This is to certify that the

thesis entitled

Changes of Surface Carbohydrates and Membrane Potential during the Encystment Process of *Blastocladiella Emersonii* Zoospores

presented by

Chaoying Jack Jen

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biophysics

Major professor

Date 23 Sept. 1980

CHANGES OF SURFACE CARBOHYDRATES AND MEMBRANE POTENTIAL DURING THE ENCYSTMENT PROCESS OF *BLASTOCLADIELLA EMERSONII* ZOOSPORES

By

CHAUYING JACK JEN

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biophysics

1980

ABSTRACT

CHANGES OF SURFACE CARBOHYDRATES AND MEMBRANE POTENTIAL DURING THE ENCYSTMENT PROCESS OF BLASTOCLADIELLA EMERSONII ZOOSPORES

by

Chauying Jack Jen

The unicellular, non-filamentous, lower aquatic fungus, *Blastocladiella emersonii* was selected as an excellent model system for developmental studies. Changes of surface carbohydrates accompanied with the encystment process were studied by using concanavalin A as a probe. The possible role of membrane potential in regulating the developmental process was investigated.

Binding of concanavalin A to the sell surface of developing *Blastocladiella emersonii* zoospores was explored by fluorescence microscopy, electron microscopy and radioactive labelling. After labelling with fluorescein isothiocyanate-conjugated concanavalin A, the fluorescence intensity of individual non-induced zoospores varies greatly. On the other hand, similarly labelled zoospores, induced with K^+ ions for synchronous development, revealed a more even dis-

tribution of fluorescence intensity. The zoospore surface contained 4.3×10^7 concanavalin A binding sites per cell which were randomly distributed and closely attached to the surface. The affinity constants range from 7.5×10^7 M^{-1} to 3.5×10^5 M^{-1}, while the Scatchard plot was typical of heterogeneous binding. Further developed cells, round cells and germlings contained 3.0×10^7 to 2.1×10^7 concanavalin A binding sites per cell which were mostly loosely associated patches protruding into the extracellular region. The affinity constants decreased appreciably compared with those measured at the zoospore stage. Specific concanavalin A binding characteristics appeared to correlate with the respective developmental stage of the zoospore.

Concanavalin A induced lysis of zoospores of the aquatic fungus *Blastocladiella emersonii*. The rapid lysis could be completely blocked by α-methyl-mannoside. At lower temperatures the cells were more tolerant towards lysis compared with cells at room temperature. Dimeric concanavalin A derivatives and *Lens culinaris* hemagglutinin A were less effective, whilst other lectins tested did not induce lysis at all. The cell lysis might be caused by the cluster formation of adjacent concanavalin A receptors.

Experiments have been performed to test the possible role of the membrane potential in the ionic induction of

encystment in *Blastocladiella emersonii* zoospores. A fluorescent, voltage-sensitive dye, 3,3'-dipropylthiadicarbocyanine iodide, was used to monitor changes in the membrane potential. Only K^+ ions, the standard developing trigger--ing agent, rapidly depolarized the membrane potential. Appli cation of an optimal K^+ ion concentration (50 mM) to a cell population depolarized the membrane potential most rapidly and induced the highest percentage of cells to encyst per unit time. The depolarization was temperature dependent and was not affected by extracellular pH changes from 5 to 9. Quinine practically prevented depolarization of the membrane potential by K^+ ions; however, the membrane potential was depolarized by subsequent valinomycin application. The K^+ induced response of the membrane potential was apparently independent upon the zoospore's age, but varied dramatically as the zoospore encysted. A strong correlation was established between the K^+ ion-induced depolarization of the membrane potential and the developmental triggering by K^+ ions. Specific K^+ carriers were proposed to be embedded in the lipid matrix, which exists in the gel + liquid-crystalline mixed lipid state.

DEDICATION

This dissertation is dedicated to my wife, daughter, and parents. Their encouragement has made this work possible.

ACKNOWLEDGEMENTS

I would like to express deepest appreciation to my major advisor, Dr. Alfred Haug, for his guidance, encouragement, stimulating discussions, and long-lasting patience through this dissertation.

Special thanks to Dr. John L. Wang for helping me during the time of great need. Thanks also to Dr. Estelle J. McGroarty and Dr. Derek T. A. Lamport for helpful discussions and endorsements. My friends in this laboratory, especially Richard Allison, provided assistance throughout the duration of this work and deserves recognition.

My sincere gratitude to my wife, Ya-Chu, for her love, faith and support during my graduate years.

The financial support from United States Department of Energy (contract number DE-AC02-76ER01338) is acknowledged.

TABLE OF CONTENTS

IV. POTASSIUM-INDUCED DEPOARIZATION OF THE TRANS-MEMBRANE POTENTIAL IN *BLASTOCLADIELLA EMERSONII* ZOOSPORES PRECEDES ENCYSTMENT

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION

An outstanding characteristic of cells is that the relationship between their internal activities and the external environment is regulated by a barrier. This barrier may exist as the plasma membrane alone or be part of a more complex cell-surface structure. Crucial activities of the cell membrane are documented in terms of transport function, receptor function, and mechanical function.

Studies of receptor function and mechanical function have led to the realization not only that components of the cell surface are mobile in the plane of the membrane but that this lateral mobility is subject to regulation (Singer and Nicolson, 1972; Nicolson, 1979). Such regulated lateral mobility of membrane components has been the basis for many hypotheses on the molecular mechanisms mediating cell recognition, cell growth control, and cell transformation (Edelman, 1976; Nicolson, 1976a, 1976b).

Investigations of transport function have led to the understanding that living things can generate electric currents and potentials by regulated, asymmetric movements of ions across membranes. Nerve fibers carry information in sequences of action potentials which consist of brief changes in membrane permeability towards Na^+ and K^+ ions (Aidley, 1971). Respiration and photo synthesis associated electric potentials which are present in mitochondria, chloroplasts, and various bacteria are believed to arise primarily from the movement of H^+ according to the chemiosmotic hypothesis

(Mitchell, 1969). Other important cellular events including energy transformation, nutrient uptake, and sensory transduction also depend on the magnitude and time course of the membrane potential.

Based on two reasons, it has become increasingly evident that the cell surface might play an important role in the regulation of development and differentiation. First, the surface functions in the transmission of information from the environment to the cell body, since it is the surface which senses the environment directly. The information can be processed as a molecular, mechanical, or electric signal. Cell surface adhesions and membrane potential changes may be associated with these signals which subsequently effect intracellular activities involved in processes of development and differentiation. Second, the surface characteristics vary as to fulfill the specific requirements of a genetically determined developmental state.

The development of higher, multicellular plants and animals involves a large number of cells and in many cases the irreversible differentiation of specialized cell types. As a result, development in multicellular eukaryotes must depend on mechanisms that extend beyond the usual notions of sequential gene activation. For example, development of an embryo requires that cells know where they are and where they should be. There must be mechanism that regulate this social behavior of cells. In this case the cell membrane is likely

to serve both as the donor and as the acceptor of developmental signals.

Cell interactions and functional specializations in multicellular eukaryotes are unavoidably associated with very complex regulatory mechanism. On the other hand, single-cell eukaryotic microorganisms are smaller and less complex; they provide experimental systems whose regulatory processes theoretically are more easily analyzed. The primary virtues of these simple organisms are ease of culture, rapid growth at high cell density, short and manipulatable life cycles, and ease of producing synchronous differentiation in large cell populations. The unicellular, non-filamentous, lower aquatic fungus, *Blastocladiella emersonii,* fulfills these criteria and serves as an excellent model system for development studies.

The growing *B. emersonii* plant follows a well-defined developmental pathway which is divided into five stages: the zoospore, cyst (round cell) and germling, exponential growth, zoosporangium formation, and zoospore differentiation. Figure 1.1 illustrates the life cycle and the respective time course of individual stages, under optimal growth conditions (Lovett, 1975). The subject of this dissertation deals with cell surface changes during the cell's development from the zoospores stage to the cyst (round cell) and germling stage.

This particular system was selected for three reasons. I) The developmental process can be manipulated by varying environmental conditions. The zoospore will encyst and begin

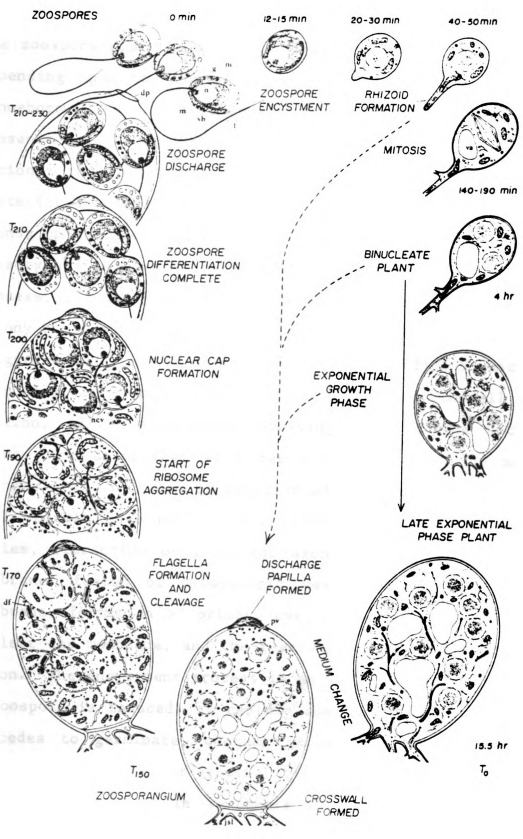


Fig. 1.1 The life cycle of *Blastocladiella emersonii*.

germination within a few minutes following its release from the zoosporangium, or it will remain motile for many hours depending upon the compo- sition of the medium (Soll and Sonneborn, 1969, 1972; Truesdell and Cantino, 1971). In the presence of 1 mM $CaCl_2$ and the absence of monovalent cations, the zoospore can be maintained in a viable motile state for extended periods. When exposed to KCl, it rapidly rounds up and encysts. Although other factors, such as cell density and incubation temperature, influence the encystment process, the zoospore exhibits excellent developmental synchrony under optimal conditions; nearly 100% of the zoospores triggered will encyst within 15 minutes. II) Pronounced morphological changes (Soll et al., 1969; Truesdell and Cantino, 1971), apparently involving cell surface alterations, are associated with rapid encystment. The major changes are: increase of cellular adhesiveness, decrease of cell volume, loss of motility, loss of cytoplasmic microtubles, retraction of flagellar axoneme, folding of plasma membrane, fusion of cytoplasmic vesicles with the plasma membrane, formation of primary cyst wall, disruption of the nuclear cap membrane, and the rearrangement of the mitochondrion. The encystment process is an all-or-none event; once a zoospore is induced, it rapidly completes the process and precedes to germinate, whether or not the conditions are suitable for subsequent growth. III) There is no detectable RNA or protein synthesis for cells at either the zoospore stage or the round cell stage. This indicates that

all of the gross changes associated with the encystment process occur in the abscence of a functioning biosynthetic machinery. Therefore, many of the changes are likely to be the result of local membrane alterations requiring rearrange ments of preexisting components. Thus, the system provides a unique opportunity for investigating 'biophysical' mechanisms of the environmental trigger without interference of subsequent 'biochemical' events.

In recent years surface carbohydrates have been implemented to play crucial roles in cellular recognition processes such as antigen-antibody interactions, pathogen-host interactions, and cell-cell/cell-substrate interactions. While cells undergo differ entiation, cell cycle propagation and malignant transformation, surface carbohydrates also exhibit specific changes involving their type, amount, distribution, and mobility. Lectins, the carbohydrate-binding proteins which are mostly isolated from plants, are excellent surface probes because they attach tightly and reversibly to specific surface carbohydrates without entering the cells (Nicolson, 1974; Sharon and Lis, 1975). Under suitable conditions, the lectin-carbohydrate interactions can be abolished by the specific saccharide(s) which bind(s) the respective lectin. The specificity of inhibition is usually taken as evidence for the presence of surface saccharide receptors whose structure is similar to that of the inhibitory sugar(s).

Considering the developmental significance of the plasma membrane, in particular that of the surface carbohydrates, one aspect of my research was concerned with the interactions of concanavalin A, Con A, with cell surface receptors of *B. emersonii* zoospores during encystment. The binding specificity of Con A, the most widely used lectin, is directed towards a variety of oligosaccharides containing α-D-Man and α-D-Glc residues. It is one of the few lectins that contains no covalently bound carbohydrate. It has been shown that Con A has 1 mole each of Ca^{2+} and Mn^{2+} per mole of protomer. This lectin is composed of identical subunits (protoomers) arragned as tetramers in its native state. A model of Con A showing metal ions and saccharide-binding sites is shown in Figure 1.2 (Becker et al., 1976). Native Con A and its chemical derivatives with a reduced number of subunits (Gunther et al., 1973) can be prepared for studying the mobility of the surface receptors. Quantitative measurements regarding Con A-receptor binding association constants and the number of binding sites per cell can be performed with radioactively labeled Con A. Fluorescent and electron microscopic methods, using FITC-Con A and ferritin conjugated Con A, allow for topographical localization of Con A receoptors on the cell surface.

In this dissertation I also report about efforts to elucidate the mechanism of the developmental triggering process. There exists abundant information concerning the biosynthesis patterns during the entire life cycle of *B. emersonii* (Lovett

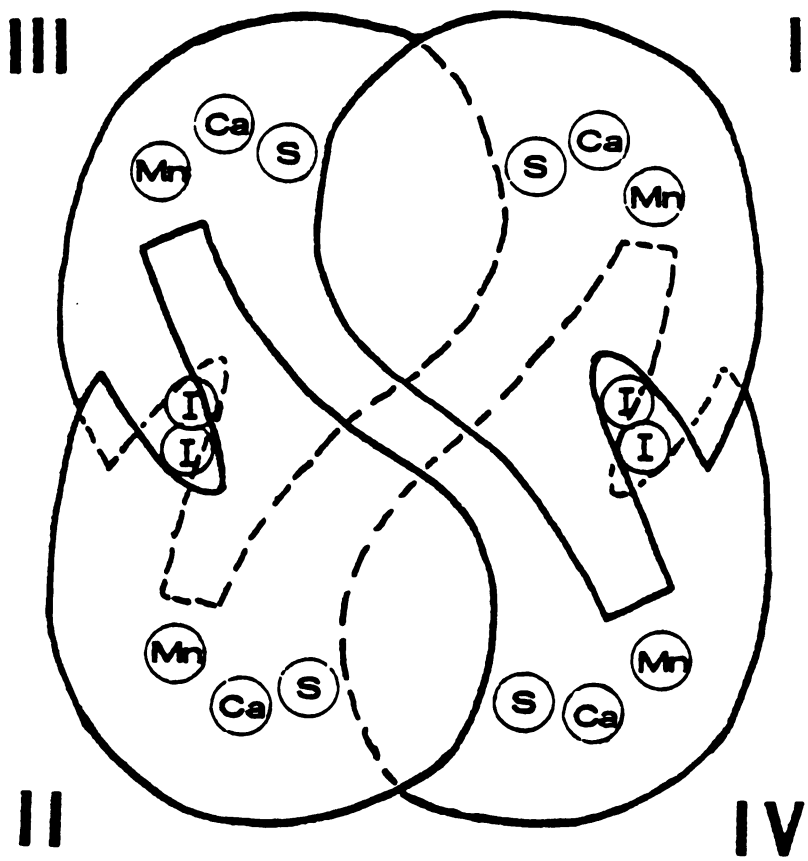
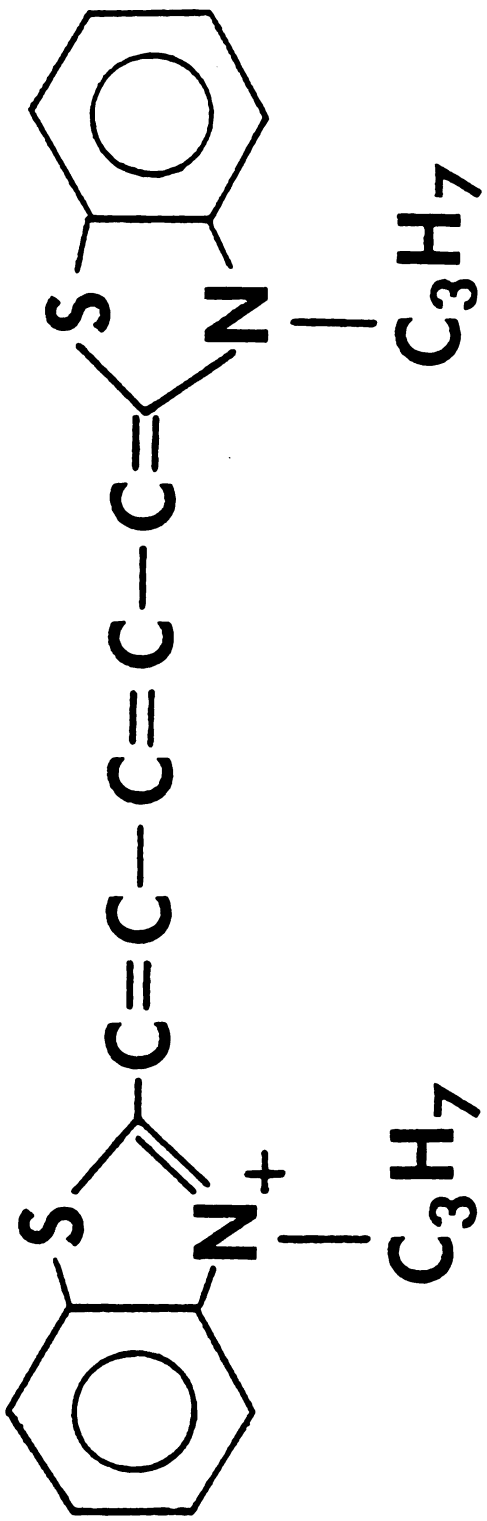


Fig. 1.2. Schematic diagram of the Con A tetramer. Binding sites for Ca^{2+}, Mn^{2+}, β-iodophenylglucose, and specific saccharides are indicated by Ca, Mn, I, and S, respetively.

1975). However, the existing information does not explain how zoospores are triggered to encyst and germinate when exposed to KCl. First, the data points collected by other investigators were measured no earlier than five to ten minutes following KCl application. Second, there is no significant change of metabolite levels within the first 10 to 15 minutes after induction. However, as previously mentioned, dramatic changes of cell morphology occur within a few minutes prior to the *de novo* synthesis of major macromolecules. Consequently, clues to the triggering mechanism are likely to be obtained if one could have access to the early events related to rapid biophysical alterations of the zoospore plasma membrane subsequent to the application of KCl.

Because the effect of electrical activity of cell membranes on growth has been recognized (Lund et al., 1947; Nuccitelli and Jaffe, 1976; Jaffee, 1976), changes in the membrane potential may constitute a conceivable trigger. The use of fluorescent, voltage-sensitive probes which respond optically to transmembrane potential changes provides a non-destructive method for the continuous monitoring of the electrical activity (Cohen and Salzberg, 1978). Therfore, a carbocyanine dye, 3,3'-dipropylthiadicarbocyanine was selected for investigating the early changes of the membrane potential when *B. emersonii* zoospores were triggered to develop. The structure of this dye is shown in Figure 1.3 (Simes et al., 1974). This positively charged dye is taken



3,3'-DIPROPYLTHIODICARBOCYANINE

DiS-C_3-(5)

Fig. 1.3 The fluorescent dye 3,3'-dipropylthiadicarbocyanine.

up by the cells upon hyperpolarization and released upon depolarization. The cell-associated dye is significantly less fluorescent than the dye in the medium and the degree of quenching depends on the amount of dye associated with the cells. This quenching probably results from the formation of dye aggregates with reduced fluorescence. In conjunction with a fluorimeter designed by the author, this dye enables the continuous measurement of alterations of membrane potential.

On the other hand, the electrical potential difference between aqueous phases separated by a natural membrane has been measured directly by suitably placed microelectrodes. However, the general use of electrodes with micro-organisms is restricted because of difficulty in implanting electrodes into the minute cell body and unavoidable cellular damage. Determination of the distribution of radioactively labeled, membrane permeant ions enables the quantitative evaluation of trans-membrane potential. This method does not allow continuous and rapid measurement which is necessary to access early events in developmental triggering.

The results of my experiments indicate that I) The Con A receptors, glucosyl/mannosyl residues, vary during the encystment process, II) mobile surface carbohydrates, presumably glycoproteins or glycolipids, are probably involved in maintaining the osmotic balance of zoospores, and III) the K^+ ions induce the depolarization of membrane potential, which precedes encystment.

CHAPTER TWO

CHANGES IN SURFACE PROPERTIES OF DEVELOPING ZOOSPORES OF *BLASTOCLADIELLA EMERSONII*: BINDING OF CONCANAVALIN A

Journal of General Microbiology (1979), **115**, 145–151. *Printed in Great Britain*

Changes in Surface Properties of Developing Zoospores of *Blastocladiella emersonii*: Binding of Concanavalin A

By C. J. JEN AND A. HAUG

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824, *U.S.A.*

(*Received* 1 *February* 1979; *revised* 15 *May* 1979)

Binding of concanavalin A to the cell surface of developing *Blastocladiella emersonii* zoospores was explored by fluorescence microscopy, electron microscopy and radioactive labelling. After labelling with fluorescein isothiocyanate-conjugated concanavalin A, the fluorescence intensity of individual non-induced zoospores varied greatly. On the other hand, similarly labelled zoospores, induced with K^+ ions for synchronous development, revealed a more even distribution of fluorescence intensity. The zoospore surface contained $4{\cdot}3 \times 10^7$ concanavalin A binding sites per cell which were randomly distributed and closely attached to the surface. The affinity constants ranged from $7{\cdot}5 \times 10^7$ M^{-1} to $3{\cdot}5 \times 10^6$ M^{-1}, while the Scatchard plot was typical of heterogeneous binding. Further developed cells, round cells and germlings contained $3{\cdot}0 \times 10^7$ to $2{\cdot}1 \times 10^7$ concanavalin A binding sites per cell which were mostly loosely associated patches protruding into the extracellular region. The affinity constants decreased appreciably compared with those measured at the zoospore stage. Specific concanavalin A binding characteristics appeared to correlate with the respective developmental stage of the zoospore.

INTRODUCTION

The life cycle of *Blastocladiella emersonii*, an aquatic unicellular fungus, constitutes a suitable eukaryotic model to investigate developmental processes (Lovett, 1975). The non-growing, motile zoospore of *B. emersonii* exhibits pronounced morphological alterations, in particular at the cell surface, while undergoing encystment and germination. The zoospore loses its motility and becomes adhesive, changes extensively in fine structure, retracts the flagellum into the main body and gradually deposits material on the cell surface for primary cell wall formation. Subsequently, a germ tube emerges from the cell and this later turns into a branched rhizoidal system. The entire process takes less than 30 min under favourable conditions and precedes *de novo* protein and RNA synthesis (Soll & Sonneborn, 1971).

Lectins are valuable tools for probing surface properties of various cell types (Gremo *et al.*, 1978; Sharon & Lis, 1975; Weeks, 1975). To obtain some insight into developmental alterations on the zoospore surface, concanavalin A (Con A) was employed. Recently, we have observed that Con A binding induced lysis of *B. emersonii* zoospores but not of round cells (Jen & Haug, 1979). In this paper, we report zoospore surface changes as monitored by Con A binding parameters. We used [^{3}H]Con A, fluorescein isothiocyanate-conjugated Con A (FITC-Con A) and ferritin-Con A.

0022-1287/79/0000 8758 $02.00

METHODS

Reagents. Con A and α-methyl-D-mannoside were purchased from Sigma, FITC-Con A and ferritin-Con A were from Miles-Yeda, [^{3}H]acetic anhydride was from Amersham/Searle, pronase was from Calbiochem, and 2,5-diphenyloxazole (PPO) and 1,4-bis-[2-(5-phenoxazolyl)]benzene (POPOP) from Research Products International Corp. (Elk Grove Village, Ill., U.S.A.).

Organism and culture conditions. The aquatic fungus *B. emersonii* was grown in standard Petri dishes at 22 °C on Difco PYG agar. Zoospores were harvested approximately 20 h after inoculation by flushing individual Petri dishes with 2 ml sporulation solution [5 mM-morpholinopropanesulphonic acid (MOPS), 1 mM-$CaCl_2$, pH 6·8] and collecting the zoospore suspension as the filtrate through a Whatman no. 1 filter. To induce synchronous zoospore germination, the procedure described by Soll *et al.* (1969) was followed. The zoospore suspension was incubated for 30 min at 27 °C, then mixed with 5 vol. germination solution (5 mM-MOPS, 1 mM-$CaCl_2$, 10 mM-$MgCl_2$, 50 mM-KCl, pH 6·8) at 27 °C. The cells underwent transformation from zoospores to round cells, and finally to germlings. Samples were taken from the suspension during the developmental process and fixed immediately in 1·5% (v/v) glutaraldehyde at 22 °C for 2 h. The cell phenotypes at the different developmental stages could be easily distinguished by light microscopy. Cells were washed three times with 0·1 M-sodium phosphate buffer, pH 7·2, containing 0·1 M-NaCl.

Binding of [^{3}H]Con A to cells. [^{3}H]Con A [specific activity 1000 c.p.m. (μg protein)$^{-1}$] was prepared using [^{3}H]acetic anhydride (Gunther *et al.*, 1973). Cells (1×10^7) were incubated for 2 h with [^{3}H]Con A in 1·0 ml 0·1 M-sodium phosphate buffer, pH 7·2, containing 0·1 M-NaCl and 0·1% (w/v) bovine serum albumin. For control experiments, 0·1 M-α-methyl-D-mannoside was present in the reaction mixture. Cells were collected on a Whatman GF/C glass-fibre filter and washed five times with 5 ml phosphate buffer supplemented as above. After drying overnight, the filters with the cells were placed in 10 ml liquid scintillation solution (4 g PPO and 0·05 g POPOP per l toluene). Radioactivity was measured with a Packard Tricarb scintillation counter (model 3390).

Labelling of cells with FITC-Con A and quantitative fluorescence measurements. Cells (2×10^6) were incubated in 1 ml sodium phosphate buffer, pH 7·2, containing 0·1 M-mannitol, 0·1% (w/v) bovine serum albumin and 200 μg FITC-Con A. For controls, 0·1 M-α-methyl-D mannoside was substituted for mannitol. After incubation at 22 °C for about 20 min, the cells were washed with the above reaction mixtures, but without FITC-Con A. The cells carrying the fluorescence label were viewed under a Leitz Orthomat-W microscope with an automatic camera. Fluorescence intensity of individual cells was determined with a Leitz photometer attached to the microscope.

Ultrastructural localization of Con A receptor sites. Fixed cells were incubated with 1 mg ferritin-Con A ml^{-1} at 22 °C for 30 min, then washed with phosphate buffered saline and postfixed with 2% (w/v) OsO_4 in phosphate buffer. The samples were washed, dehydrated and finally embedded in Spurr's resin (Spurr, 1969). Cells incubated in the presence of 0·1 M-α-methyl-D-mannoside served as controls. Thin sections, without heavy metal staining, were viewed under a Siemens Elmiskop I electron microscope.

RESULTS

Number and affinity of [^{3}H]Con A binding sites at the surface of B. emersonii

Specific Con A binding sites are those from which the bound lectin can be removed with α-methyl-D-mannoside as the hapten. Specific binding was determined by subtracting the contribution of non-specific binding from the total binding. The Con A binding sites at the zoospore surface appeared to be mostly specific. At worst, in the case of germlings, non-specific binding could account for as much as 25% of the total binding at high lectin concentrations. To ascertain the number and affinity of Con A binding sites, cells were incubated with various concentrations of [^{3}H]Con A. Results are presented in a Scatchard plot (Fig. 1). Assuming only two binding systems (Rosenthal, 1967), high and low association constants and the total number of binding sites per cell were calculated (Table 1).

The non-induced zoospore surface area was estimated as 250 μm^2 from the known size of the cell body (Lovett, 1975), an ellipsoid (7×9 μm), and the cylindrical flagellum ($15 \times 0{\cdot}2$ μm), and so the site density was calculated to be $1{\cdot}7 \times 10^5$ receptors per μm^2.

During the developmental process, when zoospores round up and germinate, a gradual decrease of Con A binding was observed. High and low association constants decreased by factors of 6 and 1·5, respectively, comparing non-induced zoospores with those within 15 min

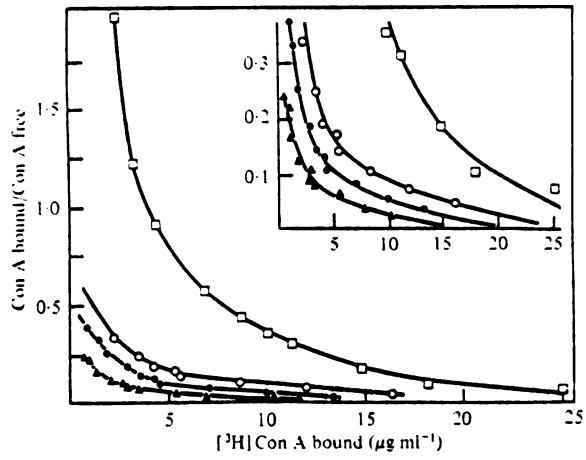


Fig. 1. Scatchard plot of specific concanavalin A binding to *B. emersonii* cells during encystment: □, non-induced zoospores; ○, 60% zoospores and 40% round cells, 15 min after induction; ●, 5% zoospores, 70% round cells and 25% germlings, 30 min after induction; ▲, 2% zoospores, 14% round cells and 84% germlings, 45 min after induction. Cells were derived from the same batch. Except for non-induced cells (□), all other cells were induced to germinate. Inset: lower part of the figure drawn to a different scale.

Table 1. *Association constants and total number of concanavalin A binding sites per B. emersonii cell*

A molecular weight of 52000 was assumed for acetylated [³H]Con A (Gunther *et al.*, 1973). The values listed were calculated from data presented in Fig. 1.

	High affinity association constant (M^{-1})	Low affinity association constant (M^{-1})	Total number of Con A binding sites per cell
Non-induced zoospores	$7{\cdot}5\times10^{7}$	$3{\cdot}5\times10^{5}$	$4{\cdot}3\times10^{7}$
Cells, 15 min after induction	$1{\cdot}2\times10^{7}$	$2{\cdot}3\times10^{5}$	$3{\cdot}0\times10^{7}$
Cells, 30 min after induction	$1{\cdot}0\times10^{7}$	$2{\cdot}0\times10^{5}$	$2{\cdot}5\times10^{7}$
Cells, 45 min after induction	$8{\cdot}0\times10^{6}$	$1{\cdot}6\times10^{5}$	$2{\cdot}1\times10^{7}$

after induction (Table 1). The number of binding sites decreased concomitantly. At the completion of encystment, i.e. about 45 min after induction, there were $2{\cdot}1\times10^{7}$ specific binding sites per cell.

Cells labelled with FITC-Con A

Labelled zoospores exhibited intense fluorescence, including the flagellum sheath (Fig. 2*a*). The fluorescence intensity seemed to be more pronounced around the nuclear cap and the 'side body' complex (for a detailed structural description, see Lovett, 1975). Following the mixing of a zoospore suspension with germination solution, the resulting elongated cells showed somewhat weaker fluorescence. Labelled round cells also fluoresced and most of them were covered with many fluorescent spots (Fig. 2*b*). The number and size of these spots varied from cell to cell. Later, as cells became adhesive and tended to form clumps, the overall fluorescence intensity decreased.

In general, fixed cells fluoresced within a few minutes after application of FITC-Con A.

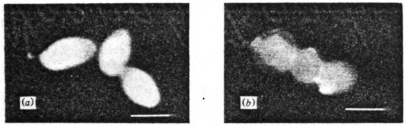


Fig. 2. Fluorescence micrographs of *B. emersonii* cells treated with fluorescein isothiocyanate-conjugated concanavalin A: (*a*) non-induced zoospores; (*b*) round cells fixed 15 min after induction. Bar markers represent 5 μm.

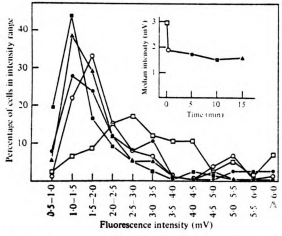


Fig. 3. Distribution of fluorescence intensity of *B. emersonii* zoospores during encystment. A total of 75 individual zoospores were randomly scored for each curve. The fluorescence intensity of individual cells was corrected for non-specific background fluorescence, determined as the average intensity from 25 control cells, in the presence of α-methyl-D-mannoside and fixed at the same time as the individual cells being studied. The ordinate shows the percentage of the individually scored cells which fell within the intensity ranges. □, Cells before induction; ○, cells fixed 15 s after induction; ●, cells fixed 5 min after induction; ■, cells fixed 10 min after induction; ▲, cells fixed 15 min after induction. Inset: the median fluorescence intensity of the 75 individually scored cells before (□) and at the respective times after induction.

Extensive washing of cells with buffer did not affect the fluorescence intensity appreciably. The fluorescence intensity value from fixed, FITC-Con A-labelled cells, in the presence of α-methyl-D-mannoside, was considered to be non-specific background fluorescence.

The fluorescence intensity of non-induced zoospores varied over a wide range (Fig. 3). Within the first 15 min after induction, elongated cells and those ready to encyst exhibited a fluorescence intensity which was weaker than that of non-induced cells, yet remained virtually constant; the intensity values of the majority of cells ranged from 1 to about 2 mV. Both round cells and germlings formed aggregates, and quantitative fluorescence measurements of single cells became impossible.

Ultrastructural localization of Con A receptor sites

Non-induced zoospores were densely labelled with ferritin-Con A which was distributed randomly over the cell surface and closely attached to the plasma membrane (Fig. 4*a*), including the sheath of the flagellum. The label density varied somewhat from cell to cell. In control samples, after application of α-methyl-D-mannoside, only occasional ferritin

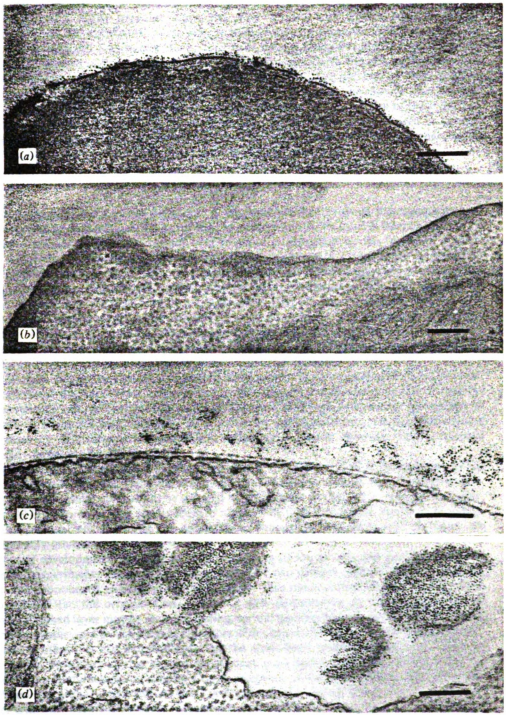


Fig. 4. Ultrastructural localization of concanavalin A binding sites of *B. emersonii cells*. (a) Non-induced zoospores; note the uniform distribution of ferritin molecules closely attached to the outer surface of the plasma membrane. (*b*) Non-induced zoospores, in the presence of 0·1 M-α-methyl-D-mannoside; only occasional ferritin molecules are attached to the cell surface. (*c*) Round cells; cells were induced and allowed to develop for 20 min before fixation and labelling; note ferritin molecules protruding into the extracellular region and forming patches. (*d*) Zoospores were labelled with ferritin-conjugated concanavalin A; γ-particles were released from zoospores following concanavalin A application and the sample was fixed after labelling; note the densely labelled γ-particles especially along the inner kidney-shaped region. Bar markers represent 0·2 μm.

molecules were attached to the membrane surface (Fig. 4*b*). Immediately after induction, zoospores had a rather irregular shape and were labelled with ferritin particles closely attached to the cell membrane. However, the particle density appeared to diminish appreciably, and it remained consistently low on later oval-shaped zoospores. But in oval zoospores fairly large patches of ferritin-Con A were loosely associated with the cell surface and protruded into the extracellular space. Later, in round cells, the number of these patches increased to cover the peripheral extracellular region: however, the closely attached cell surface layer of ferritin labels became almost undetectable (Fig. 4*c*). Some of these patches appeared to result from non-specific binding, since they could not be removed in control samples using α-methyl-D-mannoside. Compared with round cells, ferritin patches associated with germlings became less pronounced and finally disappeared.

DISCUSSION

Major changes occur at the surface of *B. emersonii* zoospores while undergoing encystment preceding detectable *de novo* synthesis of protein and RNA (Soll & Sonneborn, 1971). In the course of encystment, surface coat material is deposited by the fusion of vesicles (derived from γ-particles) with the plasma membrane (Myers & Cantino, 1974). About 30 min after induction, the cells form germ tubes and this marks the completion of encystment.

Binding of Con A to the surface of zoospores during encystment was demonstrated by three different approaches: binding of radioactive Con A, fluorescence microscopy and electron microscopy. During the entire course of encystment, Con A bound to the cell surface, although the binding characteristics varied appreciably. Moreover, at a given developmental stage, individual cells exhibited heterogeneity with respect to Con A binding as shown by fluorescence and electron microscopic experiments.

It appears that induction of synchronous development is accompanied by rapid physical alterations at the cell surface, perhaps the surface charge, membrane potential or fluidity. Observed cell volume changes (Truesdell & Cantino, 1971) are presumably not related to variations in fluorescence intensity, as observed in our experiments, because the volume decreases by 43% between 5 and 15 min following induction, whereas the fluorescence intensity remains virtually constant.

We also found that LiCl, which cannot induce encystment (Soll & Sonneborn, 1972), produced practically the same fluorescence intensity distribution as in KCl-treated cells. Synchronous development could be induced by KCl pulses as short as 2 min (Soll & Sonneborn, 1972). Therefore, our results seem to support the notion that both kinds of ion induce similar physical alterations at the cell surface. Subsequent developmental processes are presumably inhibited by LiCl.

During development from non-induced zoospores to round cells and germlings, Con A labelling experiments demonstrated the presence of two distinct surface labelling patterns, and also pronounced changes in binding affinity. This phenomenon may be interpreted in two ways. First, the original Con A binding sites participating in the formation of the closely attached layer may be removed during normal membrane turnover and then replaced by newly synthesized Con A binding receptors with a lower affinity. However, in *B. emersonii*, protein synthesis is not detectable during the short-lived encystment process. Thus, an alternative possibility is that during encystment pre-existing membrane components are modified to manufacture low affinity Con A receptors, in the absence of protein synthesis.

When the primary cell wall material is deposited, the closely attached Con A binding layer gradually becomes obscured. Meanwhile, the loosely associated ferritin-Con A patches continue to form. We propose that this pattern change is related to the fusion of γ-particle vesicles with the plasma membrane. These organelles have a high glycolipid content where glucosyl units are abundant (Mills & Cantino, 1978; G. L. Mills, personal communication). Furthermore, preliminary experiments (Fig. 4*d*) indicated that γ-particles did exhibit a

densely labelled surface of ferritin-Con A particles, following lysis of zoospores, release of γ-particles and subsequent exposure to Con A.

It is not known whether Con A binding sites play an active role in the developmental process described. Since Con A induces rapid zoospore lysis (Jen & Haug, 1979), viable cells cannot be labelled and properly examined within the short time available during encystment. Nevertheless, the lysis experiments suggest that Con A receptors, in particular those characterized by the closely attached layer of label, maintain zoospore integrity. Preliminary electron microscopic studies demonstrate that this closely attached layer is not susceptible to pronase digestion contrary to the loosely associated patches which can largely be removed. The carbohydrate moiety of the closely attached layer perhaps protects the underlying proteins from pronase attack in the zoospore stage. Pronase-treated, non-induced zoospores are capable of completing normal encystment, if pronase is removed prior to induction. It appears that later developmental stages are sensitive to pronase digestion as shown by retarded germination following pronase treatment at the round cell stage. Further experiments are necessary to clarify the physiological role, if any, of lectin receptors during zoospore development.

We thank Dr J. L. Wang for valuable discussions and Dr J. E. Wilson for allowing the use of the Leitz microscope. We are indebted to Dr E. C. Cantino for the *B. emersonii* strain. This work was supported by US-DOE Contract EY-76-C-02-1338.

REFERENCES

GREMO, F., KOBILER, D. & BARONDES, S. H. (1978). Distribution of an endogenous lectin in the developing chick optic tectum. *Journal of Cell Biology* **79**, 491–499.

GUNTHER, G. R., WANG, J. L., YAHARA, I., CUNNINGHAM, B. A. & EDELMAN, G. M. (1973). Concanavalin A derivatives with altered biological activities. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 1012–1016.

JEN, C. J. & HAUG, A. (1979). Concanavalin A induced lysis of zoospores of *Blastocladiella emersonii*. *Experimental Cell Research* **120**, 425–428.

LOVETT, J. S. (1975). Growth and differentiation of the water mold *Blastocladiella emersonii*: cytodifferentiation and the role of ribonucleic acid and protein synthesis. *Bacteriological Reviews* **39**, 345–404.

MILLS, G. L. & CANTINO, E. C. (1978). The lipid composition of the *Blastocladiella emersonii* γ-particle and the function of the γ-particle lipid in chitin formation. *Experimental Mycology* **2**, 99–109.

MYERS, R. B. & CANTINO, E. C. (1974). The gamma particle. In *Monographs in Developmental Biology*, vol. 8. pp. 15–20. Edited by A. Wolky. New York: S. Karger.

ROSENTHAL, H. E. (1967). A graphic method for the determination and presentation of binding parameters in a complex system. *Analytical Biochemistry* **20**, 525–532.

SHARON, N. & LIS, H. (1975). Use of lectins for the study of membranes. In *Methods in Membrane Biology*, vol. 3, pp. 147–200. Edited by E. D. Korn. New York: Plenum Press.

SOLL, D. R. & SONNEBORN, D. R. (1971). Zoospore germination in *Blastocladiella emersonii*: cell differentiation without protein synthesis? *Proceedings of the National Academy of Sciences of the United States of America* **68**, 459–463.

SOLL, D. R. & SONNEBORN, D. R. (1972). Zoospore germination in *Blastocladiella emersonii*. IV. Ion control over cell differentiation. *Journal of Cell Science* **10**, 315–333.

SOLL, D. R., BRAMBERG, R. & SONNEBORN, D. R. (1969). Zoospore germination in the water mold, *Blastocladiella emersonii*. I. Measurement of germination sequence of subcellular morphological changes. *Developmental Biology* **20**, 183–217.

SPURR, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31–43.

TRUESDELL, L. C. & CANTINO, E. C. (1971). The induction and early events of germination in the zoospore of *Blastocladiella emersonii*. *Current Topics in Development Biology* **6**, 1–44.

WEEKS, G. (1975). Studies of the cell surface of *Dictyostelium discoideum* during differentiation: the binding of [^{125}I]-Con A to the cell surface. *Journal of Biological Chemistry* **250**, 6706–6710.

CHAPTER THREE

CONCANAVALIN A-INDUCED LYSIS OF ZOOSPORES OF *BLASTOCLADIELLA EMERSONII*

mine the sizes of molecules that could be introduced into permeable BHK cells, replicate cultures of BHK cells were treated with increasing concentrations of lysolecithin and assayed for the ability of trypan blue (970 D) and RNases (11 000–14 000 D) to enter the cells. In addition, retention of lactate dehydrogenase (142 000 D) activity and viability were measured (table 2). At 50 μg/ml lysolecithin, 90% of the cells were permeable to trypan blue and remained viable; RNases were excluded from the cells and all lactate dehydrogenase activity was retained. Higher concentrations of lysolecithin permitted RNases to enter the cells, but viability was lost. Essentially all lactate dehydrogenase activity remained in the cells up to 100 μg/ml lysolecithin, although the cells were permeable to RNases. At concentrations of lysolecithin above 100 μg/ml, lactate dehydrogenase leaked out of the cells.

These results indicate that permeability can be controlled by adjusting the concentration of lysolecithin. Lysolecithin-permeabilized cells are being used in these and other laboratories to study processes such as DNA replication, DNA repair, mechanisms of drug action, and mechanisms of drug resistance. The results reported here extend the lysolecithin permeabilization method to a wide range of animal cells. This procedure renders many cell types reversibly permeable to small molecules following mild treatment. More severe treatment irreversibly permeabilizes cells; the degree of permeability can be controlled by altering the lysolecithin concentration.

This work was partially supported by a biomedical research grant to M. R. M., a USPHS research grant (CA-18612) to A. B. P., and a USPHS predoctoral traineeship (no. 5T32 CA 09031-02) to J. J. C. Jr. We thank David Schneider for his help in preparing the manuscript for publication.

References

1. Castellot, J J, Miller, M R & Pardee, A B, Proc natl acad sci US 75 (1978) 351.
2. Miller, M R, Castellot, J J & Pardee, A B, Biochemistry 17 (1978) 1073.
3. Kornberg, A, Meth enzymol 1 (1951) 441.
4. Lowry, O H, Rosebrough, N J, Farr, A L & Randall, R J, J biol chem 193 (1951) 265.
5. Giles, K W & Mayers, A, Nature 206 (1965) 93.

Received October 23, 1978
Revised version received December 5, 1978
Accepted December 7, 1978

Printed in Sweden

0014-4827/79/070425-04$02.00/0

Concanavalin A-induced lysis of zoospores of *Blastocladiella emersonii*

C. J. JEN and A. HAUG, *Department of Biophysics and MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA*

Summary. Concanavalin A (ConA) induces lysis of zoospores of the aquatic fungus *Blastocladiella emersonii*. The rapid lysis can be completely blocked by α-methyl-D-mannoside. At lower temperatures the cells are more tolerant towards lysis compared with cells at room temperature. Dimeric ConA derivatives and *Lens culinaris* hemagglutinin A are less effective, whilst other lectins tested do not induce lysis at all. We suggest that cluster formation of adjacent ConA receptors precedes lysis.

Concanavalin A (ConA) has been extensively used to investigate surface properties of various cell types because it binds specifically to carbohydrates. Research has been carried out with cells during differentiation [1], during different stages of the cell cycle [2], and also in malignantly transformed cells [3]. Lectin derivatives and native ConA have been also employed to study the distribution and dynamic rearrangement of carbohydrate receptors at membrane surfaces [4, 5].

Since only scattered information [6] is available on lectin receptors located at cell surfaces of aquatic fungi, we examined lectin-binding properties to the cell surface of

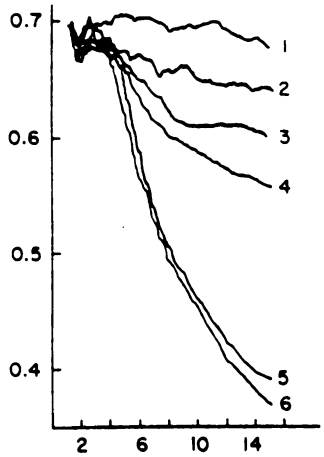


Fig. 1. Abscissa: time after mixing zoospores suspension with ConA (min); *ordinate:* absorbancy measured at 550 nm wavelength.

Absorbancy of zoospores of *Blastocladiella emersonii* exposed to various concentrations of ConA. 0.5 ml of cell suspension (1.5×10^7 cells/ml) was mixed with 1 ml water which contained (1) 100 μg/ml ConA plus 0.05 M mannitol; (2) no ConA, no sugar; (3) 25 μg/ml ConA; (4) 50 μg/ml ConA; (5) 100 μg/ml ConA; (6) 75 μg/ml ConA, final conc.

Blastocladiella zoospores. *Blastocladiella emersonii* displayed significant changes at the plasma membrane surface of motile zoospores while undergoing encystment, or forming round cells. Zoospores tend to adhere to each other and also to the bottom of the growth vessel; vesicles originating from γ-particles fuse with the plasma membrane of zoospores prior to cell wall formation; moreover, zoospores retract the flagellum within several minutes after induction with KCl [7].

In this article we report about experiments concerning cell lysis subsequent to ConA binding. The binding properties of this lectin and some of its derivatives, and those of other lectins bound to the cell surface, are also examined.

Materials and Methods

ConA and α-methyl-D-mannoside were purchased from Sigma Chemical Co., St Louis, Mo. wheat germ agglutinin (WGA) was obtained from Calbiochem, San Diego, Calif. Fucose-binding protein (FBP), *Ricinus communis* agglutinin-60 (RCA-60), and *Lens culinaris* hemagglutinin A (LCH-A) were obtained from Miles-Yeda, Elkhart, Ind. All lectins were directly used without any further purification. Chemical derivatives of ConA, viz. acetyl-ConA, and succinyl-ConA, were prepared [8].

Apart from minor modifications, *Blastocladiella* cultures and growth conditions were maintained as described [7]. Zoospores were harvested from cultures of the first generation grown on Difco PYG agar (1.25 g yeast extract, 1.25 g bactopeptone, 3.0 g dextrose, and 20 g agar per 1 000 ml of medium), and then transferred to tissue culture dishes, which contain PYG liquid medium (same as above but no agar). After 12–18 h at 22°C, growth was terminated by decanting the growth medium. At the same temperature, sporangia which were attached to the plastic bottom were rinsed and induced to sporulate by incubation with sporulation solution (10^{-3} M $CaCl_2$, 5×10^{-3} M morpholinopropane sulfonic acid, pH 6.8). The released zoospores were incubated with the respective lectin at a certain temperature, for 30 min, and were examined with a Zeiss standard microscope, model 14. Cell lysis was estimated semi-quantitatively by observation of single cells. In other experiments, after mixing the zoospore suspension with ConA, the cell lysis kinetics was measured by determining the absorbancy of the suspension at 500 nm with a Gilford spectrophotometer, model 2400.

Results

Cells in the zoospore stage began to lyse within the first few minutes after addition of ConA to the cell suspension, at 22°C. Immediately preceding cell lysis, ConA-treated cells were no longer able to swim and commenced to swell until they were incapable of maintaining osmotic balance across the plasma membrane. This swelling process did not occur at all in the presence of 0.1 M α-methyl-D-mannoside.

Measured at 500 nm, absorbancy changes clearly reflected the lytic destruction of cells. The rate of cell lysis was enhanced upon increase of the lectin concentration (fig. 1). At intermediate concentration ranges, from about 60–120 μg/ml, the zoospores were ruptured into small fragments which was indicated by dramatic absorbancy changes. However, at ConA concentrations higher than 250 μg/ml, the cells fragmented into larger pieces and the ab-

Table 1. *Temperature dependence of ConA-induced lysis of zoospores of Blastocladiella emersonii*

++++, All cells lysed; +++, approx. 75% lysis; ++, about half of the cells lysed; +, less than 25% lysis; s, cells swelled but did not lyse; ±?, effects uncertain; −, no observable effect

Temp. °C	ConA, μg/ml						
	12.5	25	50	100	250	500	1 000
22	s	+	+++	++++	++++	++++	++++
16	±?	s	++	++++	++++	++++	++++
12	±?	±?	s	s	++	++++	++++
8	−	±?	s	s	s	s	++

The zoospore suspension was incubated with ConA for 30 min at the temperature indicated. Reaction mixture contained 5×10^6 cells, ConA, 5 mM MOPS buffer, 1 mM $CaCl_2$ in 1 ml solution, pH 6.8.

sorbancy curves became flatter (data not shown). In the presence of 0.05 M mannitol, the cells were characterized by irregular shapes and failed to germinate after addition of ConA; under those conditions most of the cells did not burst.

ConA-induced cell lysis of zoospores was found to be temperature-dependent (table 1). Upon preincubation with sporulation medium and treatment with ConA at a lower temperature, the cells appeared to be more tolerant towards lysis than those treated with ConA at room temperature. For example, at 8°C, most cells were still intact although they had been exposed to 500 μg/ml ConA for 30 min. Nevertheless, these zoospores—initially treated with ConA at 8°C—lysed immediately when the temperature was raised to room temperature.

The influence of other lectins upon the zoospore surface was studied at room temperature (table 2). The dimeric ConA derivatives, *Lens culinaris* hemagglutinin A and *Ricinus communis* agglutinin-60 swelled but did not lyse the zoospores. WGA and fucose-binding protein exerted no lytic effect. LCH-A caused cells to swell at concentrations higher than 250 μg/ml. Besides native ConA, none of the other lectins tested ruptured zoospores under otherwise identical conditions.

Table 2. *Effect of various lectins on Blastocladiella zoospores, at 22°C*

	Lectin concentration, μg/ml						
	12.5	25	50	100	250	500	1 000
ConA	s	+	+++	++++	++++	++++	++++
Acetyl-ConA	−	−	−	−	−	s	s
Succinyl-ConA	−	−	−	−	−	s	s
WGA	−	−	−	−	−	−	−
RCA-60	−	−	−	−	−	−	s
FBP	−	−	−	−	−	−	−
LCH-A	−	−	−	−	s	s	s
ConA+α-methyl-D-mannoside (0.1 M)	−	−	−	−	−	−	−

Same notations as in footnote to table 1.

Discussion

Because of its size, the ConA protein will presumably not enter the cell membrane and inhibit metabolic activity within a short time period after lectin binding, at the cellular surface. We observed, however, that ConA was able to induce zoospore lysis almost instantaneously, and this process could be totally blocked by the application of α-methyl-D-mannoside. Therefore, ConA appears to affect primarily the plasma membrane. Once a sufficient number of ConA molecules is attached to the zoospore surface, the plasma membrane is altered and cannot function any more osmotically. On the other hand, mannitol stabilizes the cell osmotically, although mannitol is not a specific inhibitor for ConA binding. Preliminary experiments in our laboratory indicate that round cells, which have a primitive cell wall—in contrast to zoospores—are not susceptible to ConA lysis. They are osmotically stable and proceed to germinate in the presence of ConA concentrations which would totally lyse zoospores.

There exist at least two reasons for the diminished ConA-induced cell lysis at lower temperatures. (1) At lower temperatures ConA binding to the cell surface might be impaired. However, we found neither appreciable differences in the number of binding sites nor in their affinity, when ConA was attached to fixed cells either at room temperature or at 8°C. (2) We suggest that cell lysis requires formation of lectin receptor clusters which—to a certain extent—depends on receptor mobility in the cell membrane. The latter process is temperature dependent. The proposed cluster mechanism is triggered by native ConA which possesses four saccharide binding sites per molecule [8], which may participate in the cross-linkage of adjacent sites. Aggregation between neighbouring sites was also observed on mastocytoma cells, where a linear relationship was found between cap formation and cytolytic effect of ConA [9].

The mobility of receptor sites is reduced at temperatures below 10–12°C, where spin labelling experiments demonstrate discontinuities in the Arrhenius plots of membrane microviscosity vs temperature [10]. LCH-A has the same sugar specificity and also two binding sites per molecule as the dimeric ConA derivatives studied. Compared with dimeric ConA, LCH-A possesses a 50 times smaller binding affinity [11] and a rather similar lytic capacity towards zoospores. This seems to indicate that the crucial step in the formation of lectin clusters is the ability to cross-link and not the affinity to bind.

This project was supported by the US Department of Energy Contract EY-76-C-02-1338. We thank Dr E. C. Cantino for helpful discussions and for providing the original strain of *Blastocladiella emersonii*.

References

1. Weeks, G. J biol chem 250 (1975) 6706.
2. Shoham, J & Sachs, L. Exp cell res 85 (1974) 8.
3. Noonan, K D & Burger, M M. J biol chem 248 (1973) 4286.
4. Ash, J F & Singer, S J. Proc natl acad sci US 73 (1976) 4575.
5. Schlessinger, J, Elson, E L, Webb, W W, Yahara, I, Rutishauser, U & Edelman, G M. Proc natl acad sci US 74 (1977) 1110.
6. Lemos, E M & Lodi, W R. Exp cell res 94 (1975) 437.
7. Soll, D R, Bromberg, R & Sonneborn, D R. Dev biol 20 (1969) 183.
8. Gunther, G R, Wang, J L, Yahara, I, Cunningham, B A & Edelman, G M. Proc natl acad sci US 70 (1973) 1012.
9. Lustig, S, Asher, D, Fishman, P, Djaldetti, M & Pluznik, D H. J cell biol 75 (1977) 388.
10. Leonards, K & Haug, A. Plant physiol, suppl. 61 (1978) 113.
11. Stein, M D, Howard, I K & Sage, H J. Arch biochem biophys 146 (1971) 353.

Received November 7, 1978
Revised version received January 3, 1979
Accepted January 8, 1979

CHAPTER FOUR

POTASSIUM-INDUCED DEPOLARIZATION OF THE TRANS-MEMBRANE POTENTIAL IN *BLASTOCLADIELLA EMERSONII* ZOOSPORES PRECEDES ENCYSTMENT

POTASSIUM-INDUCED DEPOLARIZATION OF THE TRANS-MEMBRANE POTENTIAL IN BLASTOCLADIELLA EMERSONII ZOOSPORES PRECEDES ENCYSTMENT.

C. J. JEN and A. HAUG

Department of Biophysics and MSU-DOE Plant Research Laboratory

Michigan State University

East Lansing, Michigan 48824

Running title: Trans-membrane potential and developmental triggering

SUMMAR

Experi

potent

zoospo

cyanin

K^+ ion

membra

to a c

the hi

was te

from 5

potent

valinom

was ap

the zoo

K^+ ion-

trigger

lipid m

SUMMARY

Experiments have been performed to test the possible role of the membrane potential in the ionic induction of encystment in Blastocladiella emersonii zoospores. A fluorescent, voltage-sensitive dye, 3,3'-dipropylthiadicarbocyanine iodide, was used to monitor changes in the membrane potential. Only K^+ ions, the standard developing triggering agent, rapidly depolarized the membrane potential. Application of an optimal K^+ ion concentration (50 mM) to a cell population depolarized the membrane potential most rapidly and induced the highest percentage of cells to encyst per unit time. The depolarization was temperature dependent and was not affected by extracellular pH changes from 5 to 9. Quinine practically prevented depolarization of the membrane potential by K^+ ions; however, the membrane potential was depolarized by subsequent valinomycin application. The K^+ induced response of the membrane potential was apparently independent upon the zoospore's age, but varied dramatically as the zoospore encysted. A strong correlation was established between the K^+ ion-induced depolarization of the membrane potential and the developmental triggering by K^+ ions. We propose that specific K^+ carriers are embedded in the lipid matrix, which exists in the gel + liquid-crystalline mixed lipid state.

The life cycle of Blastocladiella emersonii [1], a unicellular water mold, provides a suitable model for studying the role of the plasma membrane in eukaryotic cell differentiation. During encystment, the non-growing motile zoospore of B. emersonii manifests pronounced morphological alterations, concomitant with a loss of motility, flagellar retraction, increased adhesiveness, fusion of cytoplasmic vesicles to the plasma membrane, and primary cyst wall formation. Under favorable conditions the entire encystment process requires less than 30 min after the zoospores have been exposed to KCl. These observable morphological modifications involve apparently the plasma membrane, and precede de novo detectable RNA and protein synthesis. A working hypothesis has been proposed whereby local membrane alterations result from rearrangements of pre-existing structures [2]. However, there is little information available on early (less than a few min) physico-biochemical membrane processes occurring prior to the structural changes reported. In our previous work on B. emersonii zoospores we had demonstrated a rapid change of concanavalin A binding characteristics to the plasma membrane upon KCl addition [3, 4]. Therefore, the present studies were undertaken to determine whether the developmental induction might be associated with initial changes of the transmembrane potential. To follow possible transient ion-induced responses of the membrane potential, the fluorescent, voltage-sensitive dye 3, 3'-dipropylthiadicarbocyanine iodide, diS-C_3-(5), was employed [5, 6, 7]. We observed a rapid membrane potential depolarization which occurred simultaneously with K^+ induction. At the time of the completion of this article a partially related study was published [8] which we are going to discuss.

MATERIALS AND METHODS

Reagents. The dye diS-C_3-(5) was a gift from Dr. A. Waggoner (Amherst College, Mass.). ATP, valinomycin, quinine, 9-amino acridine were purchased from Sigma; the ionophore A23187 was bought from Calbiochem; triphenylmethyl phosphonium bromide, $TPMP^+$ bromide, was bought from ICN, and tetrabutylammonium chloride was purchased from Aldrich. The amphiphilic spin probe 4-(dodecyl dimethyl ammonium)-1- oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT_{12}) was synthesized in our laboratory as described [9]. All other reagents were purchased and used without any further purification.

Organism and culture conditions. B. emersonii zoospores were prepared as described [2]. Cells were grown overnight in liquid PYG Difco medium in 100 x 20 mm tissue culture dishes (Falcon No. 3003). The medium was removed and replaced by the sporulation solution (10^{-3}M $CaCl_2$ and 5 x 10^{-3} M MOPS buffer, pH 6.7). The zoospores, released about four hours later, were maintained in sporulation solution until the experiments commenced. The cell suspension was filtered through filter paper (Schleicher and Schüll, No. 588) to remove possible cell types other than zoospores. The zoospore concentration of the filtrate was adjusted to 5 x 10^6 cells/ml for the fluorescence studies.

Fluorescence experiments. The fluorimeter used for the trans-membrane potential measurements was a modification of a phosphoroscope previously described [10]. Briefly, the output of an EMI photomultiplier was passed through a Keithley picoammeter, model 417, and recorded on a Hewlett-Packard

X-Y recorder, model 7034A. To vary the sample temperature, a holder was constructed through which temperature-controlled water could be circulated. Prior to the membrane potential measurements, the fluorimeter was tested on erythrocytes [5]. The 1 mM ethanolic stock solution of the dye was first diluted to 20 μM with sporulation solution. Typically, 25 μl of the diluted dye solution was injected into the cuvette which contained 2 ml of the zoospore suspension. In the sample cuvette, the final ethanol concentration was 0.025% (v/v). Previous control experiments had indicated that the encystment process was not affected by these minute alcohol concentrations. The fluorescence signal from the reaction mixture was continuously monitored for about 10 min at excitation and emission wavelengths of 629 and 671 nm, respectively. Above 500 nm interference from intrinsic cellular absorption, emission, and light scattering was negligible. After a steady-state fluorescence intensity level had been reached within a few min following dye injection, various agents were applied to the sample, and the fluorescence intensity change was recorded continuously. The fluorescence intensity was measured in arbitrary units, from which two parameters were derived, viz., $\Delta F/F$ (%), and the initial rate of the change, $\Delta F/F$ min^{-1}. The fluorescence ratio, $\Delta F/F$, represents the percent intensity change with respect to the steady-state level established prior to the addition of a specific reagent. The initial rate of fluorescence change is the slope of $\Delta F/F$ following application of the respective reagent. 9-aminoacridine, a fluorescent dye [11], was used in some experiments to monitor possible pH variations. Generally 1 μM 9-aminoacridine was applied to the sample, under similar conditions as described above, except that the excitation and emission wavelengths

corr

flu

this

leve

REST

Loss

(5)

of t

incr

the

for

or m

tens

the

stab

indu

trat

were

foll

init

been

[7].

thes

appa

after

corresponded to 396 and 460 nm, respectively. Although the intrinsic fluorescence and scattering of the cells interfered with the measurements, this background amounted to 10% at most, compared with the total signal level and was corrected accordingly.

RESULTS

Zoospores labelled with diS-C_3-(5). The cells were labelled with diS-C_3-(5) and the time course of fluorescence intensity recorded. Upon addition of the dye to a cellular suspension, the fluorescence intensity immediately increased, followed by a decrease (fig. 1). This behavior indicated that the cells have an interior negative membrane potential which was responsible for shifting the dye's equilibrium distribution towards the cytoplasmic side or membrane-bound state as evidenced by the decrease in fluorescence intensity [5]. The addition of 50 mM KCl to the reaction mixture intensified the fluorescence rapidly (depolarization of the membrane potential) which stabilized at a new level within a few minutes. The percentage of KCl induced fluorescence intensity change was dependent upon the dye concentration employed. Low dye concentrations displayed small changes which were difficult to measure. Too high dye concentrations suffered from the following disadvantages: (a) More than 5 min were necessary to attain the initial steady-state fluorescence level, (b) cellular injury might have been caused, and (c) the membrane potential might have been depolarized [7]. Consequently we selected a dye concentration of 2.5×10^{-7} M. Under these standard conditions, the physiological behavior of the zoospores was apparently not impaired as judged by the usual development in the cuvette after KCl addition. At this concentration the dye did not exert any

measurable influence on the observed K^+ effect since the same final fluorescence level, characteristic for the depolarized state, was reached irrespective of the sequence of K^+ or dye application. It was not necessary to add any stabilizer to our reaction mixture, in contrast to experiments described in a recent report [8], where 0.1 M sucrose was used to prevent cell lysis temporarily when the membrane potential was measured with the carbocyanine dye diO-C_6-(3). If suspended in sporulation medium (K^+ ion-free), zoospores can be maintained in that stage for up to 20 hr. The KCl induced fluorescence intensity increased but the $\Delta F/F$ values decreased when the cells were older than 15 hr.

The ion specific membrane potential depolarization. Although KCl was presumably the induction agent in the standard germination solution [12], other reagents were also tested under similar conditions (fig. 2). These reagents could be classified into three groups according to the pattern of membrane potential change produced. Type 1 salts, KCl, KBr, KI, and RbCl, depolarized the membrane potential rapidly and with practically identical efficiency. Since Rb^+ may be considered as a K^+ analogue, the results suggested that the K^+ cation, not the anions, was the effector. This was further confirmed by studying the effects of two other potassium salts. The highly permeant anion SCN^- caused no significant depolarization, whereas the impermeant anion 4-morpholinoethanesulfonate (MES^-) depolarized the membrane potential similarly as KCl. In the presence of KMES, the cells are induced to develop; they eventually lysed, however, when KSCN was applied instead. The type 2 compound, NH_4Cl, depolarized the membrane potential similarly, but to a lesser degree. In the presence of NH_4Cl, the fluorescence intensity reached a new steady-state level later than that

level reached after KCl application. Type 3 compounds, LiCl, NaCl, $CaCl_2$, choline chloride, $MgCl_2$ and CsCl, generated a weak and slow fluorescence intensity increase. We also tested the effect of LiCl, a known inhibitor for encystment [12], on K^+ induced depolarization. KCl depolarized the membrane potential irrespective of the presence of the lithium salt. The non-ionic compound mannitol did not affect the fluorescence intensity. We therefore concluded that osmolarity changes were not responsible for altering the membrane potential.

Effect of K^+ ion concentration. The fluorescence intensity change depended upon the K^+ ion concentration (fig. 3). A detectable increase in fluorescence intensity could be observed at K^+ ion concentrations as low as 0.5mM. As the K^+ ion concentration increased, both the fluorescence intensity change and the initial rate became more pronounced. The maximal intensity change was reached at about 25 mM, succeeded by a small decrease for higher concentrations. The initial rate of change reached a maximum value at about 50 mM. Further increase of the K^+ ion concentration did not enhance significantly the initial rate of change.

Effect of temperature. Samples were pre-equilibrated for 5 min at the desired temperature which was subsequently maintained throughout the experiment. Reagents were kept at the same respective temperature. For a temperature less than 15^oC, the steady-state base line fluorescence was reached at later times and was higher compared with that at room temperature. Therefore we carried out the following control experiments: $\Delta F/F$ values were unchanged, when cells were incubated with the dye, either at 4^oC for 12 min, or at 22^oC for 4 min succeeded by an additional incubation

at 4°C for 8 min, or at 4°C for 4 min. Furthermore, the ΔF/F values of cells incubated with the dye at 22°C for 12 min were virtually the same as those incubated for a 4 min period. To forestall possible cell lysis during prolonged incubation times, the reagent was injected 4 min after the dye application, over the entire temperature range tested. Below 4°C the K^+ induced fluorescence intensity change was undetectable. Parallel to a temperature raise, the K^+ effect became more pronounced and reached a plateau at about 28-30°C. At temperatures higher than 35°C, the value of ΔF/F diminished rapidly (fig. 4).

Effect of pH. The adjusted pH value of the zoospore suspension did not vary by more than 0.2 pH units within 15 min, irrespective of the presence or absence of 50 mM KCl. In the absence of KCl, the base line fluorescent level of dye-labelled cells was pH dependent and increased as the pH value of the medium decreased. Upon K^+ ion addition, however, the ratio ΔF/F remained approximately constant between pH 5 to 9 (fig. 5). The initial rate of fluorescence change remained practically constant between pH 6 and 8; beyond this range the rate was reduced. Furthermore, upon application of K^+ ions at pH 6.7, the intracellular pH stayed constant as monitored by the pH probe 9-aminoacridine (figure not shown). These results appear to indicate that membrane potential changes rather than pH gradient variations are associated with the process of KCl induction.

Effect of valinomycin and quinine. The fluorescence intensity level of cells labelled with the voltage sensitive dye changed insignificantly upon application of valinomycin, either before (curve not shown) or after K^+ addition (fig. 6a). Quinine, an inhibitor for Ca^{2+}-activated K^+ channels

in red blood cells [13], reduced the fluorescence level slightly when added to the dye-labelled cell suspension. In the presence of quinine, K^+ ions induced a much smaller depolarization of the membrane potential than that in the absence of quinine. Subsequent application of valinomycin to cells, incubated in the presence of quinine and K^+ ions, caused rapid depolarization (fig. 6b), but cells lysed eventually.

Determination of the membrane potential by the null point method [14, 15] was unsuccessful in our case because the K^+ ion-induced $\Delta F/F$ values remained essentially constant irrespective of the presence or absence of valinomycin. With the synthetic cation triphenylmethyl phosphonium ($TPMP^+$) we were able to confirm a membrane potential of about -100 mV at the resting state [8].

Response of developing cells to a second KCl pulse. We studied the fluorescence behavior of K^+ ion-induced, dye labelled cells and their response to a second KCl pulse which was applied after removal of K^+ ions from the previous induction medium. If the monovalent cation was removed within less than 10 min, the membrane potential returned to its original state, i.e., to that value prior to the application of the first K^+ pulse; then the zoospore response to a second K^+ pulse resembled that of the first ionic pulse. However, round cells responded less to the second ion pulse. By the time cells turned into germlings, about 30 min after induction, only a weak response was elicited as reflected by a small fluorescence intensity increase following the second pulse (fig. 7).

The effect of calcium/ionophore and synthetic permeant cations. Although our results agree in principle with those reported recently [8], we attempted to test whether zoospore encystment can be induced by membrane potential

depolarization generated by ions other than K^+ ions, in particular Ca^{2+} ions. In the presence of the ionophore A23187 (2.5×10^{-6}M), low Ca^{2+} concentrations (1-10 mM) could neither depolarize the membrane potential nor induce zoospore encystment. This is in agreement with recent observations describing the lack of zoospore encystment following the application of the ionophore A23187 at low external calcium concentrations; under these conditions a Ca^{2+} ion efflux occurred [16]. However, at high Ca^{2+} concentrations (> 25 mM), in the presence of the ionophore, we observed a membrane potential depolarization similar to that induced by equivalent K^+ concentrations, but they were not effective in inducing synchronous encystment. This depolarization may be caused by inhibition of a membrane-bound ATPase by Ca^{2+} ions, which influxed via A23187 in exchange for protons [17]. Indeed, preliminary experiments with zoospore plasma membrane vesicles suggest that a membrane potential can be established in the presence of ATP.

High concentrations (50-100 mM) of synthetic permeant cations ($TPMP^+$ and tetrabutylammonium) depolarized the membrane potential (data not shown). However, the cells treated with those synthetic cations lysed eventually, possibly as a consequence of cytotoxic effects.

DISCUSSION

These results demonstrate that induction of zoospores by K^+ ions is accompanied by a rapid depolarization of the plasma membrane potential. This partial depolarization is practically completed within the first minute after application of the monovalent cation, and precedes observable morphological alterations of the cell.

More

morp

achi

with

a pl

a te

read

15°

ency

nei

ind

men

Bey

man

and

syn

dec

The

con

pro

Ca(

dye

af

en

in

th

Moreover, at later times after induction, our results correlate well with morphological alterations observed [12, 18]: (a) A 50 mM KCl concentration achieved optimal induction of synchronous encystment. This is consistent with our findings that at the same concentration the rate of $\Delta F/F$ reached a plateau value. (b) The fluorescence ratio $\Delta F/F$ became maximal within a temperature range from 26°C to 35°C, where the synchrony of encystment reached an optimum and was rather insensitive to temperature shifts. Below 15°C, $\Delta F/F$ decreased rapidly and the time span for 50% of the zoospores to encyst increased considerably. Above 39°C, the vast majority of the cells neither germinated nor exhibited significant $\Delta F/F$ values. (c) Following K^+ induction, both the initial rate of fluorescence change and the developmental synchrony were not influenced by pH variations between pH 6 and 8. Beyond this pH range, both kinds of parameters decreased in a parallel manner. (d) Zoospores, less than 15 hr old, showed similar $\Delta F/F$ responses and developmental kinetics upon K^+ induction. Still older cells lost synchrony, some cellular destruction occurred, and the value of $\Delta F/F$ decreased upon induction.

These physical and developmental responses can perhaps be elicited by compounds other than K^+ ions. Under certain conditions the encystment process can be induced by reagents such as sulfonic acid azo dyes, $MgCl_2$, $CaCl_2$, NaCl, CsCl, and choline chloride [8, 12, 19]. The sulfonic acid azo dyes inhibit growth in the early germling stage. Whether these azo dyes affect the membrane potential is unknown. Both $MgCl_2$ and $CaCl_2$ initiate encystment but with less synchrony as KCl does, germ tube formation is inhibited. These divalent cations modify the membrane potential much less than K^+ ions (fig. 2). However, NaCl, CsCl, and choline chloride generated

minor potential changes, and induced encystment but with less synchrony as compared to that induced by K^+ ions, under our experimental conditions. On the other hand, it has been reported [12] that these three monovalent cations induce encystment similarly as compared with K^+ ions. Presently we do not know the reason for that discrepancy.

Although both LiCl and KCl screen the surface charge (Jen and Haug, unpublished results) and affect concanavalin A binding to the cell surface similarly [4], these salts display pronounced differences in regard to the physiology of encystment and membrane depolarization. LiCl is a known inhibitor; KCl is an inducer of encystment [12]. LiCl depolarizes the trans-membrane potential slightly in contrast to KCl. Since LiCl did not prevent the K^+ induced depolarization (in contrast to quinine), we conclude that LiCl acts at a later time in blocking the developmental process.

One of these subsequent processes might involve biochemical signals. Indeed, the germination process was reported to depend upon the intracellular cyclic AMP levels [20]. In spite of the fact that both cyclic AMP and adenine induced germination [20], our preliminary experiments indicate that neither compound caused a depolarization of the membrane potential. It is therefore conceivable that membrane potential depolarization can be bypassed in triggering the developmental process, provided that the secondary biochemical signals can be manipulated directly.

If one takes into account the individual heterogeneity within a cell population, then the K^+ concentration-dependent changes of the membrane potential (fig. 3) may be interpreted in at least two ways. First, K^+ ions primarily affect the rate of individual cell transformation, whereby

individual cells may respond differently to the respective monovalent cation concentration. This assumption might be consistent with the fact that lectin binding characteristics of the cell surface vary appreciably from cell to cell. However, upon treatment with K^+ ions, the individual heterogeneity is significantly reduced which results in a rather defined synchrony of the cell population [4]. Moreover, heterogeneous membrane potential responses to lectins were reported recently in lymphocytes [21]. Consequently we favor the following second interpretation as to the K^+ effect. Application of an optimal K^+ ion concentration to a cell population induced the highest percentage of cells to encyst per unit time, hence the largest rate of fluorescence intensity change. Our results are also in accord with the observation that cells, which undergo rapid germination, apparently do so at the same rate [12].

As to the mechanism of K^+ ion depolarization, we propose the following hypothesis: Specific K^+ carriers which permit passage of K^+ ions across the membrane are embedded in the mixed lipid state of the plasma membrane. The K^+ carriers are presumably proteins, perhaps different from those Ca^{2+} activated K^+ channels reported elsewhere [13]. This hypothesis is supported by the following observations. 1.) The developmental induction process and the transmembrane potential depolarization are effected by specific ions. In preliminary electron paramagnetic resonance experiments with the amphiphilic surface charge sensitive spin probe CAT_{12}, we ruled out the possibility of specific K^+ surface charge screening effects (Jen and Haug, unpublished). 2.) Without quinine, the K^+ ion-induced depolarization was not measurably affected by the presence or absence of valinomycin. Consequently the membrane may contain already existing K^+ carriers.

This is further supported by the fact that quinine application resulted in a virtual block of K^+ passage across the membrane. From transport studies the existence of similar channels had been inferred in Neurospora and animal cells [13, 22]. Moreover, B. emersonii zoospores accumulate K^+ ions from the medium [8]. 3.) The existence of a gel + liquid crystalline mixed lipid state between 4^o and 35^oC had been deduced from spin labelling experiments on intact zoospores and isolated plasma membrane vesicles [23]. 4.) The growth range of B. emersonii [18] is limited to that of the mixed lipid state. 5.) The temperature-dependent changes of $\Delta F/F$ exhibit discontinuities at 4^oC and 35^oC (fig. 4).

The K^+ carrier structure is perhaps maintained by the specific bulk lipid state, or a specific lipid domain structure, or a specific physical state of boundary lipids around the channel protein. Below 4^oC, the B. emersonii zoospore membrane is in the rigid lipid state and above 35^oC the membrane is in the liquid crystalline state. Both states are presumably not conducive to the proper carrier functioning. A somewhat similar situation has been found for the outer membrane of E. coli which contains pore proteins. This bacterium can only grow in a temperature range where the cell can maintain the outer membrane lipids in a mixed lipid state [24].

From the effect of a second K^+ ion exposure (fig. 7) on the membrane potential in developing cells we conclude that a functional K^+ carrier can only exist during the zoospore stage. The disappearance of this carrier apparently coincides with the commencement of amino acid incorporation in later developmental states [25]. Moreover, zoospores exposed to a K^+ pulse for about 2 min, then resuspended in K^+ -free sporulation medium, developed with the

same synchrony as those continuously exposed to K^+ [12].Also in amphibian oocytes the K^+ channel in the plasma membrane can be turned off after a certain time coinciding with long term membrane potential depolarization and meiotic division[26]. These carriers may process environmental information for cellular differentiation. Once the information has been transmitted, the cells are triggered to develop and the carriers are no longer needed.

ACKNOWLEDGEMENT

We are indebted to Dr. A. S. Waggoner for a gift of the dye. We are grateful for helpful discussions by Dr. J. L. Wang. The original strain of the organism was provided by Dr. E. C. Cantino. We also gratefully acknowledge the work by Mr. C. R. Caldwell in preparing the spin probe CAT_{12}. This research was supported by Department of Energy contract No. DE-AC02-76ER01338.

REFERENCES

1. Lovett, J S, Bacteriol rev 39 (1975) 345.

2. Soll, D R, Bromberg, R & Sonneborn, D R, Devel biol 20 (1969) 183.

3. Jen, C J & Haug, A, Exp cell res 120 (1979) 425.

4. Jen, C J & Haug, A, J gen microbiol 115 (1979) 145.

5. Sims, P J, Waggoner, A S, Wang, C -H & Hoffman, J F, Biochem 13 (1974) 3315.

6. Doughty, M J & Dodd, G H, Biochim biophys acta 451 (1976) 592.

7. Rink, T J, Montecucco, C, Hesketh, T R & Tsien, R Y, Biochim biophys acta 595 (1980) 15.

8. Van Brunt, J & Harold, F M, J bacteriol 141 (1980) 735.

9. Mehlhorn, R J & Packer, L, Methods enzymol LVI (1979) 515.

10. Priestley, E B & Haug, A, J chem phys 49 (1968) 622.

11. Schuldiner, S, Rottenberg, H & Avron, M, Eur j biochem 25 (1972) 64.

12. Soll, D R & Sonneborn, D R, J cell sci 10 (1972) 315.

13. Lew, V L & Ferreira, H G, Curr top membr transp 10 (1978) 217.

14. Hoffman, J F & Laris, P C, J physiol 239 (1974) 315.

15. Laris, P C, Pershadsingh, H A & Johnstone, R M, Biochim biophys acta 436 (1976) 475.

16. Gomes, S L, Mennucci, L & Maia, J C C, Devel biol 77 (1980) 157.

17. Reed, P W & Lardy, H A, J biol chem 247 (1972) 6970.

18. Soll, D R & Sonneborn D R, Devel biol 20 (1969) 218.

19. Truesdell, L C & Cantino, E C, Curr top devel biol 6 (1971) 1.

20. Gomes, S L, Mennucci, L & Maia, J C C, Cell differen 9 (1980) 169.

21. Shapiro, H M, Natale, P J & Kamentsky, L A, Proc natl acad sci USA 76 (1979) 5728.

22. Slayman, C L, Membrane transport in plants (eds Zimmerman, U & Dainty, J) p 107 Springer-Verlag, Berlin (1974).

23. Leonards, K S & Haug, A, Biochim biophys acta (1980) in press.

24. Janoff, A S, Haug, A & McGroarty, E J, Biochim biophys acta 555 (1979) 56.

25. Soll, D R & Sonneborn, D R, Proc natl acad sci USA 68 (1971) 459.

26. Morrill, G A & Ziegler, D, Devel biol 74 (1980) 216.

Li

Fi

(a

Ti

of

re

1 r

was

car

Fig

(ar

Tim

aft

to

and

and

inte

fluc

of t

Fig.

High

K^+ co

expe

LEGENDS

Fig. 1. *Abscissa* = time (min); *ordinate* = fluorescence intensity (arbitrary units)

Time course of fluorescence intensity at various concentrations of the voltage sensitive dye diS-C_3-(5). Prior to dye addition the reaction mixture contained 5 x 10^6 zoospores/ml, 5 mM MOPS (pH6.7), and 1 mM $CaCl_2$. The dye and KCl (50mM) were added as indicated. Fluorescence was recorded at 671 nm, with excitation at 622 nm. Experiments were carried out at 22°C.

Fig. 2. *Abscissa* = time (min); *ordinate* = fluorescence intensity (arbitrary units)

Time course of fluorescence intensity; the effect of various salts. 4 min after injection of the dye (2.5 x 10^{-7}M) various salts (50mM) were added to the same reaction mixture as given in fig. 1. Curve 1: KCl, KBr, KI, and RbCl; curve 2: NH_4Cl; curve 3: NaCl, CsCl, choline chloride, $CaCl_2$, and $MgCl_2$. The symbols are defined as, F: the steady state fluorescence intensity base line level before KCl addition; ΔF: the change of fluorescence level following KCl addition; $\Delta F/F$ min^{-1}: the initial slope of the fluorescence change upon KCl addition.

Fig. 3. *Abscissa* = K^+ concentration (mM); *ordinate* = *left* $\Delta F/F$ (°/o) (●); *right* initial rate (°/o min^{-1}) (□)

K^+ concentration dependent fluorescence intensity changes. The same experimental conditions were maintained as in fig. 2.

Fig. 4. *Abscissa* = temperature (^{o}C); *ordinate* = $\Delta F/F$ (%)

Temperature dependent fluorescence intensity changes upon KCl addition (50mM). The same conditions were used as in fig. 2, except that the reaction temperature was adjusted to the respective temperature.

Fig. 5. *Abscissa* = pH; *ordinate* = (*left*) $\Delta F/F$ (%) (●); (*right*) relative baseline fluorescence intensity $\bar{F} = F(pH)/F_{6.7}$ (□)

pH dependent fluorescence intensity changes upon KCl addition (50mM). The same conditions were maintained as indicated in fig. 2, except that the extracellular pH was adjusted to the desired value by adding 0.1N HCl or NaOH to the reaction mixture.

Fig. 6. *Abscissa* = time (min); *ordinate* = fluorescence intensity (arbitrary units).

The time course of fluorescence intensity; the effect of K^{+} (50mM), valinomycin (1μM) and quinine (1mM). Various reagents were added as indicated. The same experimental conditions as in fig. 1 were maintained prior to the application of reagents.

Fig. 7. *Abscissa* = time (min); *ordinate* = (*left*) $\Delta F/F$ ($^{o}/_{o}$) (●—●); (*right*) CpM ($x10^{-3}$) (□---□)

Flourescence intensity changes upon a second K^{+} pulse (50mM) at the respective time of cellular development. Zoospores were first induced by a 50 mM KCl pulse at room temperature (t=0), and then resuspended into

KCl-free sporulation solution before fluorescence experiments were performed. Abscissa indicated the time for development after initial KCl induction, including 2 min wash and 4 min dye equilibration. The right ordinate (□) represents the cpm for the incorporation of ^{3}H-leucin into TCA precipitable material, (data adapted from [21]). Symbols for the major cell types at the respective developmental time are indicated on the top of the figure: o—, zoospore; o, round cell; C⇒, germling.

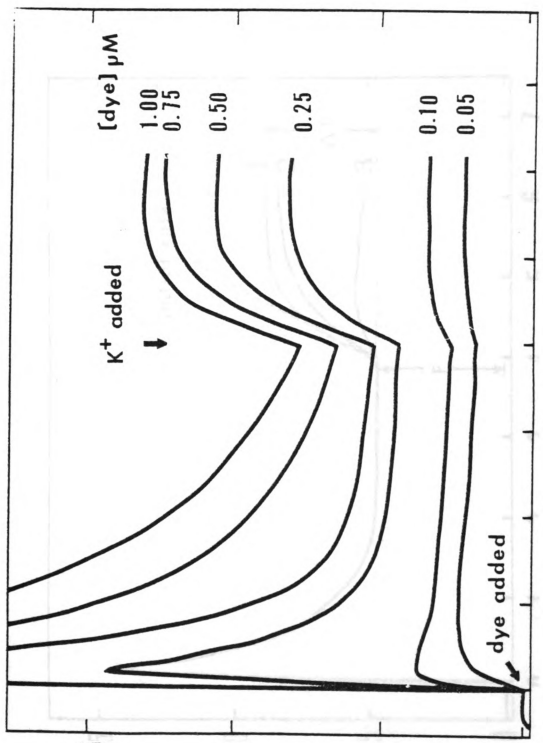

[dye] μM
1.00
0.75
0.50
0.25
0.10
0.05
K+ added
dye added
15
10
5
0
0
1
2
3
4
5
6
7

Figure 4.1

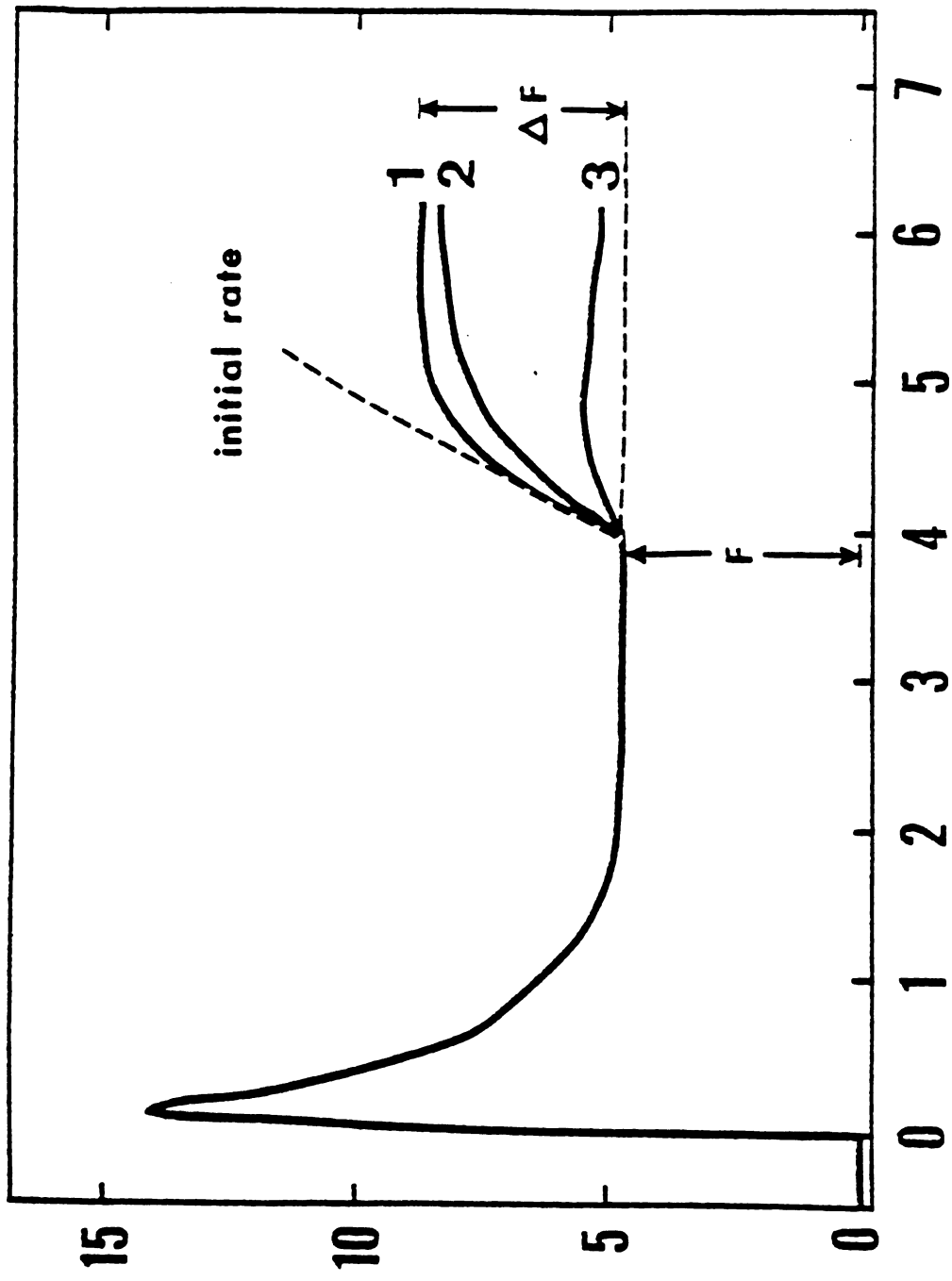


Figure 4.2

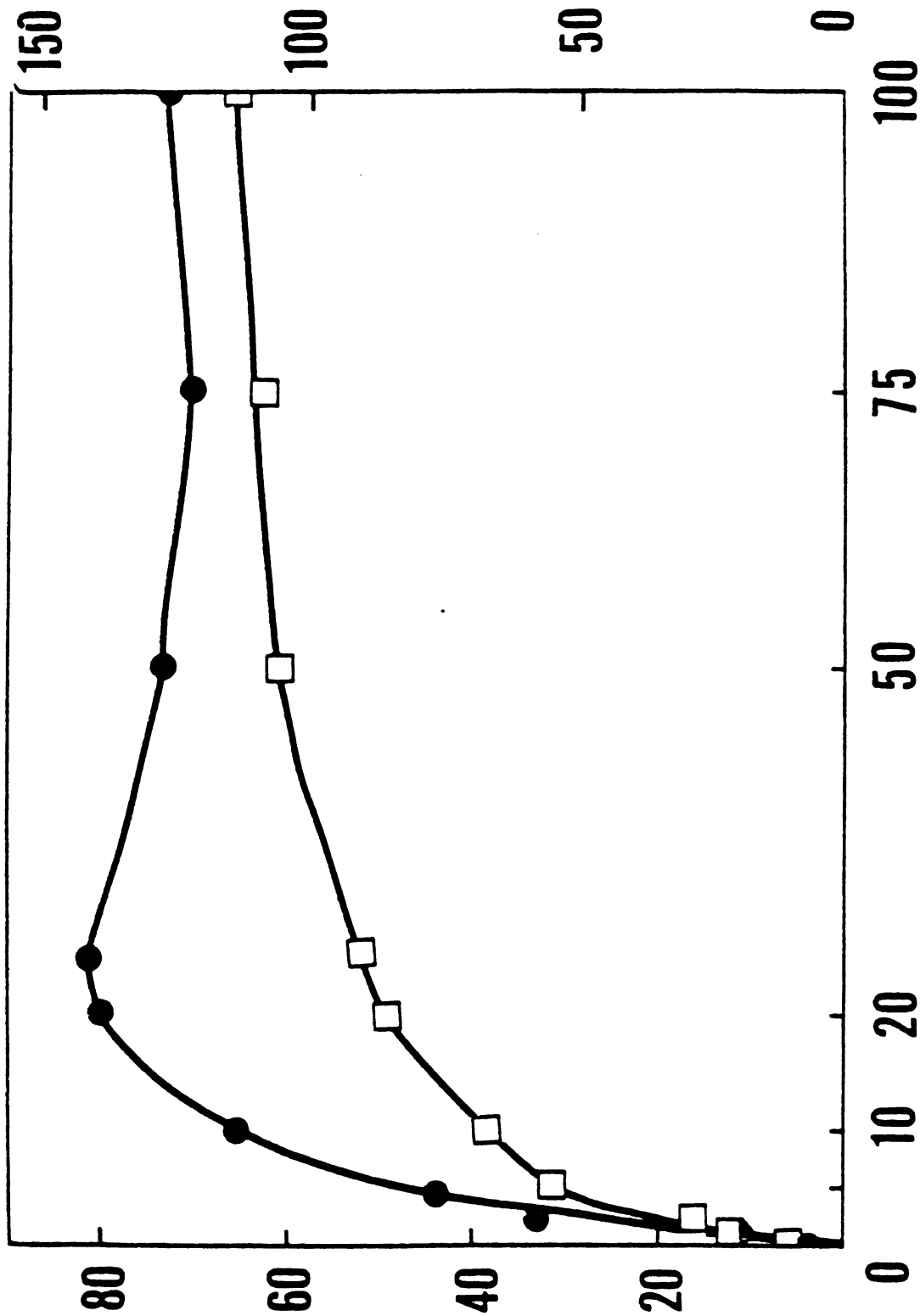


Figure 4.3

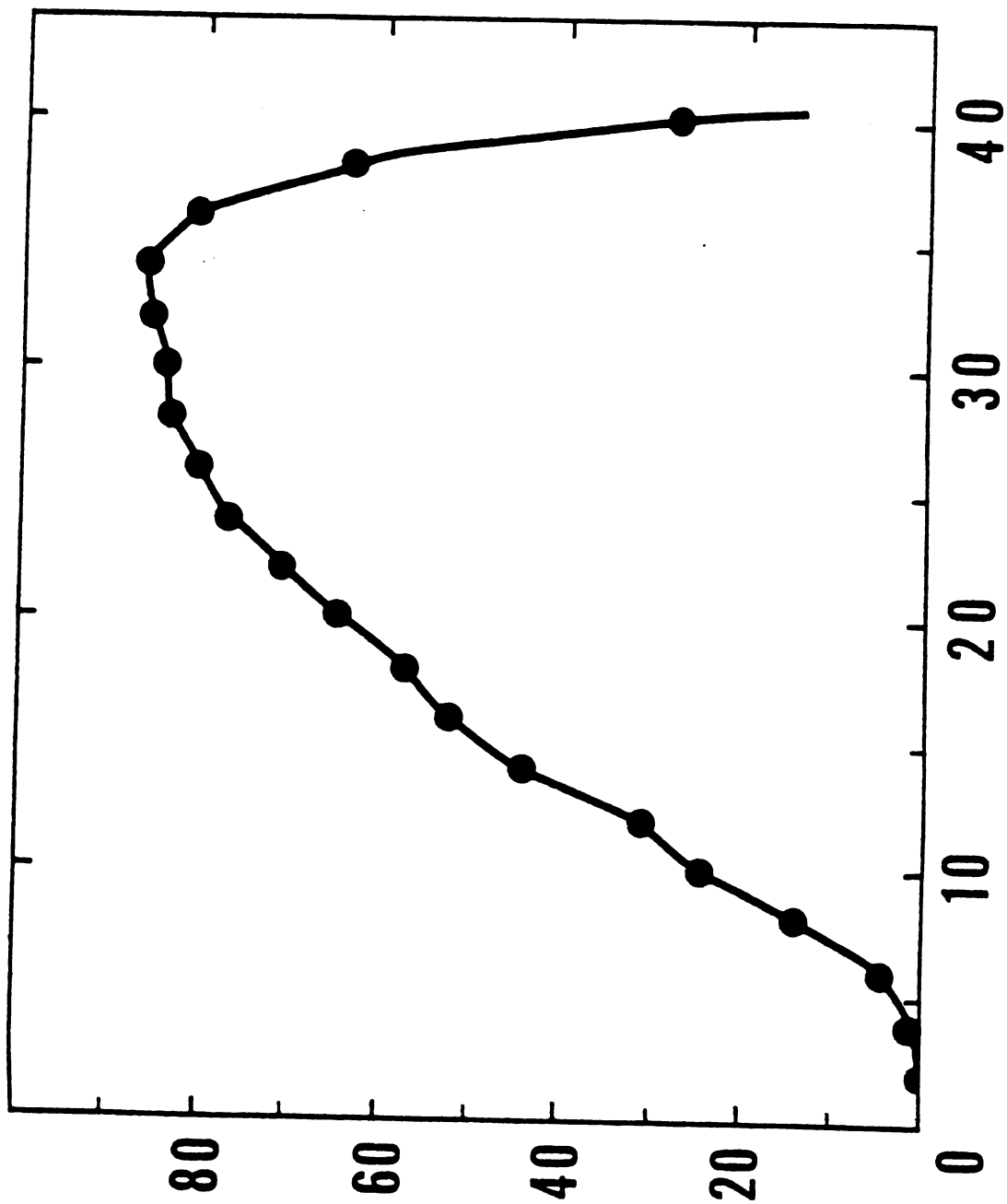


Figure 4.4

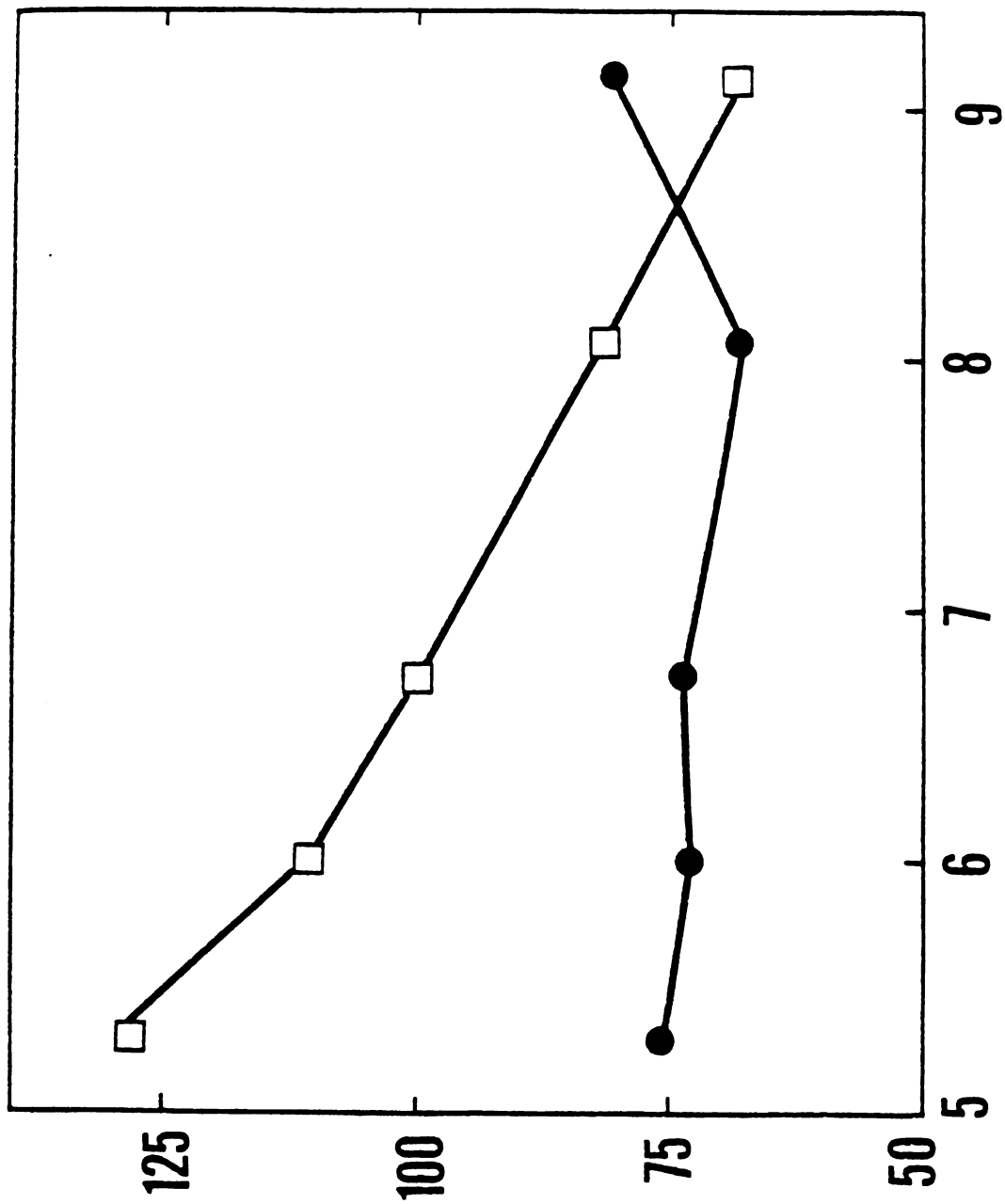


Figure 4.5

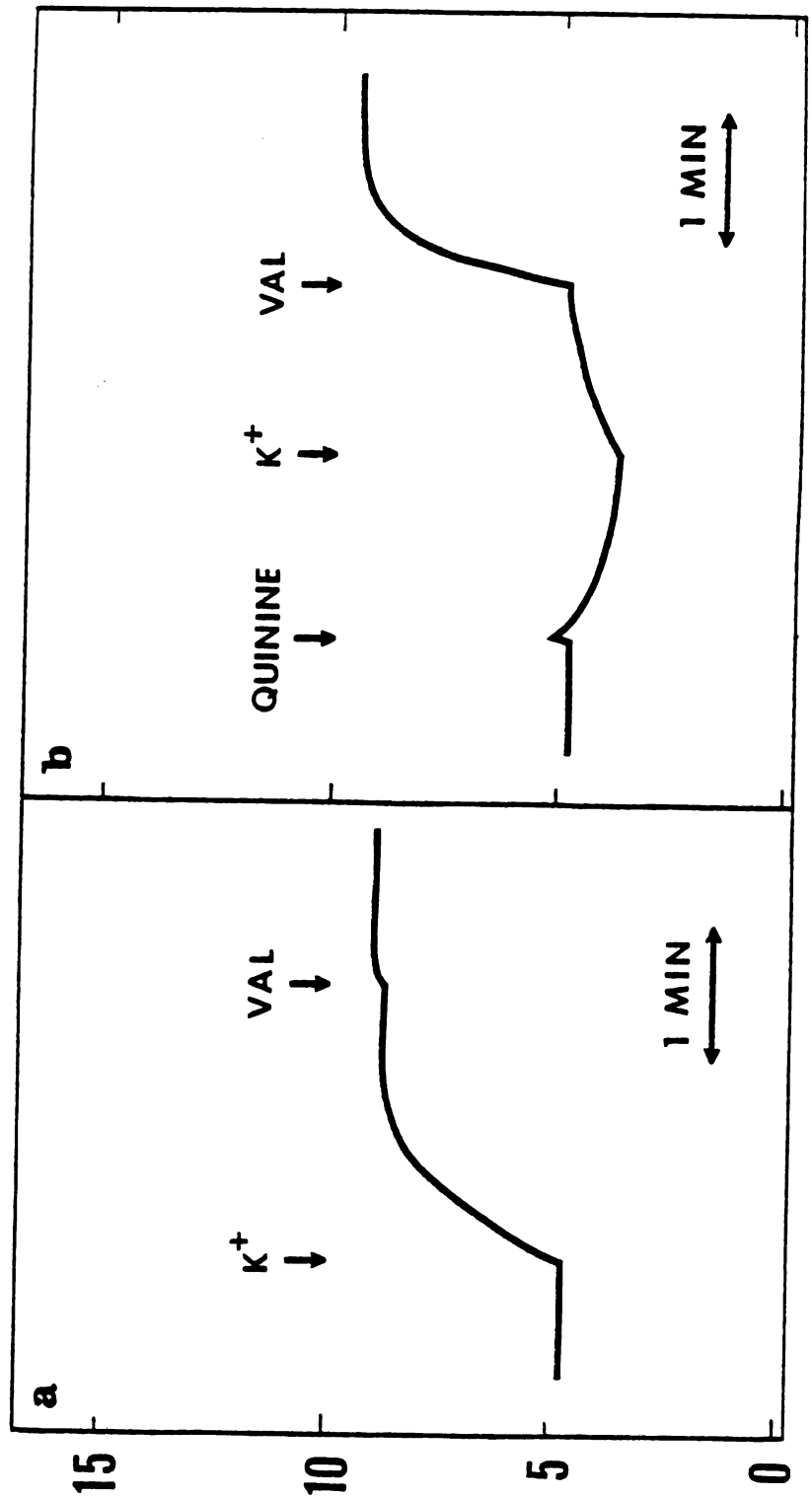


Figure 4.6

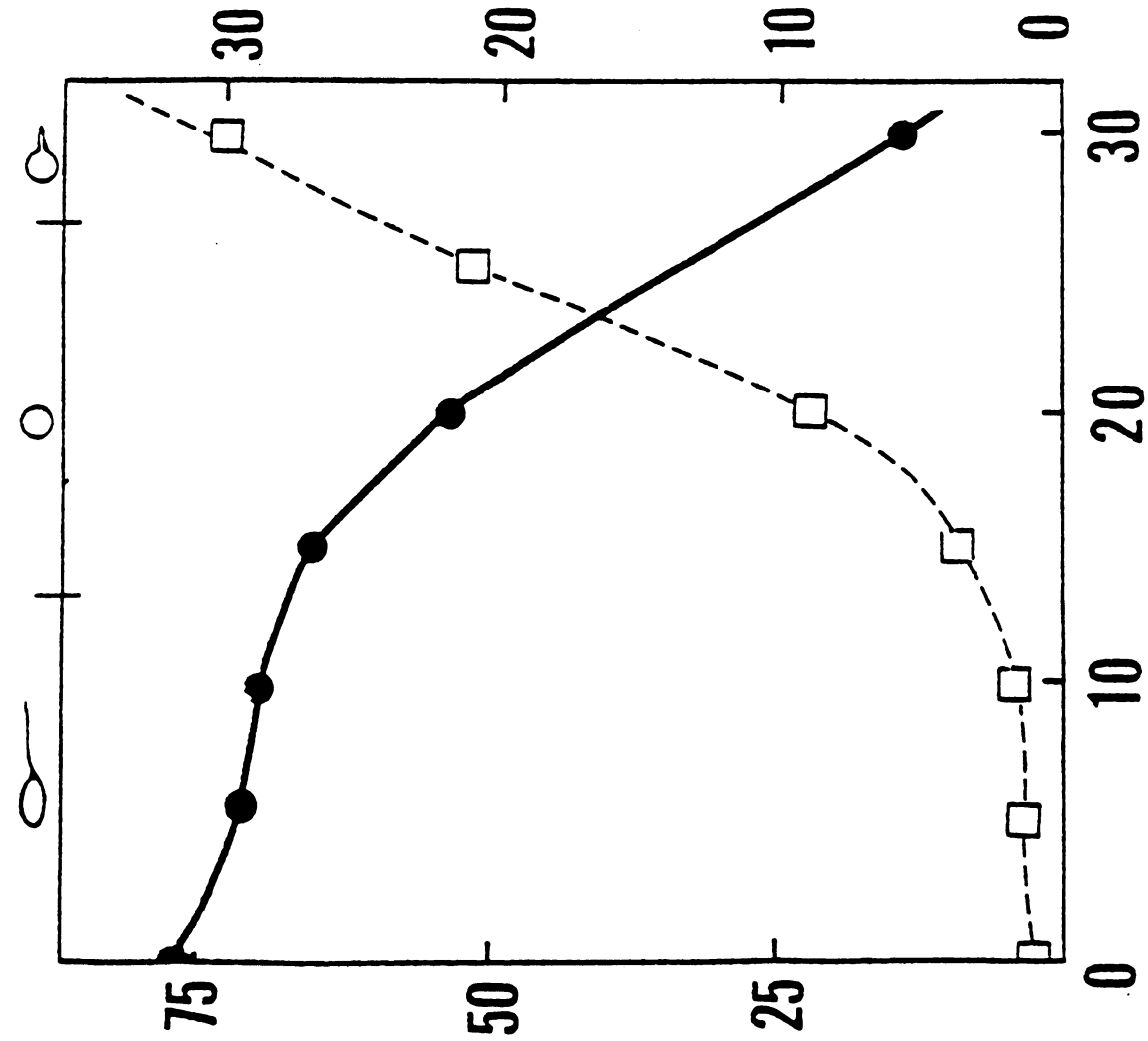


Figure 4.7

GENERAL REFERENCES

1. Aidley, D. J. (1971) 'The Physiology of Excitable Cells' Cambridge University Press.

2. Ash, J. F., & Singer, S. J. (1976) Proc. Nat. Acad. US 73:4575.

3. Becker, J. W., Cunningham, B. A., Reeke, G. N. Jr., Wang, J. L., & Edelman, G. M. (1976) In 'Concanavalin A as a Tool' Eds. Bittiger, H., & Schnebli, H. P. p. 33. Wiley.

4. Cohen, L. B., & Salzberg, B. M. (1978) Rev. Physiol. Biochem. Pharmacol. 83:35.

5. Doughty, M. J., & Dodd, G. H. (1976) Biochim. Biophys. Acta 451:592.

6. Edelman, G.M. (1976) Science 192:218.

7. Gomes, S. L., Mennucci, L., & Maia, J. C. C. (1980a) Devel. Biol. 77:157.

8. Gomes, S. L., Mennucci, L., & Maia, J. C. C. (1980b) Cell Differen. 9:169.

9. Gremo, F., Kobiler, D., & Barondes, S. H. (1978) J. Cell Biol. 79:491.

10. Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., & Eldemann, G. M. (1973) Proc. Nat. Acad. Sci. US 70:1012.

11. Hoffman, J. F. & Laris, P. C. (1974) J. Physiol. 239:315.

12. Jaffe, L. A. (1976) Nature 261:68.

13. Janoff, A. S., Haug, A. & McGroarty, E. J. (1979) Biochim. Biophys. Acta 555:56.

14. Jen, C. J., & Haug, A. (1979a) Exp. Cell Res. 120:425.

15. Jen, C. J., & Haug, A. (1979b) J. Gen. Microbiol. 115:145.

16. Laris, P. C., Pershadsingh, H. A., & Johnstone, R. M. (1976) Biochim. Biophys. Acta 436:475.

17. Lemos, E. M., & Lodi, W. R. (1975) Exp. Cell Res. 94:437.

18. Leondards, K. S., & Haug, A. (1978) Plant Physiol., Supp. 61:113.

19. Leonards, K. S., & Haug, A. (1980) Biochim. Biophys. Acta (in press).

20. Lew, V. L., & Ferreira, H. G. (1978) Curr. Top. Membr. Transp. 10:217.

21. Lovett, J. S. (1975) Bacteriol. Rev. 39:345.

22. Lund, E. J. et al. (1947) 'Bioelectric Fields and Growth' University of Texas Press.

23. Lustig, S., Asher, D., Fishman, P., Djaldetti, M., & Plunznik, D. H. (1977) J. Cell Biol. 75:388.

24. Mehlhorn, R. J. & Packer, L. (1979) Methods Enzymol. LVI:515.

25. Mills, G. L. & Cantino, E. C. (1978) Exp. Mycol. 2:99.

26. Mitchell, P. (1969) Theo. Exp. Biol. 2:159.

27. Morrill, G. A., & Ziegler, D. (1980) Devel. Biol. 74:216.

28. Myers, R. B. & Cantino, E. C. (1974) In 'Monographs in Developmental Biology' ed. Wolky, A. 8:15 Karger Press.

29. Nicolson, G. L. (1974) Intl. Re. Cyto. 39:89.

30. Nicolson, G. L. (1976a) Biochim. Biophys. Acta 457:57.

31. Nicolson, G. L. (1976b) Biochim. Biophys. Acta 458:1.

32. Nicolson, G. L. (1979) Curr. Top. Devel. Biol. 13:305.

33. Noonan, K. D., & Burger, M. M. (1973) J. Biol. Chem. 248:4286.

34. Nuccitelli, R., & Jaffe, L. F. (1976) Devel. Biol. 49:622.

35. Priestley, E. B. & Haug, A. (1968) J. Chem. Phys. 49:622.

36. Reed, P. W., & Lardy, H. A. (1972) J. Biol. Chem. 247:6970.

37. Rink, T. J., Montecucco, C., Hesketh, T. R., & Tsien, R. Y. (1980) Biochim. Biophys. Acta 595:15.

38. Rosenthal, H. E. (1967) Anal. Biochem. 20:525.

39. Schlessinger, J., Elson, E. L., Webb, W. W., Yahara, I., Rutishauser, U., & Edelman, G. M. (1977) Proc. Nat. Acad. Sci. US 74:1110.

40. Schuldiner, S., Rottenberg, H., & Avron, M. (1972) Eur. J. Biochem. 25:64.

41. Shapiro, H. M., Natale, P. J., & Kamentsky, L. A. (1979) Proc. Nat. Acad. Sci. US 76:5728.

42. Sharon, N., & Lis, H. (1975) In 'Methods in Membrane Biology' Ed. Korn, E. D. 3:147 Plenum Press.

43. Shoham, J. & Sachs, L. (1974) Exp. Cell Res. 85:8.

44. Simes, P. J., Waggoner, A. S., Wang, C.-H, & Hoffman, J. F. (1974) Biochem. 13:3315.

45. Singer, S. J., & Nicholson, G. L. (1972) Science 175:720.

46. Slayman, C. L. (1974) In 'Membrane Transport in Plants' Eds. Zimmerman, U., & Dainty, J. p 107 Springer-Verlag Press.

47. Soll, D. R., Bramberg, R., & Sonneborn, D. R. (1969) Devel. Biol. 20:183.

48. Soll, D. R., & Sonneborn, D. R. (1969) Devel. Biol. 20:218.

49. Soll, D. R., & Sonneborn, D. R. (1971) Proc. Nat. Acad. Sci. US 68:459.

50. Soll, D. R., & Sonneborn, D. R. (1972) J. Cell Sci. 10:315.

51. Spurr, A. R. (1969) J. Ultrastruc. Res. 26:31.

52. Stein, M. D., Howeard, I. K., & Sage, H. J. (1971) Arch. Biochem. Biophys. 146:353.

53. Talmadge, K. W., & Burger, M. M. (1975) MTP Intl. Rev. Sci., Biochemistry Series 1, 5:43.

54. Truesdell, L. C. & Cantino, E. C. (1971) Curr. Top. Devel. Biol. 6:1.

55. Van Brunt, J., & Harold, F. M. (1980) J. Bacteriol. 141:735.

56. Weeks, G. (1975) J. Biol. Chem. 250:6706.