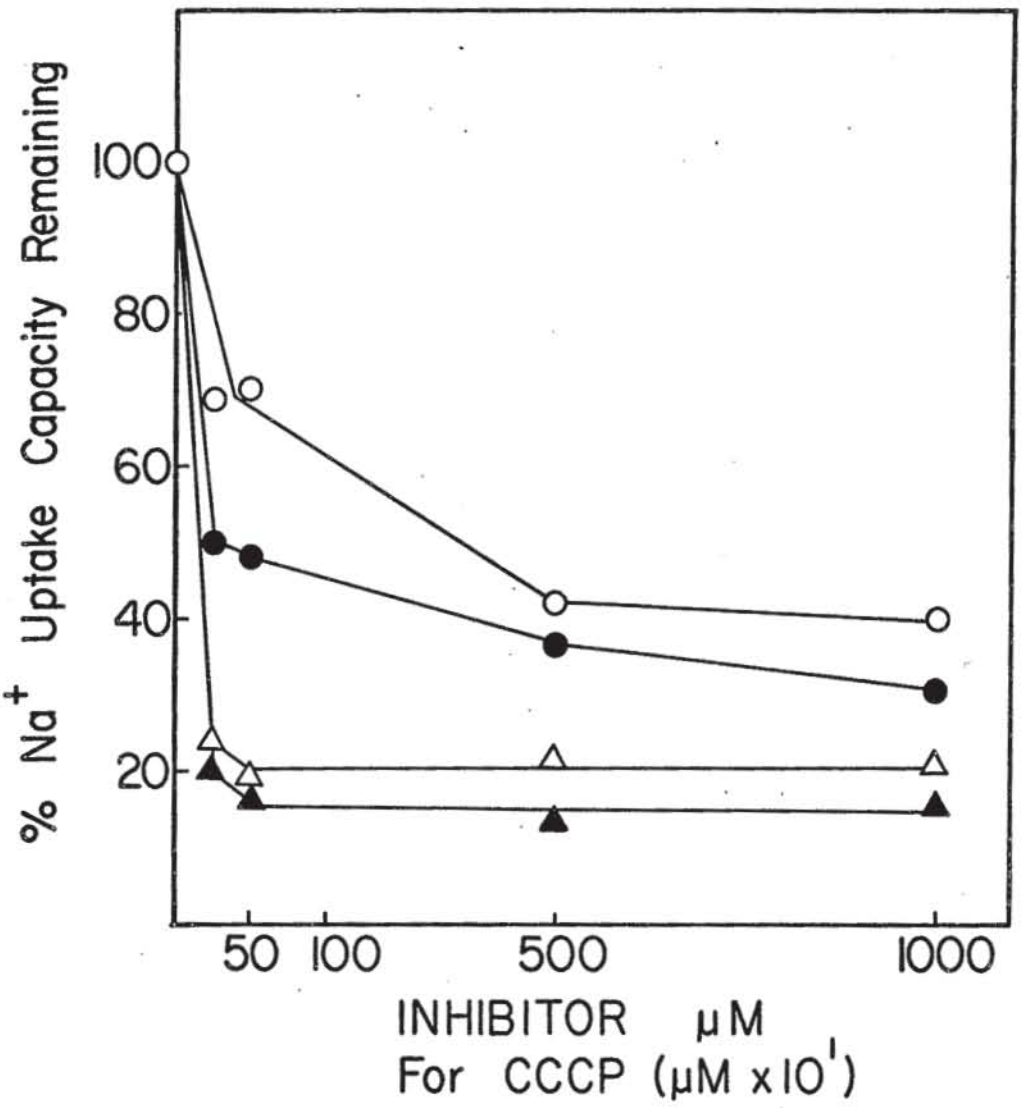
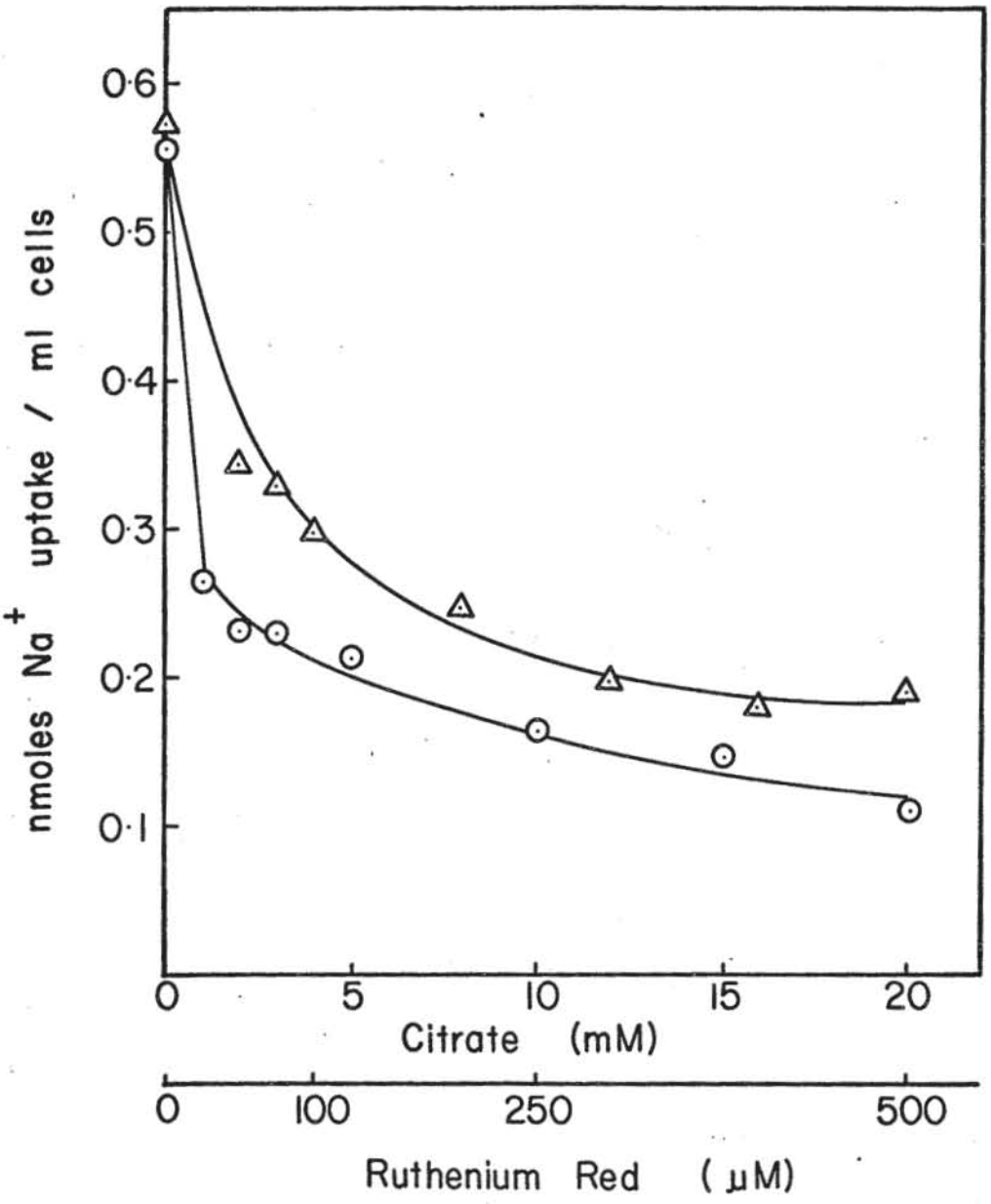

% Na+ Uptake Capacity Remaining
100
80
60
40
20
50
100
500
1000
INHIBITOR μM
For CCCP (μM x 10')

Figure 90: Inhibition of Na^+ uptake by citrate (⊙) and ruthenium red (△).


0·6
0·5
0·4
0·3
0·2
0·1
nmoles Na+ uptake / ml cells
0
5
10
15
20
Citrate (mM)
0
100
250
500
Ruthenium Red (μM)

DISCUSSION

Amino Acid Transport and Protein Synthesis Linkage

In Achlya, protein synthesis and amino acid uptake were found to be separable processes. Cycloheximide, an inhibitor of protein and RNA synthesis in Achlya, had no effect on the initial amino acid uptake rate. When amino acid uptake in the presence of cycloheximide was followed over a period of time under growth conditions, the drug eventually became inhibitory. Of interest was the finding that in growing cells, cycloheximide slightly enhanced uptake prior to cumulative inhibitory effect, reminiscent of results obtained by Hunter and Segel (1973 a,b) for enhanced calcium uptake in growing cells by cycloheximide.

Additional proof that amino acid uptake and protein synthesis are separate processes was shown by the data of Fig. 74. Amino acids entered a cellular pool before incorporation into proteins. The amino acid pool size increased in the presence of cycloheximide, but synthesis of protein was blocked.

Inhibition of Amino Acid Transport

Results obtained for calcium effect on protein synthesis showed that the major part of calcium antagonism of citrate action may yet

be at another level. One logical site of action is at the membrane and this led to a study of amino acid transport. Amino acids are transported in germinated sporangiospores by specific energy-dependent carrier-mediated processes. Inhibitors that disrupt the generation of metabolic energy interfere with amino acid transport and cause an efflux of amino acids.

Citrate: Citrate was found to inhibit energy-related amino acid transport, but there was no corresponding efflux of the solute. In addition, citrate had no adverse effect on reducing the size of the endogenous amino acid pool (Fig. 69). Upon further investigation, citrate effect on amino acid transport was found to be indirect. Citrate, being a chelator of calcium, inhibited calcium transport as a competitive inhibitor of intact and osmotically shocked cells and as an inhibitor of calcium binding by the glycopeptide. In agreement with these results, citrate inhibition of amino acid transport was shown to be non-competitive, reducing the maximum velocity while not interferring with the K_m of the carrier systems. Furthermore, all amino acids were affected in an analogous manner by citrate; if citrate was binding individual transport systems, one would expect that citrate would differentially affect amino acid transport.

At low concentrations (50 to 100 μM) of citrate amino acid uptake was enhanced and calcium reduced this activation. For this situation, citrate may form a irreversible complex with Ca^{++} on the

cell surface desensitizing the calcium transport system to the regulatory controls of energy-linked solute transport producing maximum uptake of such solutes.

Ruthenium red: The calcium transport system in Achlya, as calcium transport processes in other organisms, was specifically inhibited by ruthenium red. Ruthenium red competitively inhibited the uptake of calcium in whole and osmotically shocked cells, and, in addition, antagonized glycopeptide calcium binding. The inhibition results observed for amino acid uptake by ruthenium red, confirmed previous studies using citrate. Ruthenium noncompetitively inhibited amino acid transport reducing both the binding affinity and maximum velocity of amino acid transport. Accordingly, citrate and ruthenium red seem to inhibit amino acid transport by directly blocking calcium transport.

Calcium Enhancement of Amino Acid Transport

The inhibition of amino acid transport by ruthenium red was specifically antagonized by calcium (Fig. 79), as shown by rate-concentration time-course kinetic analyses. Other divalent cations, Mg^{++} and Mn^{++} were unable to reverse ruthenium red inhibition of amino acid transport. Moreover, ruthenium red inhibited protein synthesis and this effect was also antagonized by calcium.

Under normal assay conditions, calcium had no apparent effect on amino acid uptake by germinated sporangiospores of Achlya. Furthermore, calcium effect on protein synthesis could only be demonstrated by its reversal of the inhibitory effects of chelators. However, when the cells were first incubated in the presence of citrate, Ba^{++}, or 2,4-DNP, calcium enhancement of amino acid transport was demonstrated, and a slight increase in protein synthesis was observed (Fig. 73). The antagonism of citrate and ruthenium red inhibition of amino acid transport by calcium confirms the importance of an operational calcium transport system.

Active and Facilitated Transport

As calcium appears to be involved in the energy-related transport of amino acids, calcium effects on another energy-related process, nucleoside transport were studied and results similar to those for amino acid transport were obtained. Citrate inhibited the transport of nucleosides causing an efflux, and inhibition was shown to be non-competitive (Fig. 82). The energy-related transport system of D-glucose was also shown to be non-competitively inhibited by citrate (Goh, 1975).

Na^{+} was accumulated by a concentrative process in Achlya as determined by kinetic analysis, pH studies, and inhibition by metabolic energy antagonists. Both ruthenium red and citrate inhibited Na^{+} transport, and Na^{+} transport was negligible in osmotically shocked cells.

The calcium transport inhibitor, citrate, inhibited only energy-dependent metabolite transport as shown by its negligible effect on the uptake of nucleobases that are transported by a carrier-mediated process but a direct energy source is not involved. Surely, it appears that the calcium transport process is linked to active accumulation of metabolites.

Confirmation of these observations came after a study of metabolite transport in osmotically shocked cells. Osmotically shocked cells accumulated calcium but with a decreased maximum velocity. However, transport of all accumulated metabolites that are concentrated was abolished, only metabolites normally transported by facilitated diffusion could be taken up to some degree by osmotically shocked cells. These results in conjunction with earlier studies of the calcium binding glycopeptide released by osmotic shock treatment support the conclusion that an intact calcium transport system and/or glycopeptide-associated calcium are required for optimal operation of energy-dependent transport processes. Glycopeptide-associated calcium may act by maintaining the integrity of the membrane resulting in correct conformation of the active transport processes for optimal transport and may maintain an energetic membrane potential gradient reminiscent of potential gradient generated by an active extrusion of H^+ ions and secondary uptake of K^+ that was Mg^{++}- Ca^{++} dependent (Fisher & Albersheim, 1973). Evidence that supports calcium association with H^+ ion extrusion in Achlya came from the observation that acid conditions induced calcium efflux. The effect that

calcium has on the electropotential gradient was not carried out, as it was beyond the scope of the present study.

Calcium Interaction with Purines

As previously discussed, N^6-(substituted)adenine compounds (cytokinins) are known to bind to the calcium binding glycopeptide causing the release of calcium, and probably the subsequent uptake of calcium by the intrinsic calcium transport component. In addition, cytokinins inhibited the energy-related transport of cell metabolites. Cytokinin inhibition, as that of citrate and ruthenium red, appear to be mediated via the calcium transport system. Cytokinin may either inhibit concentrative metabolite transport by inciting the release of calcium from the glycopeptide which has been accorded as necessary for active metabolite transport in Achlya, or else, may directly affect the membrane or components thereof.

CONCLUSIONS

CONCLUSIONS

Studies presented in this thesis have demonstrated that calcium is transported in Achlya by a complex carrier-mediated process that is regulated. Calcium, itself, is involved in the regulation of all facets of cellular metabolism that involve energy generation. The most obvious is regulation at the physiological level for in the absence of calcium there is no growth, and successive removal of calcium throughout the asexual ontogenic cycle immediately arrested growth.

Calcium regulation at the macromolecular level of synthesis is apparent from specific calcium reversal of citrate inhibited protein, RNA and DNA syntheses. Results showed that calcium may be associated with the regulation of protein synthesis, but a direct role, if any, of calcium in DNA and RNA synthesis remains unexplored.

The results presented in this thesis showed that the major role of calcium is to facilitate energy-linked solute transport at the plasma membrane. Only when the calcium transport system is intact and operative can energy-linked transport of amino acids, nucleosides, sugars and ions proceed. As calcium was shown to be linked to only energetically-linked solute transport not facilitated uptake processes, calcium must in some manner be associated with the solute transport either by maintaining the proper conformation of the individual transport carriers or by being associated with or an integral part of an electropotential proton gradient that controls

energy-linked transport of solutes.

In some form of coordination with the calcium transport system is a membrane-located calcium-binding glycopeptide. Of significance was the large number of calcium atoms bound to the glycopeptide (14 atoms per mole). Removal of this glycopeptide led to a loss of the ability of the membrane to actively accumulate solutes. When considering the importance and absolute necessity of calcium throughout the entire asexual life cycle the most probable conclusion is that surface bound calcium is necessary for the proper operation of an active proton pump that maintains an electropotential gradient across the membrane essential for all energy-linked solute transport. Calcium merely maintains a proper membrane configuration that allows proton flux to occur. This conclusion is based on indirect evidence and a further study of the speculative proton pump would be necessary to substantiate this latter conclusion.

The singular importance of calcium for energy-linked accumulation of solutes was strengthened by the discovery that the calcium transport system was affected in a complex manner by a variety of physiologically active compounds, cations, cytokinins and auxin. As to the exact involvement of these two plant hormones or some yet to be discovered regulatory compound specific for water moulds, no substantiated conclusion can be drawn. However, the potential significance of these compounds in this organism was shown by a study of the action of cytokinins and auxin on the movement of calcium to and from the cell surface glycopeptide. The resulting effects were found to be identical.

Of interest was the chemical and physical nature of this calcium binding glycopeptide. After initial chemical and physical analysis of the composition of this glycopeptide, it seemed impossible that such complex regulation could occur on such a low molecular weight molecule. But upon further study, in conjunction with physiological results, this was resolved. All modulators could bind to that glycopeptide, and calcium in large quantities (14 moles of Ca^{++}/mole glycopeptide). In addition, all control mechanisms need not be directed at the glycopeptide per se, as it seems that calcium bound by the glycopeptide indirectly maintains an electropotential gradient across the surface membrane. Of importance is the finding that the glycopeptide could bind calcium reversibly. Pertinent to this is the finding of Stevenson (1974) that cytokinins bind to osmotically shocked cells that have lost the calcium binding glycopeptide. Cytokinins may therefore interact also at the membrane level by destroying the electropotential gradient.

The ability of the glycopeptide to bind large amounts of calcium may also be useful as a storage site of calcium necessary for cell growth and differentiation. The fresh water habitat of this oomycete makes it such that calcium cannot always be readily available. The glycopeptide molecule would serve as an efficient means of collecting and storing calcium from the surrounding medium for future use. Another similar function of the glycopeptide may be the binding of extruded calcium thereby preventing its loss to the surrounding medium. This would lead to a recycling process. The high affinity binding exhibited by the glycopeptide for calcium

makes this molecule a likely candidate for such "scavenger" and storage functions.

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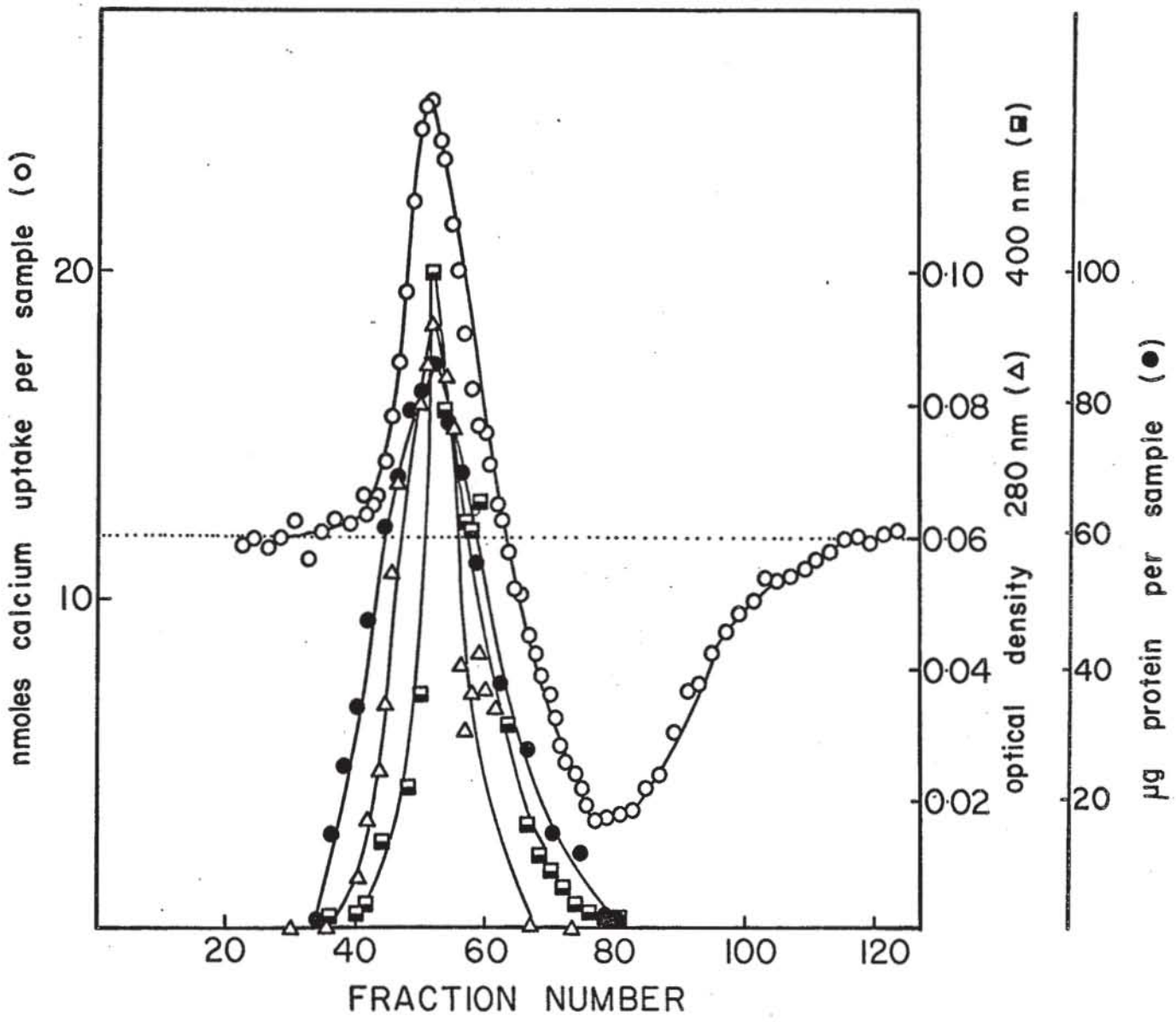

nmoles calcium uptake per sample (o)
optical density 280 nm (Δ) 400 nm (■)
μg protein per sample (●)
FRACTION NUMBER
20
10
0·10
0·08
0·06
0·04
0·02
100
80
60
40
20
20
40
60
80
100
120

Figure 43: Determination of the molecular weight of Achlya calcium binding glycopeptide.

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