THE EFFECT OF SODIUM ON THE GROWTH AND DEVELOPMENT OF AN ESTUARINE SPECIES OF PHLYCTOCHYTRIUM (CHYTRIDIALES)

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Doctor of Philosophy

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by

James P. Amon

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

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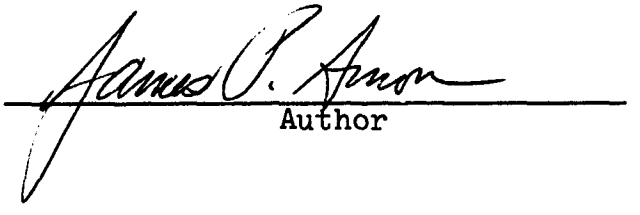

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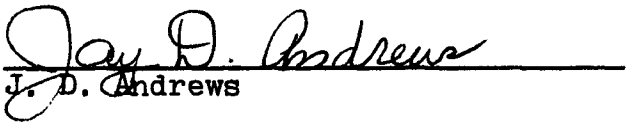

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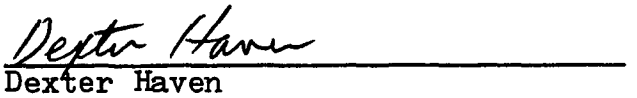

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ABSTRACT

Studies on a species of Phlyctochytrium isolated from the York River Estuary showed that the fungus required at least 21 mM sodium for minimal growth and development. The uptake and incorporation of amino acids was affected by the concentration of external sodium. Little incorporation occurred in the absence of external sodium. The requirement for sodium was not entirely osmotic. It was found that cells 15 hours past the cyst stage could develop in a sodium free medium and it was shown that this might be related to their ability to retain sodium during the same developmental period. The concentration of sodium in the medium was also shown to have affects on the size of the cells at maturity, the rate of development, and the general morphological aspects of the cell. Preliminary studies of enzyme activities showed that some enzymes of this fungus might be sensitive to the concentration of sodium. The implications of the transient requirement for sodium and the ability to retain sodium in an environment where Na^+ fluctuates are discussed.

THE EFFECT OF SODIUM ON THE GROWTH AND DEVELOPMENT OF AN ESTUARINE SPECIES OF PHLYCTOCHYTRIUM (CHYTRIDIALES)

GENERAL INTRODUCTION

The concept of categorizing the biota of the earth into broad habitat-associated groups such as freshwater, terrestrial, arctic, and marine is familiar to biologists. One of the implications of these assignments is that the organism is best suited to one habitat, and that other habitats are less desirable. However, it is not clear from these descriptions exactly what aspects of that habitat make it desirable for a particular organism. It is the purpose of this dissertation to examine one organism from the marine environment and to elucidate some of the features which make it "marine".

The organism chosen has been tentatively described by Kazama (1972) as a species of Phlyctochytrium, isolate 71-1-E, a saprophytic, eucarpic, monocentric, epibiotic, posteriorly uniflagellate fungus. It was isolated from the York River estuary, a tributary to Chesapeake Bay. It grows well in pure cultures in nutrient media composed of estuarine water or synthetic sea water. Large populations of cells developing in a nearly synchronous manner can be obtained making it possible to determine the effect of experimental variables on a selected stage of development. Synchrony is shown to be of prime importance to this work.

The work of W. Hohnk (1939, 1952, 1956) centered on the distribution of various classes of fungi in the marine environment. Some of his findings indicated that the development of both asexual and sexual structures in the marine environment might be used to determine if a fungus was truly marine. In other words, the inability to form these structures in marine waters was an indication that the environment was limiting. Controlled laboratory experiments using marine and nonmarine fungi which are known to produce sexual structures should be performed to assess the effect of various salinity regimes. While the probable correctness of Hohnk's interpretation cannot be discounted it does need further supporting evidence. Furthermore, this approach does not identify any specific factors unique to the marine environment which can be implicated in making a fungus marine. Vishniac (1955a, 1955b, 1960) used pure cultures and partially or totally defined media to determine how the concentration of sea salts would effect the growth of fungi isolated from marine environments. Her findings showed that certain obligately marine fungi required salinities of about 5 o/oo or higher and that some organisms needed NaCl in amounts far exceeding that found in freshwater environments. Other physiologists (Goldstein, 1963; Siegenthaler, Belsky and Goldstein, 1967; Sykes and Porter, 1973; Kazama and Fuller, 1973; Jennings, 1974; and MacLeod, 1965) have given additional evidence that salinity

is a limiting factor and by studying the mineral nutrition of these marine isolates have determined that sodium is an important limiting factor. Further evidence has shown that the requirement for sodium is specific and cannot be entirely replaced by potassium or an osmoticum (Vishniac, 1955a; Kazama and Fuller, 1973; Siegenthaler, Belsky, Goldstein, and Menna, 1967). It has been possible to ascribe a role for sodium in facilitating the uptake of phosphate (Sykes and Porter, 1974; and Siegenthaler, Belsky, Goldstein and Menna, 1967). In addition it has been shown that in some marine fungi there are specific interactions between the cation composition of the medium, the uptake of glucose, (Allway and Jennings, 1970b), and the sodium-potassium content of the mycelium. The treatise of Johnson and Sparrow (1961) provides additional insights into the nature of marine and estuarine fungi.

In the following sections there will be an attempt to determine the influence that sodium has in the development of this isolate Phlyctochytrium. In these sections it will be shown that 1) Na^+ and other ions at concentrations found in estuarine water are required for growth, 2) the concentration of Na^+ influences the morphological development of the fungus, 3) Na^+ is related to uptake and incorporation of protein precursors and, 4) Na^+ is retained by the cell at certain stages of development.

SECTION I THE DEVELOPMENT OF AN ARTIFICIAL SEAWATER MEDIUM AND PRELIMINARY OBSERVATIONS

INTRODUCTION

In the initial description of Phlyctochytrium isolate 71-1-E, Kazama (1972) termed it an "estuarine" fungus and stated that it would grow on a nutrient medium containing estuarine water having a salinity of 18-20 o/oo. It was not mentioned whether this fungus actually required estuarine water for development and survival. To determine if this were true the organism was inoculated into flasks containing nutrient media made from either distilled water, pond water, or various dilutions of 30 o/oo seawater and growth was observed. These results indicated that the cells could not grow well in media made either with distilled water or pond water; and that where growth did occur, in the seawater based media, the amount of growth was related to the salinity.

Since little is known of the factors which make a fungus obligately marine or estuarine (Johnson and Sparrow, 1961), it was felt that this organism could be used as a tool to pursue this question. The evidence available in the few studies which have been done indicates that sodium was the factor in the marine environment most likely to limit the growth of the fungus (MacLeod, 1965; Vishniac, 1960). Other cations, such as K^+, Mg^{++}, and

Ca^{++}, are in relatively high concentrations in the marine environment and they may also influence the cell. To determine if this was the case for Phlyctochytrium, the effect of each ion alone had to be determined, and to do this it was desirable to work with a synthetic sea-water medium. A defined medium similar to that developed by Kazama and Fuller (1973) was tested and found to support good growth. In this section information is reported which indicates the levels of Na^{+}, K^{+}, Ca^{++}, Mg^{++} which best support the growth of the fungus and the role of Na^{+} as an osmoticum is investigated.

MATERIALS AND METHODS

General Procedures

Dr. F. Kazama provided his Phlyctochytrium isolate 71-1-E (Kazama, 1972) for this investigation. The cultures were maintained on YPD or SYA (see section on "Preparation of Media") plates or slants at 4°C, or in seawater with pine pollen. To obtain an inoculum 100 mm petri dishes of YPD were spread with a zoospore suspension and incubated at room temperature (24°C) for about 50 hours. Plates showing cleaved sporangia at that time were flooded with approximately 8 ml sterile York River seawater and allowed to stand about 20 minutes. The released zoospores were immediately pipetted into an equal volume of 2X thioglycollate broth, briefly agitated by inversion and allowed to stand for 5 minutes. This treatment caused the cells to encyst. Cysts were centrifuged to pellets at about 1000g for 2 minutes, resuspended in the appropriate defined medium by a vortex mixer, recentrifuged and resuspended a second time. This suspension of cysts was then used for an inoculum. Germination and subsequent development was nearly synchronous as indicated by morphogenesis. The rinsing medium used for suspension of the inoculum was made to exclude only the ion to be used as the experimental variable. Composition of the media is found under a separate heading.

Liquid media were sterilized by filtration through 0.20 micron membrane filters (Nalgene Filter units) and 50 ml was dispensed into sterile, aluminum foil-capped Erlenmeyer flasks. Media in these flasks was shaken for at least 8 hours prior to inoculation to insure uniform aeration. Enough of a cyst suspension was added to each flask to give a final concentration of about 5×10^5 cells per ml (usually 0.20 ml). Cultures were then incubated in the dark at 24°C on a New Brunswick Gyrotary model G-2 shaker at 150 RPM. Growth was measured by increase in Lowry Protein (Lowry, et al., 1951) and in preliminary work it was found that these values were closely reflected in measurements of dry weight (see section IV for dry weight procedures). Parallel experiments were done in 5 ml aliquots contained in 30 ml disposable tissue culture flasks (Falcon 3012 Bioquest) which permitted microscopic observation of cell development during the growth period. These flasks were inoculated and set aside in the dark for one hour prior to agitation to permit cells to attach to the optical surface of the flask. Cells were agitated at 110 RPM which gives a growth rate comparible to that obtained in 50 ml flasks on the G-2 shaker.

Reagent grade chemicals were dissolved in glass distilled, deionized water to make stock solutions and were combined to formulate the final medium. No attempt was made to further purify the chemicals. The results represent two separate sets of experiments. The first

determined the approximate optimal levels of each ion tested. The second incorporated that information to better design the basic medium in which the variable was tested in the final experiment. The concentrations of compounds used ranged from zero to somewhat higher than would be expected in the normal marine environment. The highest concentration of calcium used was limited by its solubility in the basal medium.

Where the osmotic pressure of the growth medium had to be manipulated, mannitol was used as an osmoticum and the omsolarity of the final solution was determined by freezing point depression on the Osmette osmometer (Fisher Scientific).

Salinities were determined using a Model RS-7B induction salinometer (Beckman Instruments Inc.).

Composition and preparation of media used

1. YPD - autoclaved under standard conditions

Yeast extract	1 g
Peptone	1 g
Dextrose	10 g
Agar	15 g
York River Estuarine water 18 o/oo	1 liter

2. SYA - autoclaved under standard conditions

Yeast extract	2 g
Soluble starch	7.5g
Agar	15 g
York River Estuarine water 18 o/oo	1 liter

3. 2X Thiglycollate Broth - autoclaved under standard conditions

Bacto Casitone	30 g
Yeast extract	10 g
Dextrose	11 g
L - Cystine	1 g
Na Thioglycollate	1 g
York River Estuarine water 18 o/oo	1 liter

4. OS, a defined medium for "optimal" growth. The medium was prepared from stock aqueous solutions (W/V) of the following salts.

NaCl	25%
KCl	2.25%
$MgSO_4 \cdot 7H_2O$	13%
$CaCl_2 \cdot 2 H_2O$	1.5%

KH_2PO_4	2.72%
KOH	1 normal

Trace metal stock

Na_2EDTA	250 mg
$FeSO_4 \cdot 7H_2O$	150 mg
$ZnSO_4 \cdot 7H_2O$	40 mg
$MnSO_4 \cdot H_2O$	60 mg
$CoSO_4 \cdot 5H_2O$	15 mg
$CuSO_4 \cdot 5H_2O$	10 mg
H_3BO_3	100 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg
Glass Distilled Water	100 ml

To prepare the OS medium

Add, in sequence, the following to 375 ml of glass distilled water, mixing well at each step.

NaCl stock	27.5 ml
KCl stock	10.5 ml
KH_2PO_4 stock	5.0 ml
Tricine Buffer (SIGMA)	1.79 g
INKOH	4.5 ml
Trace metals stock	0.5
$MgSO_4$ stock	10.0 ml
$CaCl_2$ stock	17.5 ml
Monopotassium glutamate (L form)	1 g
Glucose	5 g

Bring to final volume of 500 ml with glass distilled water. The medium cannot be autoclaved and is ster-

ilized by filtration through a 0.20 microns or 0.45 microns membrane filter.

5. LS, a defined medium containing levels of sodium just sufficient for growth, was prepared in the same manner as OS but only 2.5 ml of the NaCl stock was used.
6. HS, a defined medium containing sodium near the maximum tolerable level, was prepared in the same manner as OS but 60 ml of NaCl stock was used.
7. Pondwater was obtained from Haynes Mill Pond located in Gloucester County, Virginia, USA.

RESULTS

The results of the experiments are presented in Figure 1a-d. In each experiment the inoculum was a cyst suspension and cells were harvested at 69 hours which allowed no more than one generation to develop.

Sodium and Potassium Requirements

The sodium chloride concentration required for maximum growth appeared to be about 0.237 M (Figure 1a). In the absence of Na^+ there was little growth and cells did not mature. It was demonstrated in a separate experiment that the need for sdoium chloride could be replaced by an equimolar amount of sodium as sulfate, but in an experiment using equimolar amounts of potassium chloride no growth was obtained. This result indicates that the requirement was for sodium alone and was not dependent on the chloride ion. At levels higher than 0.2 to 0.3 M there was a decrease in growth rate. The sodium levels used were similar to the range found in the estuarine environment and growth indicated the organism was best suited to environments with sodium concentrations lower than oceanic seawater. The broad range of tolerance indicated that the fungus was well suited to euryhaline habitats. In another experiment the result obtained above was duplicated by substituting natural seawater for artificial seawater at various sodium

concentrations (Figure 2). Maximum growth occurred at 12-20% salinity. The seawater was prepared by diluting $30^{o}/oo$ natural seawater with pond water.

The response of Phlyctochytrium to various concentrations of potassium chloride is shown in Figure 1b. Maximum growth was at a concentration above $6x10^{-3}$M K^{+} and higher concentrations, up to $3x10^{-2}$M, did not increase the yield. Cells at the lowest concentration did not grow. If the concentration was $3x10^{-3}$M the development was completed to the point of cytoplasmic cleavage but no further. The concentration required for growth was considerable higher than normally found in fresh water (Table 1) and approximated the levels present in estuarine water. Concentrations above $3x10^{-3}$M gave erratic results and it appeared that high concentrations might have inhibited growth.

Sodium and potassium both had an effect on the ultimate size of the mature cell as well as on the amount of protein produced (Tables 2 & 3). Note that the sporangial size increased as Na^{+} or K^{+} increased. The sporangial size also increased as Na^{+} ot K^{+} increased together. At the highest values the combined effect seemed to have decreased the ultimate cell diameter slightly. The amount of protein produced followed the same pattern without exception. Similar changes in size of the mature sporangium were noted in media prepared with diluted seawater (Table 4).

The difference between the summarized experiment in Table 4 and the one in Figure 1 is that it represents cells

harvested at maturity rather than at a particular time. The results indicated that concentrations of Na^+ and K^+ affected not only the rate of production of protein but also the extent of growth during the life cycle.

Magnesium and Calcium

Magnesium at a level of $1x10^{-2}$M gave near maximal growth and increases up to $3.5x10^{-2}$M did not greatly increase the final yield (Figure 1c). Two sets of experiments are represented in Figure 1c. The upper curve is for $MgCl_2$ in Na_2SO_4-based medium and the second is for $MgSO_4$ in NaCl-based medium. There is a difference between the curves but the same trends appear. Since there was a relatively low concentration of SO_4^{--} in the trace metals mix portion of the medium, the lack of growth indicated by the $MgSO_4$ curve at the zero level of $MgSO_4$ could also mean that not enough SO_4^{--} was present. The experiment on $MgCl_2$, where Na_2SO_4 provides excess SO_4^{--}, eliminates this problem. The cause of the decreased growth at higher concentrations of Mg^{++} is not known. The Mg^{++} levels required for good growth appeared to be somewhat lower than the concentrations in the estuary where the organism was isolated and a trace of Mg^{++} apparently supplied in the inoculum was sufficient to sustain moderate levels of growth. Concentrations of Mg^{++} higher than those expected in freshwater (Table 1) did stimulate increased growth. Such concentrations were higher than reported for media used to grow terrestrial fungi. In this respect the organism has nutritional requirements which

can best be satisfied by an estuarine habitat. The levels of calcium provided during the experiment were maximal and did not seem to replace the need for Mg^{++} to support good growth.

Trace amounts of calcium, probable provided by the inoculum, allowed complete development of the organism (Figure 1d) but much better growth was obtained at levels representative of those one would expect to find in the estuarine environment (Table 1). Further increases in the levels of Ca^{++} above $5x10^{-4}$ M resulted in a gradual increase in the yield up to the point where no more $CaCl_2$ could be dissolved in the medium. Cells at maturity did not vary in size with changes of Mg^{++} or Ca^{++} concentrations, as they did in varying Na^{+} or K^{+} concentrations, the average size sporangia being about 35μ.

Figure 1 Change in amount of cell material produced at different levels of Na^{+}, K^{+}, Mg^{++}, and Ca^{++}. In each experiment the concentration of the three major cations not tested was held at the level found in the OS medium.

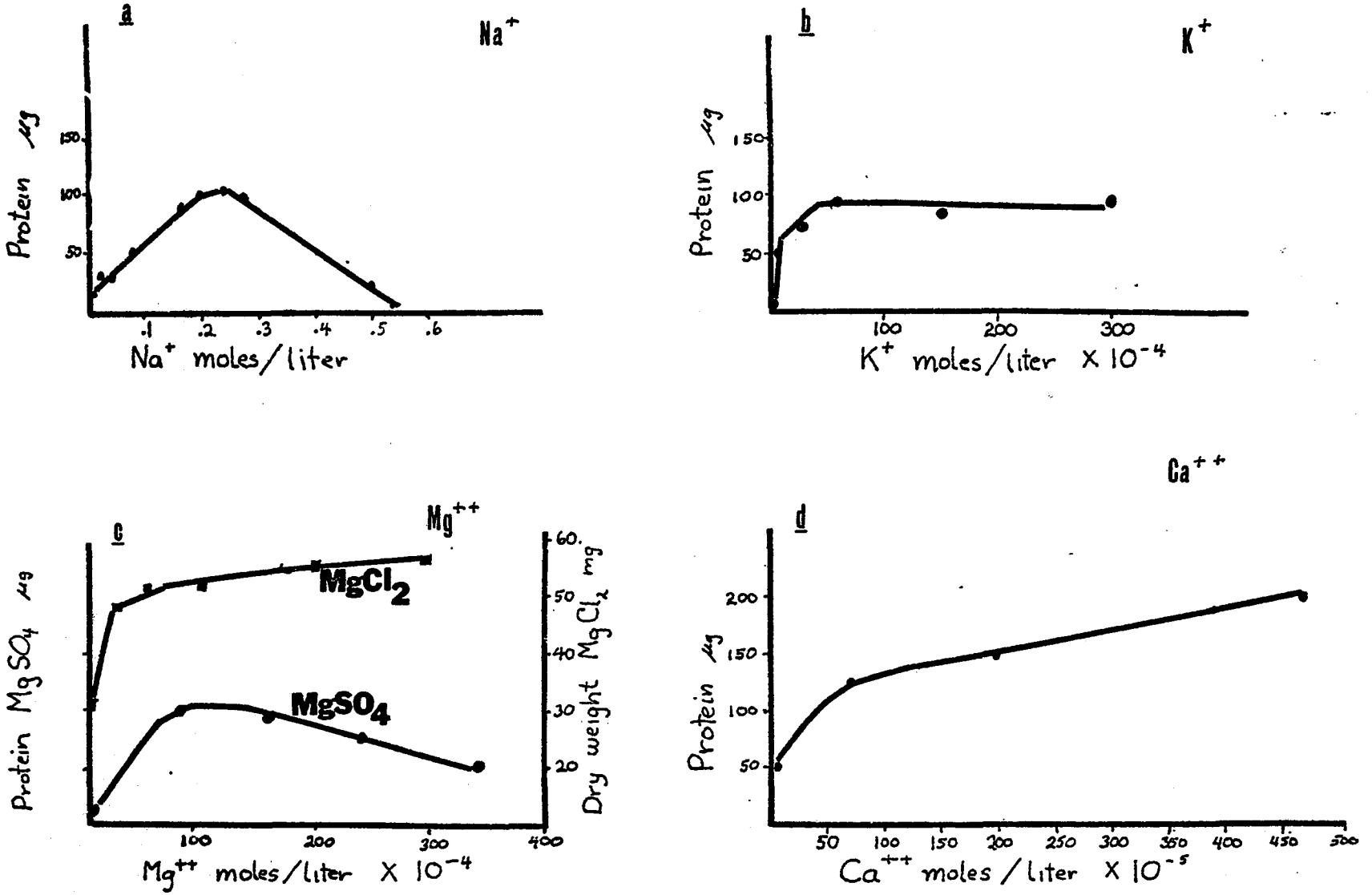

a
Na+
Protein µg
150
100
50
.1
.2
.3
.4
.5
.6
Na+ moles/liter
b
K+
Protein µg
150
100
50
100
200
300
K+ moles/liter X 10-4
c
Mg++
Protein MgSO4 µg
MgCl2
MgSO4
60.
50
40
30
20
Dry weight MgCl2 mg
100
200
300
400
Mg++ moles/liter X 10-4
Ca++
d
Protein µg
200
150
100
50
50
100
150
200
250
300
350
400
450
500
Ca++ moles/liter X 10-5

Figure 2 Increase in protein produced from a four day old culture at different salinities. Dilutions of 30°/oo seawater with pondwater, 0°/oo is pondwater.

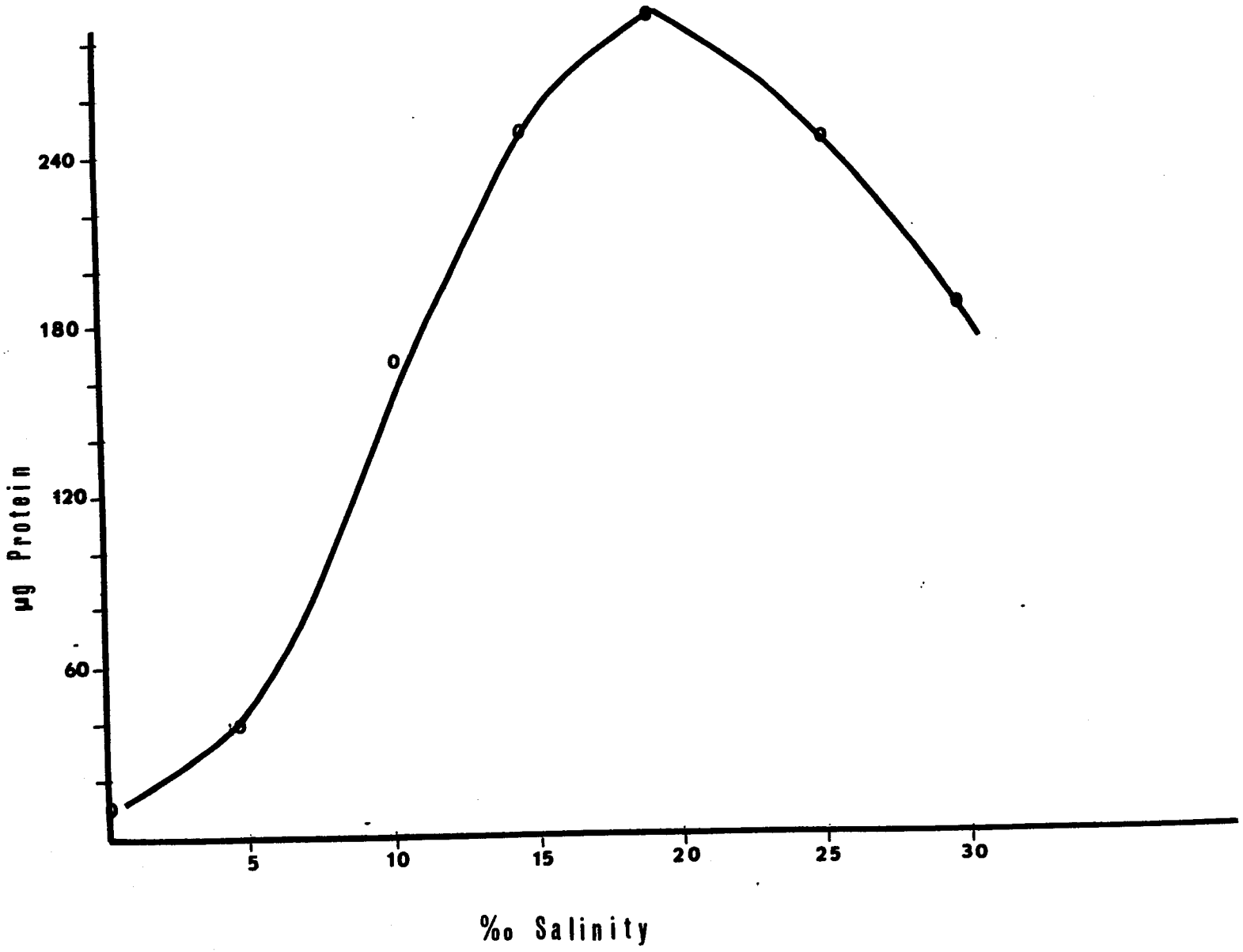

240
180
120
60
µg Protein
5
10
15
20
25
30
‰ Salinity

TABLE 1

Selected Cations in Various Media and Natural Waters
Concentrations
in Millimoles

Fluid and Reference	Na^+	K^+	Mg^{++}	Ca^{++}
Phlyctochytrium medium (OS)	237.00	6.00	22.00	4.70
"Na^+ Free" *Phlyctochytrium* medium (SF)	0.32	6.00	22.00	4.70
Seawater at approximately 13°/oo analysis by atomic absorption spectrophotometry	169.00	6.00	23.00	3.20
Freshwater from Haynes Mill Pond, Va., analysis by atomic absorption spectrophotometry	0.16	0.03	0.05	1.00
Freshwater - Biological Data Handbook 2nd edition	0.274	0.058	0.168	0.375
Euallomyces medium (Machlis, 1953)	-	10.00	1.00	0.200
Thraustochytrium medium (Goldstein, 1963)	428.00	14.10	20.30	2.00
Labyrinthula medium (Vishniac, 1955a)	459.00	14.00	20.00	2.50
Pythium medium (Kazama & Fuller, 1973)	353.00	13.00	26.00	1.00
Marine pseudomonad (Thompson & MacLeod, 1973)	220.00	10.00	26.00	-

TABLE 2

Effect of Na^+ and K^+ Concentrations on Size of Mature Cells

(Sizes in Microns)

Na^+ Concentration	Average diameter of mature sporangium at different K^+ concentrations Range indicated by ( )		
	0.0015 M	0.0030 M	0.0150 M
0.0216 M	9.6 (7.2-168)	16.8 (12-21)	19.2 (17-22)
0.230 M	31.2 (24-38)	33.6 (24-48)	45.2 (31-55)
0.460 M	48.0 (43-72)	50.4 (43-72)	40.8 (24-48)

Based on 50 measurements.

TABLE 3

Effect of Na^+ and K^+ Concentration on Increase of Lowry Protein

Cells were harvested at maturity. Concentrations are in mg of Lowry Protein.

Na^+ concentration	K^+ Concentration .0015 M	.0030 M	.0150 M
.0216 M	120	130	145
.230 M	180	220	225
.460 M	185	245	275

TABLE 4

Mature Size *vs* Salinity in Natural, Concentrated Seawater Diluted With Pondwater

Salinity °/oo	Average diameter of mature sporangium Microns	range in microns
0	9.6	8-11 (never discharged spores)
5	21.6	19-24 (complete development)
10	21.0	19-24 " "
15	21.2	19-24 " "
20	21.0	19-24 " "
25	24.0	19-29 " "
30	26.4	20-29 " "
35	27.0	21-29 " "

Averages based on 50 - 100 measurements.

Sodium Chloride and Osmotic Pressure

Since sodium chloride must be provided in large amounts it was thought that its effect was largely osmotic. To test this hypothesis the KCl concentration was brought to 0.45M in the absence of NaCl and in 0.0216M NaCl. In both instances no growth or germination occurred. With no sodium present and in 0.025M KCl, initial development only to the germling stage was observed. In 0.0216M NaCl and 0.006M KCl good growth was obtained. This result indicated that KCl could not replace NaCl at equivalent osmolarities and that it might have an inhibitory effect at low sodium concentrations as noted for *Pythium marinum* by Kazama and Fuller (1973).

In another group of experiments a neutral osmoticum, mannitol, was used to simulate the osmotic effect of NaCl. Table 5a shows that the requirement for NaCl could not be replaced by an osmoticum and that the growth response in Figure 1a was probably due to Na^+ levels at least at the lower concentrations. Table 5b shows that the decrease in the growth rate at high levels of Na^+ could be related to the increased osmotic pressure. Preliminary work showed that mannitol could not be used as a sole carbon source by the fungus.

The "Optimal" Medium

As an end result of these experiments a defined medium was designed which provided good growth. The concentration of the four major inorganic components of

the medium were based on observed experimental optima and closely approximat the ionic make-up of the environment from which the organism was isolated. The composition and preparation is given in the preceeding section on preparation of media under section 4.

TABLE 5A

Growth in varying concentrations of Na^+

Osmolarity was held at a salinity equivalent of 30°/oo with mannitol.

(Range 1050-1200 milliosmoles)

Na^+ in mM	Approximate °/oo Salinity	µ g Protein in Sample
0	0	25 (cells died)
61	5	75
122	10	85
184	15	100
245	20	115
306	25	125
372 (no Mannitol used)	30	125

TABLE 5B

Effect of osmolarity on growth using the mannitol osmoticum

Na^+ Concentration = 230 mM

Osmolarity (Milliosmoles)	Approximate °/oo Salinity	µg Protein in Sample
830 (no osmoticum)	15	150
920	20	145
1000	25	150
1090	30	150
1185	35	130
1295	40	125
1380	45	115

DISCUSSION

Nutritional studies of this organism indicate that it is different from its terrestrial and freshwater counterparts in that it requires relatively high concentrations of each of the four major cations of seawater for optimum growth (Table 1). This relationship is also demonstrated for several "marine" fungi such as Pythium marinum (Kazama & Fuller, 1973), Labyrinthula spp. (Vishniac, 1955a) and Thraustochytrium roseum (Siegenthaler, Belsky, Goldstein, & Menna, 1967; Goldstein, 1963). Some have suggested that Na^+ is related to the uptake of $PO_4^{\equiv}$ (Siegenthaler, Belsky, Goldstein, & Menna, 1967; Kazama, 1969; and Sykes & Porter, 1974) and the respiration rate (Kazama, 1969; Davidson, 1969). Siegenthaler, Belsky, Goldstein, & Menna show that in Thraustochytrium the differences in respiration rates are largely attributable to osmotic effects. Among the Chytridiales only a few species are considered to be entirely marine and to my knowledge only Phlyctochytrium mangrovii has been demonstrated to have a growth requirement for NaCl (Ulken, 1972). Nutritionally Phlyctochytrium mangrovii differs from the Phlyctochytrium in the present study in that $CaCl_2$ does not promote growth of the former.

In marine bacteria Na^+ is required for uptake of specific molecules (Thompson & MacLeod, 1971, 1973; Sprott & MacLeod, 1972) and is also required for the synthesis of specific proteins (Webb & Payne, 1971). It is

believed that Na^+ functions in some way to maintain the integrity of the cell wall of marine bacteria (Buckmire & MacLeod, 1964).

The present work further validates the generally accepted concept that the requirement for high levels of Na^+ and possibly Mg^{++} is characteristic of marine microorganisms (MacLeod, 1965; Kazama & Fuller, 1973; Vishniac, 1960; Ulken, 1972; Goldstein, 1963). In addition it is shown that at least in Phlyctochytrium (71-1-E) K^+ and Ca^{++} at levels representative of the marine environment stimulate growth. When the optimal levels of each of the four major cations are compared to the concentrations found in the estuary where the organism was isolated it appeared that the estuary can meet the requirements of the fungus. The inability of the fungus to propagate in pondwater media or sodium free media demonstrates that it would be severely limited in most terrestrial or freshwater environments.

There are several ways to state what characterizes a marine fungus based on mineral nutrition. Since some non-marine terrestrial fungi can tolerate high levels of NaCl or other salts (Tresner & Hayes, 1971) it is believed that many fungi recovered from the marine environment are of terrestrial origin (Johnson & Sparrow, 1961) and are capable of withstanding the elevated ionic concentration. There is evidence that the development of sexual stages in phycomycetous fungi may be reduced with increasing salinities (Hohnk, 1956). Other fungi such as the members

of the Blastocladiales have not been reported from marine waters (Sparrow, 1960) and are apparently intolerant of the environment. In some "terrestrial" species which are known to exist in the marine environment it has been demonstrated that the ability to produce cellulases is enhanced by increased salinity (Kazama, 1974). Booth (1971) suggests that terrestrial species found in the marine environment may represent ecotypes. It has also been suggested that some organisms may be limited to the marine environment because in addition to nutritional requirements there are close relationships to other obligately marine organisms as in the case of Pythium marinum, parasitic on Porphyra miniata (Kazama & Fuller, 1973). Remaining are those which cannot tolerate the freshwater environment and require salts at levels which can only be supplied by the marine or marine-like environment. The organism used in the present study is obviously a member of this group. The only group of fungi exclusive in this respect is the Labyrinthulaceae-Thraustochyriaceae complex and it even appears that there may be some exceptions to this generalization since some workers have shown Labyrinthula spp. capable of growth on freshwater media. (Aschner, 1958; Perkins, 1974, personal communication; and my own observations). Johnson and Sparrow make a point that there can be no all-encompassing definition of what makes a fungus marine since each individual or group interact differently with the marine environment.

The present study demonstrates that Na^+ in sufficient quantity to support growth of this estuarine Phlyctochytrium species cannot be provided by most terrestrial or aquatic habitats. Given this relationship, two major questions stand out. First, what effect does sodium have on the synthesis, growth, and ultimately morphogenesis of the organism? Second, can a functional role be assigned to sdoium in this fungus? The following sections represent an attempt to resolve these questions, and may lead to a better understanding of this obligately marine fungus.

SECTION II THE EFFECT OF SODIUM ON GROWTH AND DEVELOPMENT

INTRODUCTION

It has long been recognized that the morphology of fungi can be affected by its nutritional environment (Cochrane, 1958; Sparrow, 1960; and Hawker, 1957, 1966). Nutrition is not however, the only factor which affects the morphogenesis of fungi. Cantino (1966) has stated that an endless list of factors; physical, chemical, and biological, will effect the direction and completion of morphogenesis. In the marine and estuarine environment the affect of salinity is logically suspected of having major effects on morphogenesis. The tonicity of seawater could cause terrestrial or aquatic species to plasmolyze, and the high concentration of chloride and sodium ions might be toxic to some organisms. Cells may be obligated to expend great amounts of energy to prevent entry of excess Na^+. Yet, no structures have been identified which are peculiar to marine fungi and which would augment their survival in marine waters (Johnson & Sparrow, 1961). There have been studies which identified morphological characteristics which seem to be correlated with salinity of the marine environment. In studying the effects of seawater and NaCl on several chytrids, Scholz (1958) found that in some species the

increased salinities caused larger sporangia, enlargement and protrusion of exit papillae, and a repression of resting spore formation. Although NaCl and seawater had definite effects on the morphogenesis of the fungi he studied, none of the organisms required the presence of NaCl or seawater for their development. Ulkèn (1972) found that Phlyctochytrium mangrovii not only required NaCl for growth but that the ornamentation of its thallus could be correlated to the salinity at which it was grown. According to Hohnk (1952, 1956), the salinity of the environment has also been related to the production of sexual stages in phycomyceteous fungi and to the distribution of Phycomycetes, Ascomycetes, and Fungi Imperfecti.

In general it is thought that the Ascomycetes and Fungi Imperfecti are broadly tolerant of high salt concentrations and will grow well in hypersaline conditions (Vishniac, 1960). Basidiomycetes seem to be less tolerant of high salt concentration (Tresner and Hayes, 1971) and within the aquatic phycomycetes there may be some groups which are more suited to the marine environment than others. For example, Hohnk (1956) found that more biflagellate than uniflagellate forms could be isolated from brackish or marine waters and that the reverse was true in freshwater and terrestrial environments. As a result of these and many other observations relative to the marine environment, one must conclude that the marine environment must influence the morphogenesis of some fungi.

The importance of morphologically conservative features in classifications is well known and the variability of features used to classify some members of the Phlyctidiaceae (e.g. Phlyctochytrium & Rhizophydium) has been noted by several workers (Barr, 1969; Kazama, 1972; Koch, 1957; Sparrow, 1969; Chong & Barr, 1974; Couch, 1932). Therefore, it is of considerable importance that the changes which are caused by the normal variation in the environment be elucidated, especially in the estuarine environment where there is such a great range of salinities. It is also important that the range of changes in the growth and development which are caused by environmental parameters be established before workers attempt to assign ecological implications to their presence in that environment.

To further understand how salinity affects the development and morphogenesis, Phlyctochytrium cells in synchronous culture were observed throughout a single life cycle while growing at several different concentrations of sodium. The sodium ion was chosen because 1) preliminary experiments have shown that it does have morphogenic effects, 2) it is the major cation in seawater, and 3) previous workers have found reason to believe it has direct effects on the metabolism of obligately marine microorganisms (MacLeod, 1965; Siegenthaler, Belsky, Goldstein, & Menna, 1967). The major trends of morphogenic development, which relate to the concentration of sodium, are reported in this section.

MATERIALS AND METHODS

Cultures were established either in 30ml tissue culture flasks (5 ml medium) or in a 5 ml Zeiss plankton counting chamber (1 ml medium) as designed for use on the Zeiss inverted microscope. Except where otherwise stated the inoculum was a suspension of cysts prepared as previously described. The development of the cells in both of these chambers can be documented photographically although the optical quality of the latter is far superior. Agitation was delayed about 1 hour until most cells attached to the optical surface, after which agitation at 110 RPM was begun on the G-2 shaker. The defined medium was previously described and the variations of Na^+ content are noted in the results. The basal medium without sodium is referred to as the sodium-free (SF) medium, the medium containing 0.0216M NaCl is the low sodium (LS) medium, the one with 0.237M NaCl is referred to as the "optimal sodium" (OS) medium, and the one with 0.517M NaCl is referred to as the high sodium (HS) medium. (See section I for preparation.) The methods conditions for each experiment are stated in the text. Sizes given are averages based on 50 to 100 measurements.

RESULTS

Developmental cycle vs. concentration of sodium.

Times necessary to complete the life cycle at three concentrations of sodium were determined and the results are found in Table 6. It was shown that, within the usual limits of sodium concentrations encountered by this organism, the length of time necessary to complete the life cycle increased as sodium concentrations increased. As previously indicated (Table 2) the size of the mature sporangium increased with sodium. The life cycle as it takes place in LS, OS, and HS is shown in Figures 3, 4, and 5. The development was essentially as described by Kazama (1972) but with a few additions and exceptions. The growth here represented only that observed in the defined medium and some of the points of departure between this description and that of Kazama (1972) are possibly due in part to the nature of the medium. The main features I wish to describe here are those relating to sodium content of the medium. The cyst germinated producing an unbranched germ tube at about 1 - 2 hours in LS, and OS, and at about 3 hours in HS. Germination took place in SF at about the same time as in LS. By the fourth hour there was a difference in the average lengths of the germ tube representing different rates of development. At four hours the germ tube was 33μ long in SF or LS, 22μ long in OS, and

11μ long in HS. In SF there was often a slight swelling at the terminus of the germ tube and in cultures extensively washed with SF this was the maximum extent of development (Figure 6). The rhizoid produced the first branch at about +6hours in LS, at 7 hours in OS, and at 8.5 to 9 hours in HS. Further branching took place at irregular intervals of time and could occur at any point along the main rhizoid. By the ninth or tenth hour the main rhizoid had reached what appeared to be its maximum length before it became indistinguishable from the new secondary and tertiary branches. At this time it was approximately 55μ long in LS and OS, and 35μ long in HS. During the next 10-12 hours the thallus enlarged from a diameter of about 7μ to 8μ in LS, 12μ in OS, and 12μ in HS. The branching pattern appeared to differ with the sodium level. In LS there was a tendency for many secondary rhizoids to form a right angle to the primary rhizoid during this period. At higher Na^+ concentrations the tendency was toward branching at an angle of less than 90^o to the distal portion of the rhizoid (Figures 3b, 4c,d, and 5d-g). By the time development was complete the maximum extent of branching was much less in LS than in OS or HS. During development of the thallus there was a gradual increase in the number and appearance of inclusion bodies. With a brightfield or phase contrast microscope the zoosore and cyst showed a single highly refractile globule.. This structure was the only easily recognizable structure until

about the 15th to 24th hour of development. By using phase contrast microscopy it was possible to see what appeared to be a single nucleus and the other observable inclusions increased rapidly in number. The timing of these events also changed with the concentration of sodium. In LS it begins at about 15-22 hours, in OS at 18-22 hours, and HS at 22-24 hours. The proliferation of these inclusions continued and eventually the cell was filled with vacuoles, nuclei, and additional inclusions in a homogeneous pattern. Later, the inclusion bodies became concentrated toward the basal portion of the cell and the portion above it contained clear cytoplasm (a stage not recorded by Kazama, 1972). This occurred at approximately 42 hours in LS, 48 hours in OS, and 65 hours in HS. Observations have shown that the formation of exit papillae can begin once the cytoplasm has taken on this divided appearance. The formation of the exit papillae appeared to begin at an earlier developmental stage in HS where it was noted soon after the clear area formed (60-70 hr.). In OS it was not evident until just prior to cleavage (65-75 hr). The size and form of the exit papillae also varied with the sodium content of the medium. In LS and OS the papillae were small and did not protrude greatly above the circumference of the sporangium wall (Fig. 7) but in HS the wall of the sporangium expanded as if under pressure in the location where the papillae would form and remained so until prior to cleavage when the plug apparently became fully differentiated. Prior to cleavage the clear

area underlying the papillae was filled with granular material once again. The redistribution of granular material prior to cleavage also occurred in LS and OS. The time at which papillae were formed is discussed by Kazama (1972) who stated that papillae were formed during or after cleavage whereas Blastocladiella formed them before cleavage. The present observations have shown that the onset of this event may vary with the salinity (Na^+) of the medium and this made it difficult to ascribe any phylogenetic relationship based on the time of exit papilla formation. Porter and Smiley(1974) have determined that in the marine Phlyctochytrium they examined the sites of exit papillae were determined prior to cleavage.

Figure 3. Life history in LS

Micrographs show features of development as represented in LS. a) Germling at 4 hours showing exceptionally elongate germ tube and single refractile inclusion. b) Formation of numerous perpendicular branches along germ tube. At 18 hours some cells just beginning to show more than one inclusion, nucleus visible (may be dividing), c) By 29 hours numerous inclusions and vacuoles are visable. Rhizoid network has increased. d) Thallus at 40 hours has enlarged and cytoplasm is segregates to granules in basal part and few in the area above, extensive, but not dense rhizoids. e) At 55 hours granulation of the cell is again homogeneous, cleavage has begun and exit papillae are beginning to form. f) At 60+ hours cleavage is complete and exit papillae are well differentiated. Zoospores will be released within about 1 hour. Phase contrast microphotographs. Bar = 20 μ .

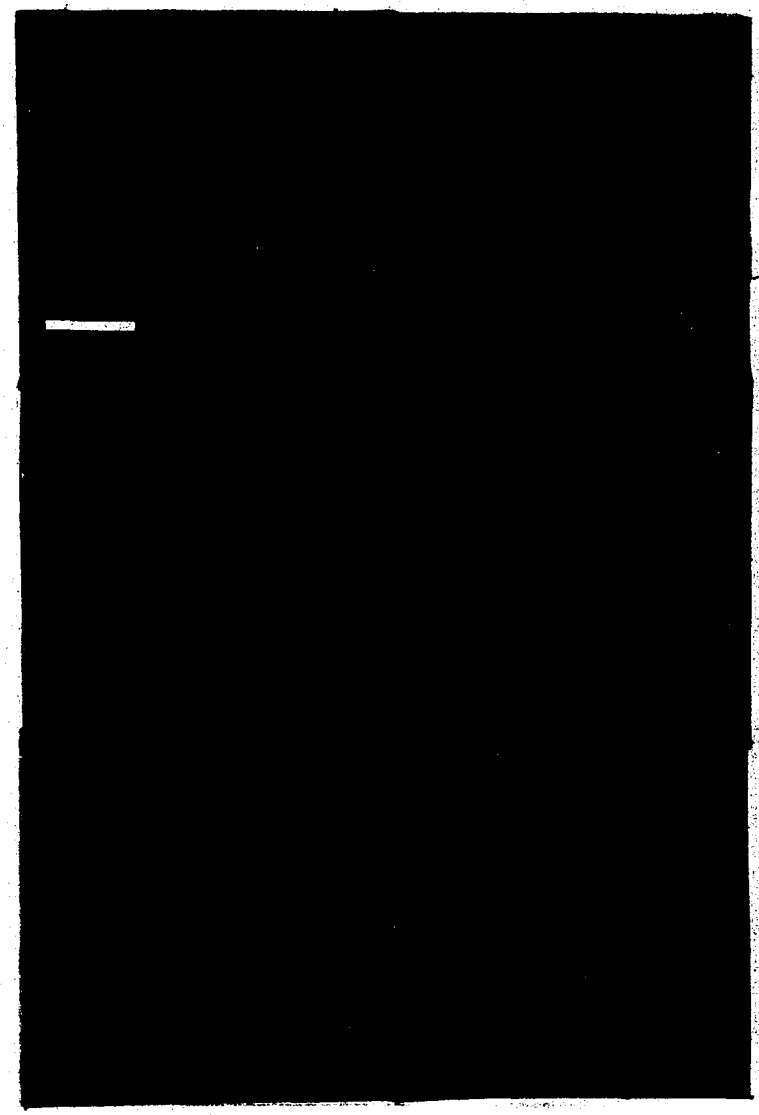


Figure 4. Life history in OS

a) Cyst and germling each showing one refractile inclusion less than 1 hour after inoculation. b) At 1 hour germ tube has developed to about ½ its maximum length. c) At 4-8 hours the first branching is observed and the germ tube is not as elongate as in LS, note the less perpendicular nature of branching. d) Secondary branches are forming at 12 hours; only a single bright inclusion and 1 nucleus are visable. e) At 12 to 18 hours the cell becomes binucleate. f) More extensive branching and thallus enlargement noticeable by +25 hours, inclusions increasing and 4 or more nuclei present. Vacuoles present but not obvious in photo. g) Many inclusions by 32 hours; rhizoids have increased in complexity, being already much more dense than in LS. h) At about 50 hours the cytoplasm is segregated to basal granules and an overlying area; rhizoid pattern has reached maximum development. i) A few hours later papillae begin to form. j) At 70 hours cleavage beginning after redistribution of granulation. k) cleavage complete and exit papillae differentiated, 1) empty sporangium after zoospore discharge at 75 hours. Bar=20 μ, a,d,f,g,i,k,l taken using 40x phase contrast objective; e,h,j, 100x phase contrast objective.

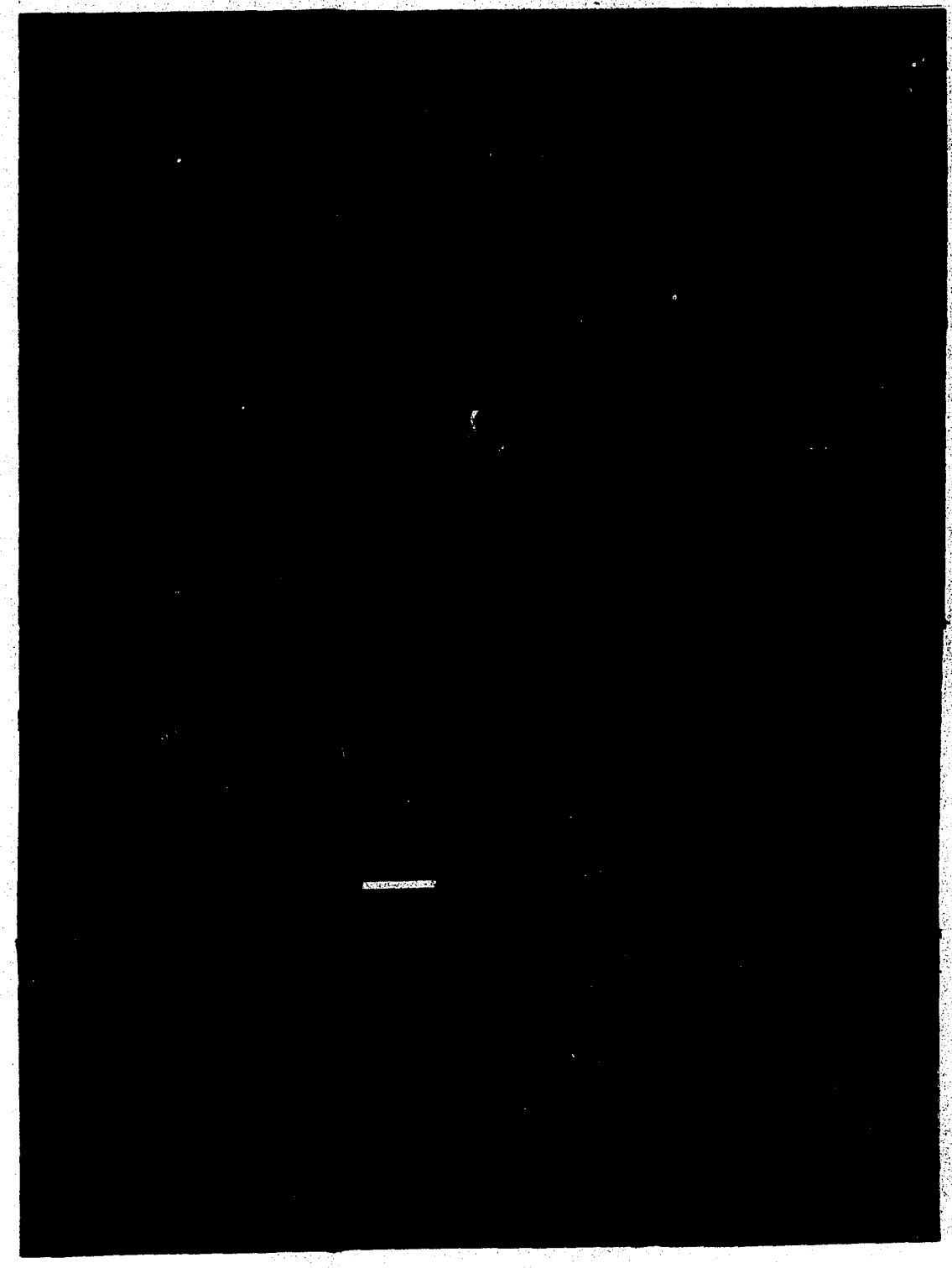


Figure 5. Life history in HS

a) Cyst with single refractile inclusion at 0 time, b) Germination beginning at 2 hours, c) Germ tube at 5 hours. d) Lateral branches forming at 8 hours, single inclusion still present. e) More extensive branching, still 1 nucleus at 19 hours. f) Two nuclei, vacuoles and additional inclusions appear at 22 hours. g) By 24 hours four nuclei present. h) At 32 hours granulation has increased and rhizoids have begun stronger development. i) By 49 hours there is still greater enlargement of thallus and greater density of rhizoids. j) At 68 hours the cytoplasm shows basally granulated cytoplasm. k) Redistribution of cytoplasmic granulation just prior to cleavage. l) At 74 hours beginning of differentiation of exit papillae and cytoplasmic cleavage, and completion at about 80 hours. m & n) The protruding exit papillae which become noticeable at about 70-80 hours at this Na^+ concentration.

Photos a-d are with 100x objective, e-m with 40x phase objective, n enlargement from 16x objective. Bar= 20μ .

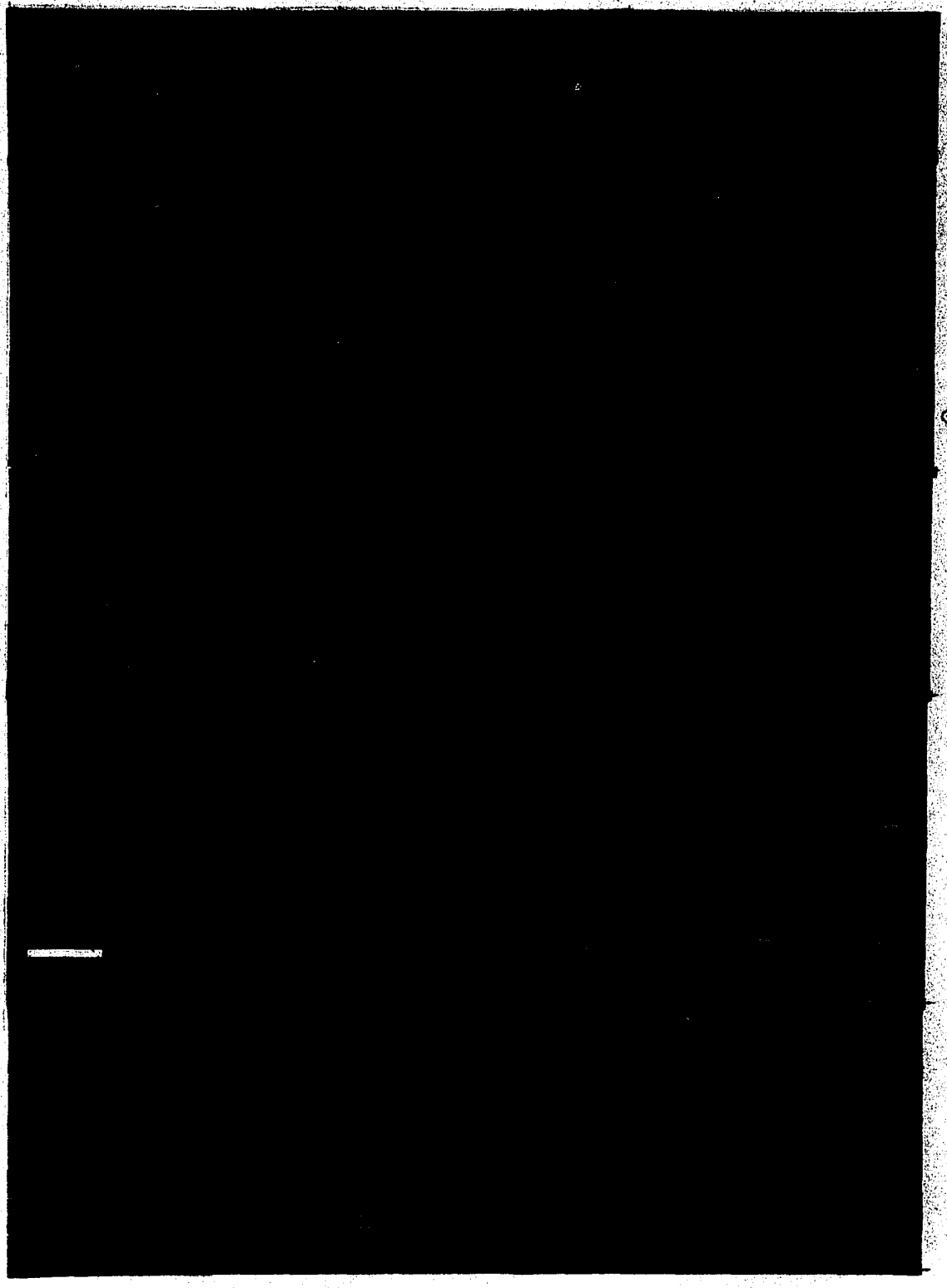
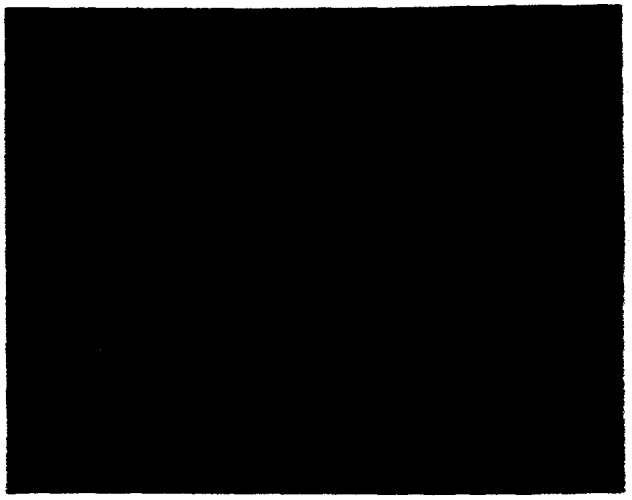


Figure 6. A photograph from a tissue culture flask showing the maximum extent of development in SF. Note the knoblike structure at the end of the germ tube. The knob is always seen under these conditions. Bar=20 μ.



Development in a sodium free medium (SF)

Cells grown in OS for various times were placed in SF and their development was observed. Table 7 shows how the ability to complete development in SF changes with the time of incubation in OS. After about 15 hours in OS the cell attained the ability to survive and produce zoospores in SF. These zoospores do not however, produce another generation in SF. In an expanded experiment it was found that the concentration of sodium in the initial growth medium had an effect on the time required for the cell to attain this ability to develop in SF (Table 7). Cells completing development in SF were somewhat smaller and had less extensive rhizoids than the controls in OS and in most respects the development was like those developing in LS. Zoospores produced from cells maturing in SF could swim and apparently were not affected by the absence of sodium. The zoospores could encyst and usually a germ tube could develop in this second generation but the number of cells germinating was low and complete development was never achieved. Repeated attempts to obtain a population of cells which could complete two generations in SF were unsuccessful.

TABLE 6

Time Required to Complete Life Cycle at Different Na^+ Concentrations (Cyst Inoculum)

mM Na^+	
0	does not complete cycle
21.6	60 - 70 hours
237	70 - 80 hours
417	80 - 100 hours

TABLE 7

Effect of Incubation in LS, OS, and HS on the Subsequent Development in SF

Time of change to SF (hrs.)	% Completing development if from:		
	LS	OS	HS
+ 2	0	0	0
+ 4	0	0	0
+ 8	0	0	0
+ 12	0	0	0
+ 16	<1	25	50
+ 20	1	50	>95
+ 24	20	>95	>95
+ 28	50	>95	>95
+ 34	nearly all	>95	>95

The effect of the osmotic pressure of the medium on the development of enlarged exit papillae and other features.

To test the effect of osmotic pressure on the formation of exit papillae, OS (831 milliosmoles) was brought to approximately 1590 milliosmoles with a mannitol osmoticum. Cells grown in this medium were compared to those in HS and OS without osmoticum and it was found that in HS and OS with osmoticum, enlarged exit papillae were formed (Fig. 7). In addition observations during the experiment reported in Table 5b showed that at the 1185 to 1380 milliosmoles range enlarged papillae were observed (not illustrated).

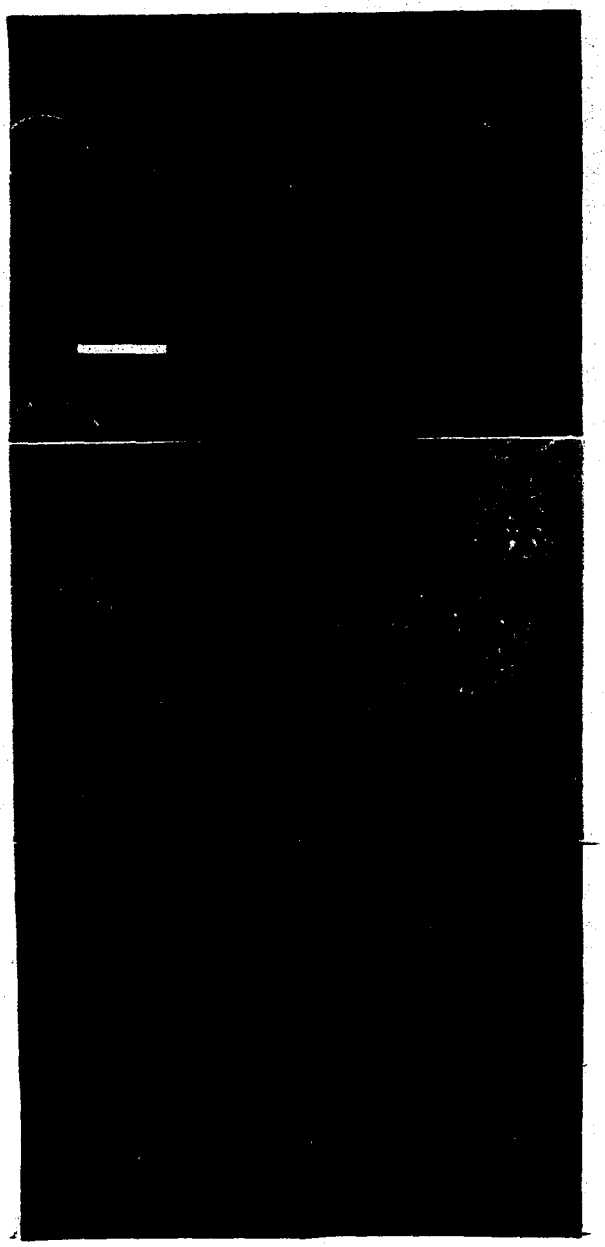
At exceptionally high osmotic pressure (1590 milliosmoles) the rhizoids became shorter and appeared thickened and blunted. This same effect was noticed only in media with 0.560M NaCl and not at lower concentrations. The other features of development seemed to be independent of osmotic pressure and responded to changes in Na^+ regardless of the osmolarity of the medium.

Figure 7. Protrusions and salinity or osmolarity.

a) Cells grown in HS and which show the large protrusion characteristic of this salinity.

b) Cells grown in OS osmotically adjusted with mannitol to the tonicity of the medium in a. Note the same large protrusions where exit papillae are forming.

c) Cells grown in OS and having no large protrusions during formation of exit papillae.



DISCUSSION

It has been demonstrated that the rate of growth and the form of growth are influenced by the concentration of NaCl and in some cases by osmotic pressure of the medium. With the exception of the time of papilla formation the events of development seem to occur in the same sequence with time as the only modifying factor. Lower concentrations of NaCl permit rapid cycling but the extent of growth is reduced considerably. There are also differences in the branching pattern and extent of development of the rhizoids but this is a relatively subtle difference. The time of exit papillae formation is more obvious and the initiation of this event appears earliest at the highest concentration of sodium. It also appears that this feature of development is more closely related to the osmotic pressure of the medium than to its sodium contents.

Morphogenic effects due to changes in the marine environment are not unexpected. In the chytrids similar effects on the size of sporangia and the morphology of exit papillae have been noted by Scholz (1958). Separation of the effects of Na^+ from the osmotic effects has not been previously elucidated. The enlargement of sporangia cannot be attributed directly to osmotic pressure, since

a hypertonic solution would result in plasmolysis, but the increased size is instead a result of growing in a medium having a high osmotic pressure.

Similar circumstances may be responsible for the observed decreases in morphogenesis of sexual stages of Oomycetes in brackish water and seawater, but investigations of this kind are not available for comparison. Kazama's (1969) investigation of a marine Pythium indicates that sexual stages are produced in an artificial medium with a relatively high salinity, but his fungus has been shown to be "marine" by its growth response to Na^+. Further comparison of osmotic pressures and Na^+ levels on marine and non-marine forms would be useful.

The form of exit papillae and size of the cell at maturity is also important from a taxonomic standpoint. In the Phlyctidiaceae, members of the genus Rhizophydium are often differentiated on the basis of the type of exit papillae and the size of the sporangium (Sparrow, 1960). While it is true that this organism is tentatively identified as a species of Phlyctochytrium (Kazama, 1972), it is possible that it would fit into the genus Rhizophydium or some other group as implied by Chong & Barr (1974). A problem arises when it is recognized that htere is a broad variation in the form of this fungus in a simple defined medium when only one significant variable such as Na^+ is manipulated. Compounding the taxonomic problems is the tendency for the investigator, who isolates a fungus from the marine environment, to taxon-

omically characterize it in media or on substrates combined with waters representative of the salinity from which the organism was isolated - regardless of the effects on the morphology of the organism. It should become obvious that in order to characterize new isolates or reinvestigate the established genera we must be aware of the morphological plasticity inherent in the organism we are describing and of the organism to which we compare it. It is also important to recognize that the nutritional value of the growth medium is a combination of both organic and inorganic factors and that slight variations of the inorganic fraction may have major effects on the fungus.

Morphogenic response to the range of salinities is also important for ecological reasons. The size of the sporangium will determine the number of reproductive units which will be produced and survival may be dependent upon a high reproductive rate. The combined effect of the length of the cycle and the ultimate size of the sporangium (and thereby the number of spores) would indicate that up to about 0.5M NaCl (35 to 40°/oo salinity) the reproductive potential increases with the salt content. In addition this "obligately marine" fungus has the ability to tolerate the absence of NaCl for an extended period of time during the mid portion of its life cycle. This probable increases the survival potential of the organism in the intertidal estuarine environment where salinities fluctuate and occasional inundation with freshwater is probable.

The ability to grow in Na^+ free media suggests that either the organism changes its nutritional requirements as it develops or that it acquires the ability to somehow satisfy those requirements, possible by retention of Na^+. Since Na^+ has been implicated in uptake phenomena in Labyrinthula and Thraustochytrium (Siegenthaler, Belsky, Goldstein, & Menna, 1967; Sykes & Porter, 1973), and protein synthesis in marine bacteria (Webb & Payne, 1971), it is possible that the level of Na^+ effects the morphogenesis of Phlyctochytrium through regulation of these parameters.

It is also possible that the rate of morphogenesis could be controlled by some salinity dependent factor. Griffiths and Morita (1973) report that salinity has an effect on the hexose monophosphate pathway in a marine bacterium. In a system of this sort the rate of development could be controlled by the availability of ribose for RNA synthesis. Demonstration of a similar system in Phlyctochytrium would represent a major finding. The finding that development continues in the absence of sodium begins to shed light on other problem areas. For example, it was previously supposed that the effect of environmental factors like Na^+ were directly influencing the cytoplasmic activities, but now it must be suspected that this is not the case and that other relationships must be investigated.

Without the use of synchronously growing populations of cells, as has been done in the present work, it would be impossible to recognize if the relationships of Na^+ to developmental stages existed. Synchronously developing cells

are also important in the present investigation where the cells ability to retain Na^+ (Section IV) is investigated. It is also useful in the investigation of the way in which Na^+ effects the pattern of protein and RNA synthesis and how synthetic events may relate to the uptake and incorporation of exogenously supplied substrates (Section III).

SECTION III ROLE OF SODIUM IN THE UPTAKE AND INCORPORATION OF PRECURSORS TO PROTEIN AND RNA

INTRODUCTION

Since sodium has been shown to influence the morphogenesis of this fungus it is probable that it in some way affects the synthetic events which produce that developmental change. However, there are several biochemical steps which are involved in producing a morphogenic result from an exogenously supplied source of nutrient. Any of these steps may be affected by the conditions of the cellular environment and result in a distinct mode of development. Basically, the cell must bring nutrients into the cell, chemically alter them, and incorporate them into the structural or functional protein of the cell. Halvorson and Cowie (1961) have described a system of amino acid pools in yeast cells based on extractability in cold trichloroacetic acid (TCA) and cold water. I have used these fractions to describe how Na^+ influences the entry of precursors into each pool. In this scheme substances extractable in cold water without TCA are presumed to be loosely bound or unbound inside the cell and are not held on one side of the membrane against a concentration gradient. Halvorson and Cowie (1961) call this the "expandable pool". This pool is also extracted by cold TCA. Their

"internal pool" is extractable with cold TCA but not water alone. The meaning of and reasons for this compartmentalization by extractability are not entirely clear. Since larger molecules of RNA and protein are precipitated by TCA (their third pool) it is assumed that the internal pool must represent some change in the activity of the molecule which means that it is retained against a concentration gradient. The cold TCA extract must also represent molecules which are too small to be precipitated by TCA.

MATERIALS AND METHODS

Experiments were carried out in 30 ml tissue culture flasks with 5 ml of the defined medium. The inoculum was prepared as previously stated. For most of the experiments it was important that the inoculum was kept low, 1×10^5 cells per ml or less, to prevent overcrowding. Overcrowding caused the cells to detach from the surface of the flask and also contributed to a measureable decrease in synchronous development.

At the end of the labelling period (10 minutes unless otherwise noted) the cells were harvested and extracted to obtain the label in each of the previously described pools. First the cells were "chased" with an unlabelled form of the isotope being used. This nearly stopped the uptake of label. The liquid over the cells was drained away, the cells were rinsed quickly with 2 ml of unlabelled medium, and then flooded with 5 ml of the same fluid. The cells stood in the solution for 5 minutes at room temperature (24°C) and then the supernatant (the water soluble pool) was placed in a 20 ml scintillation vial and evaporated to dryness at 50 to 70°C. The cells remaining in the flask were briefly rinsed as before and flooded with 5ml of ice cold 5% TCA and were refrigerated at 4°C overnight for extraction of the cold TCA soluble pool. Glass beads, 3mm in diameter, were then added to the flask and

agitated to dislodge and disrupt the cells. Agitation was continued just long enough to insure removal of all the cells from the surface of the flask and then the flasks were allowed to remain in the cold for an additional hour. The suspension was then separated into the TCA precipitate and TCA soluble (filtrate) fractions by filtration through a Millipore type EH 0.5 micron cellulose acetate filter. Both fractions were placed in scintillation vials and evaporated to dryness. After drying, each sample was wetted with one or two drops of distilled water and then the sample was solubilized with NCS (Nuclear Chicago Solubilizer) and dissolved in 10 ml toluene scintillion counting cocktail: toluene 1000 ml, PPO (2,5-diphenyloxazole) 6g, POPOP (triphenyloleoxazole) 0.5g. After sitting overnight, samples were counted on a Beckman Model LS-50 Liquid Scintillation Counter. The LS-50 employs an external standard to assess the relative quench in each sample and has an automatic quench compensator, both of which were used in all counts.

In single label experiments counts were made using the widest counting windows for the appropriate isotope. In dual labelling experiments the narrow H-3 and C-14 windows were used so that spillovers from tritium into C-14 would be negligible (Bransome, 1970). Curves for the percent efficiency and percent spillover of C-14 into H-3 were established relative to the external standard

ratio. Counting time was made long enough to insure no more than 3% counting error. The following formula could be used to compute the DPM*of each isotope.

$$\text{C-14 DPM} = \frac{\text{CPM C-14}}{\text{efficiency C-14}}$$

$$\text{H-3 DPM} = \frac{\text{CPM H-13 - C-14 spillover CPM}}{\text{efficiency H-3}}$$

Isotopes were obtained from ICN and were used at the following concentrations:

C-14 valine (UL)	2.5×10^{-8} mM/ml
C-14 dextrose (UL)	5.0×10^{-7} mM/ml
C-14 glutamic acid (UL)	5.0×10^{-6} mM/ml
H-3 uridine (5-H-3)	2.5×10^{-9} mM/ml
H-3 thymidine (methyl H-3)	2.17×10^{-9} mM/ml

Throughout an experiment the development and morphological detail of the cell could be observed in the flasks at 400x on a bright field microscope. In this way it was possible to precisely ascribe a particular level of isotope uptake to a certain developmental stage.

Measurement of respiration was done with a Yellow Springs Instruments Oxygen Meter, Model 54 in a 150 ml vessel. The medium was saturated with oxygen by strong agitation prior to introduction of a constant inoculum of cells. The temperature for all experiments was maintained at 24°C and the incubation was in the dark except during the periods of observation.

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* Disentegrations per minute

RESULTS

Figure 9a shows the change in the rate of protein synthesis as the synchronized cells go through their development when grown under optimal conditions in the OS medium. Figure 9b represents the same experiment except that RNA synthesis is being measured. The pattern of peaks produced in both graphs can be used as a base line on which to compare results from experiments where the Na^+ concentration is greater or less than optimal.

With both kinds of label it was found that there was a very low rate of synthesis immediately after the cells had been induced to encyst, but that during the next 35 hours this rate increased greatly. During this time there were two readily definable peaks, one at approximately 4 hours and the other at about 30 hours. The first peak corresponded closely with the elongation of the initial rhizoid and the second with initiation of branching, rapid cell enlargement, increased number of granular inclusions and possibly with onset of nuclear divisions as indicated by incorporation of labelled thymidine (See section below). As this second peak declined the branching of rhizoids was nearly complete. The next period of development seemed to be one of

growth and differentiation, rather than growth alone. It was characterized by lower rates of labelled RNA and protein precurser incorporation. The trough at the beginning of this period seemed to be correlated with the stage of development in which the cytoplasmic granules were all located in the basal portion of the thallus. It must be also noted that the clear cytoplasmic area above the granules was underlying the area where exit papillae would be produced at a later stage. The segregation of the cytoplasm in the above manner lasted only until the next peak of synthesis began. This peak reached its maximum during the cleavage of the cytoplasm into zoospore precursors. In some experiments on protein synthesis there may have been a small peak just preceeding the one just described but its relationship to morphological change was not obvious. In the RNA synthesis measurements a larger peak was noted prior to the cleavage (Fig. 9b). There was some correlation between this peak and the redistribution of cytoplasmic granules. However, since there does not seem to be a corresponding peak of protein synthesis, it may be well to consider another alternative. The RNA may not represent RNA as is usually measured in this experiment. Instead, it may be RNA being synthesized for incorporation into the "nuclear cap" (Kazama, 1973) of the zoospore which forms at that time. Exit papillae formation was not associated with a particular peak and initiation of this event was observed both before and

after cleavage. It usually occurred before cleavage when the cells were grown in HS and after cleavage when they were grown in LS.

The above information is based on the combined data from eight experiments using duplicate or triplicate samples and forms a baseline of information based on development in the optimal (OS) medium. The patterns of synthesis which were produced by cells grown in LS are shown in Figure 8, and those grown in HS are shown in Figure 10. The general pattern of three protein peaks and four RNA peaks was found in each medium. Growth and development of cysts did not occur in SF and therefore was not illustrated. The major difference between synthesis in OS and that at LS, or HS was the time at which the successive peaks were formed. Generally, the succession relative to OS was shorter in LS and longer in HS.

To determine if the observed patterns were due to the osmotic pressure of the growth medium, the first 32 hours of the experiments were repeated with the following variations. LS was brought to the same osmolarity as OS with mannitol (765-827 milliosmoles) and OS was brought to the same osmolarity as HS (1300-1380 milliosmoles). They were then compared to controls in LS, OS, and HS. While there were variations in the magnitude of synthesis the patterns closely reflected the Na^+ concentration of the medium rather than the osmolarity (Figures 11 and 12).

Figures 8, 9, and 10. Change in the rate of protein and RNA synthesis* at different concentrations of Na^+. DPM are given for a constant number of cells pulse labelled for ten minutes. Curves presented are composites from several overlapping experiments.

The developmental stages which correspond to the various peaks are shown in Figure 9. a= protein synthesis, b= RNA synthesis.

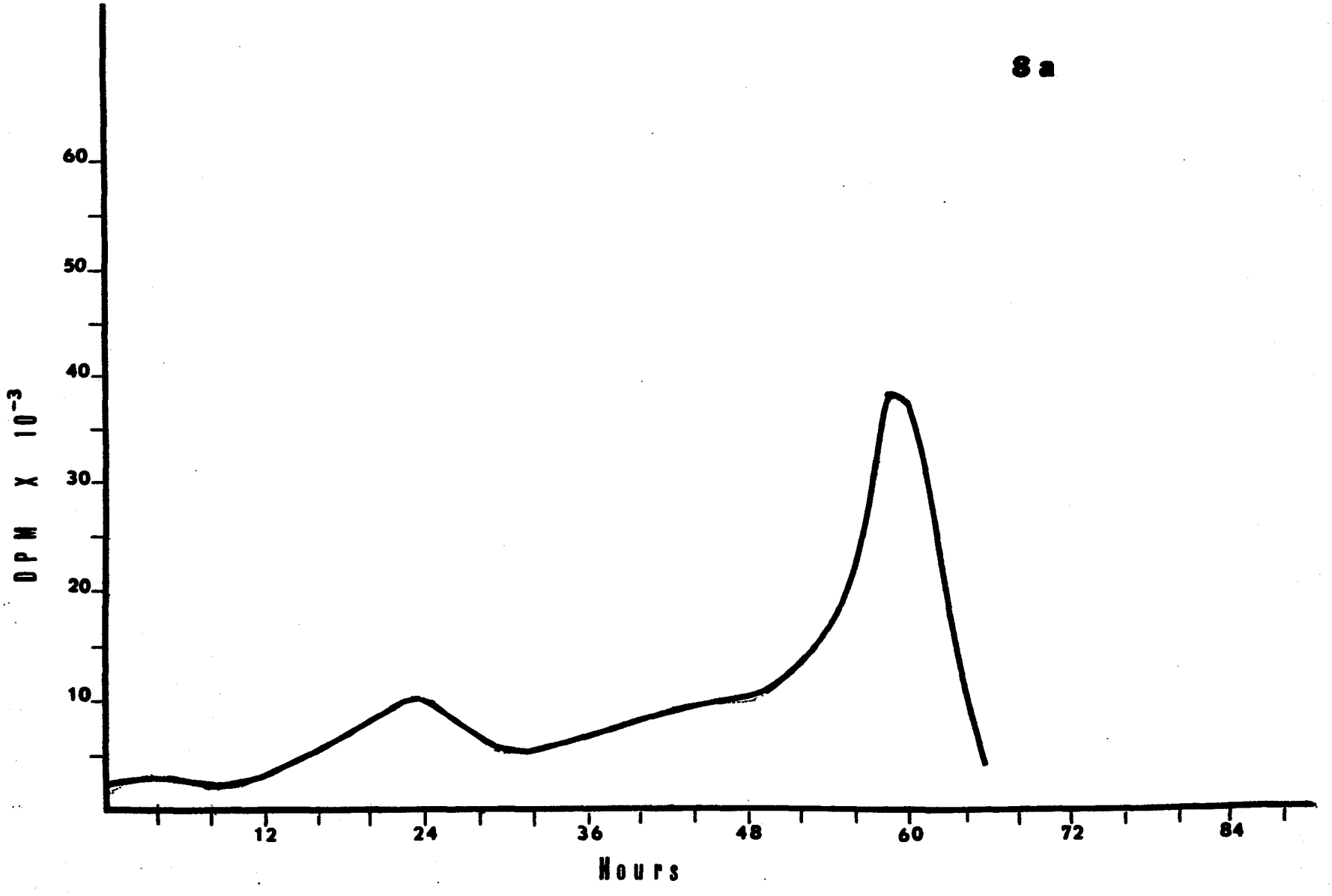
Figure 8. 21.6 mM Na^+

Figure 9. 237 mM Na^+

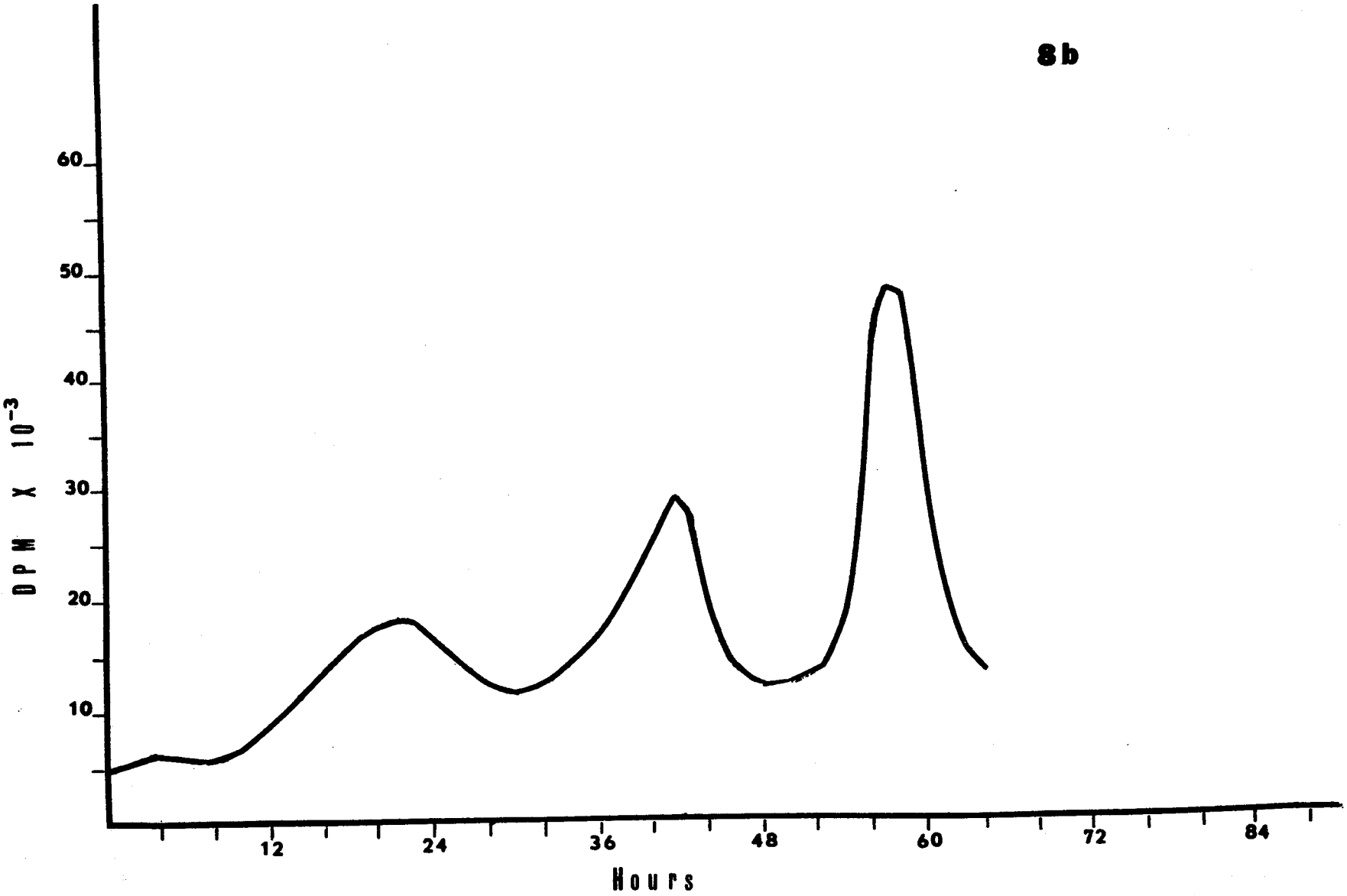
Figure 10. 517 mM Na^+

* As TCA precipitates

8a

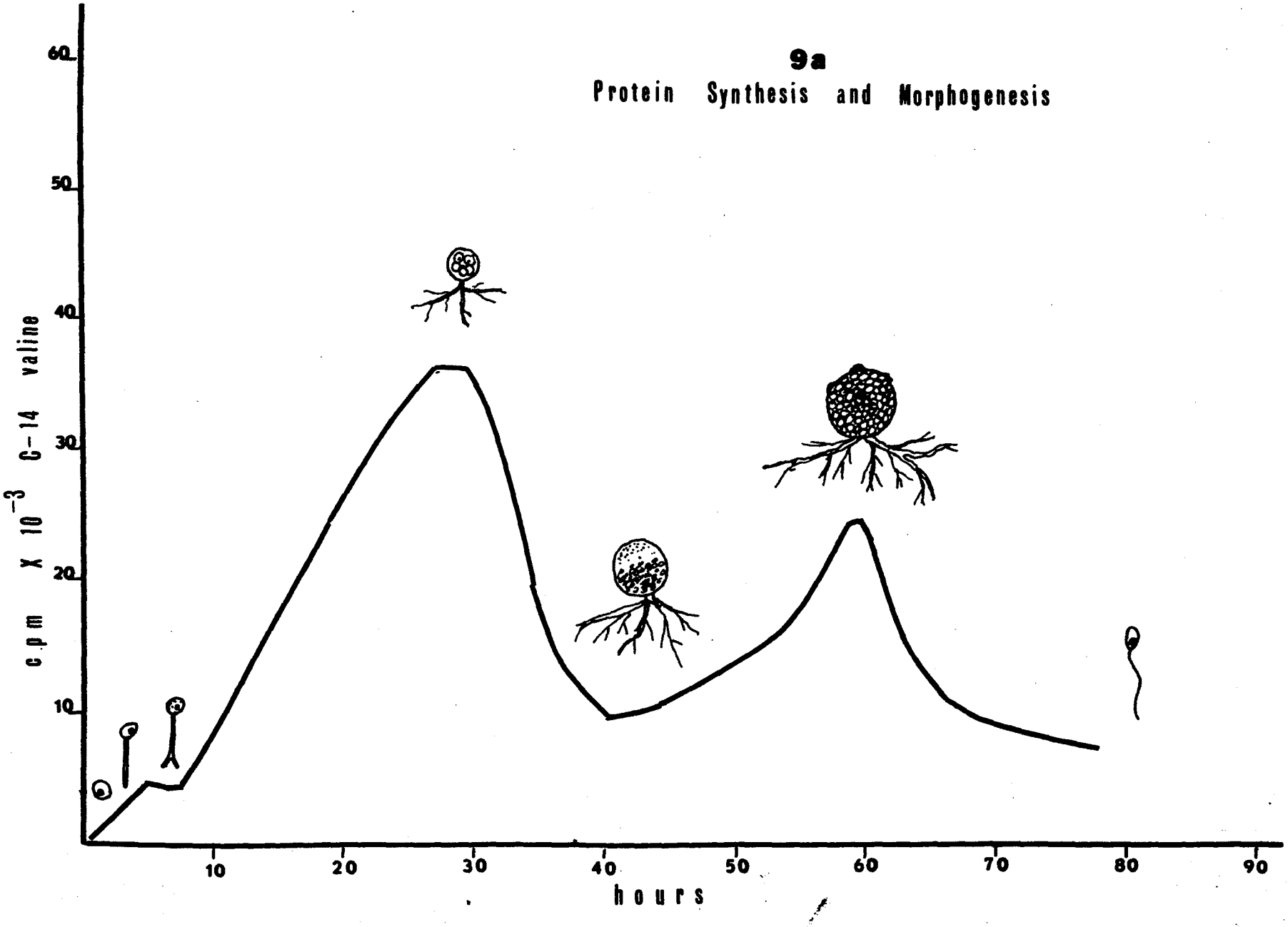


8b

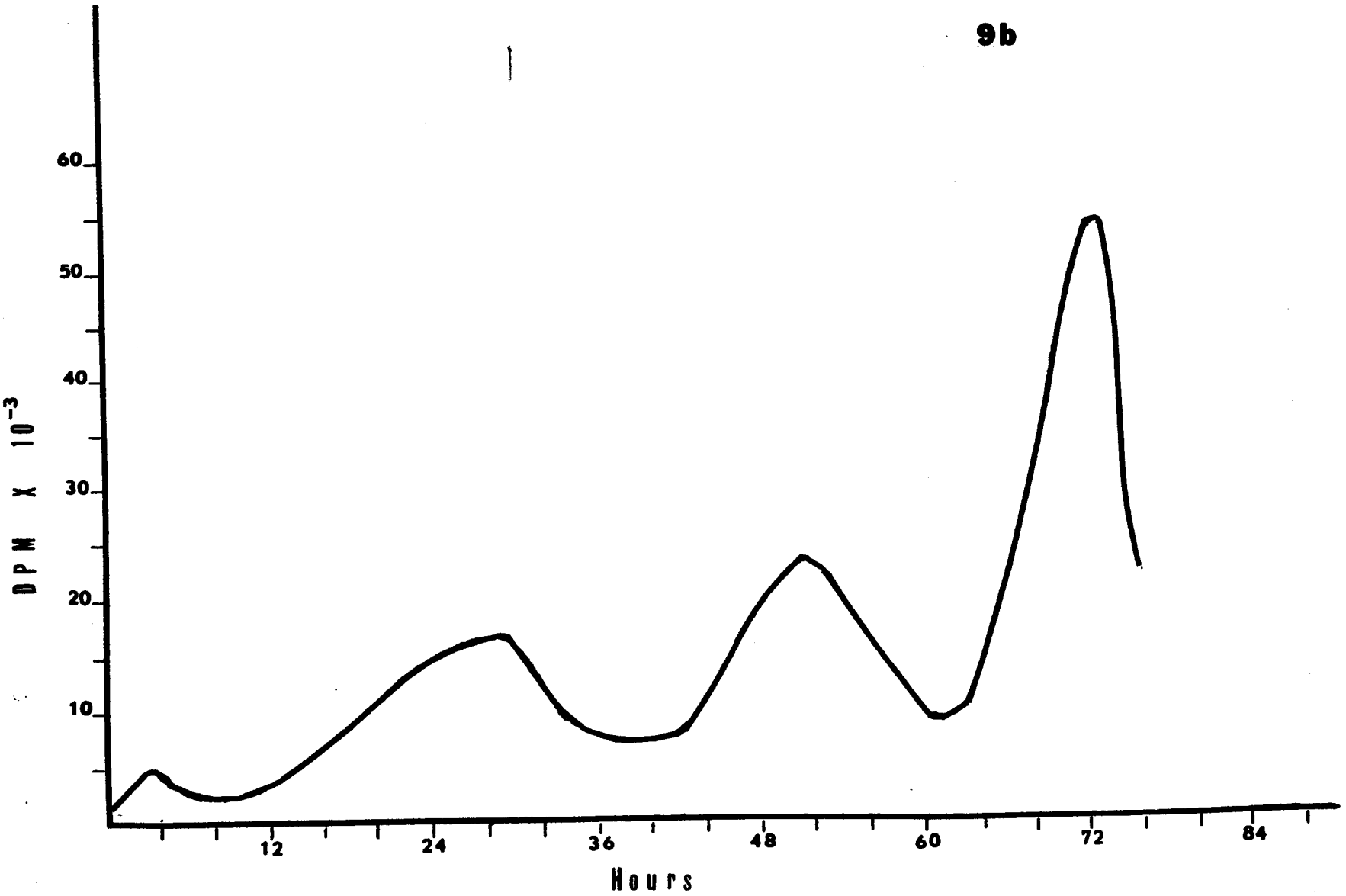


9a

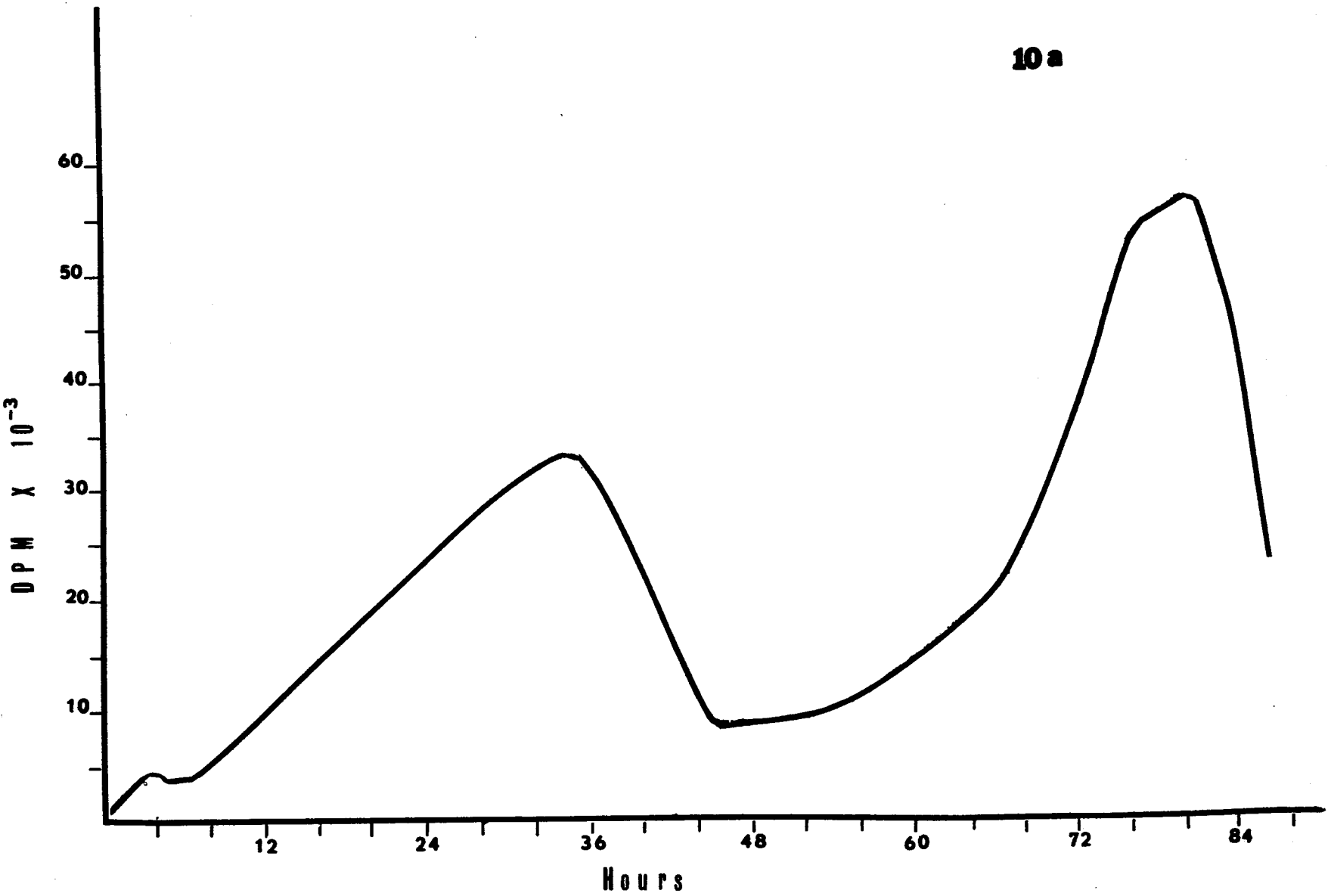
Protein Synthesis and Morphogenesis



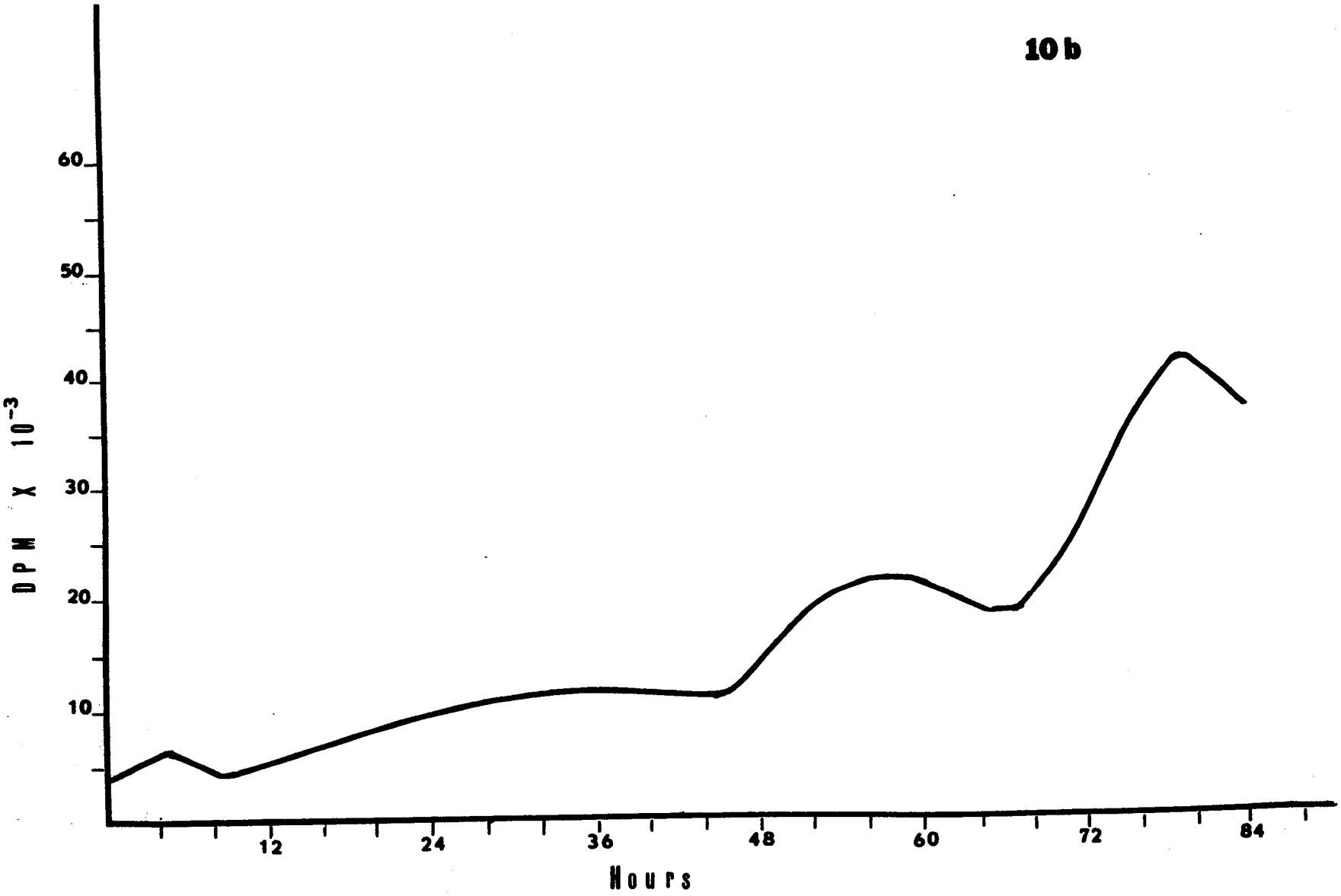
9b



10 a

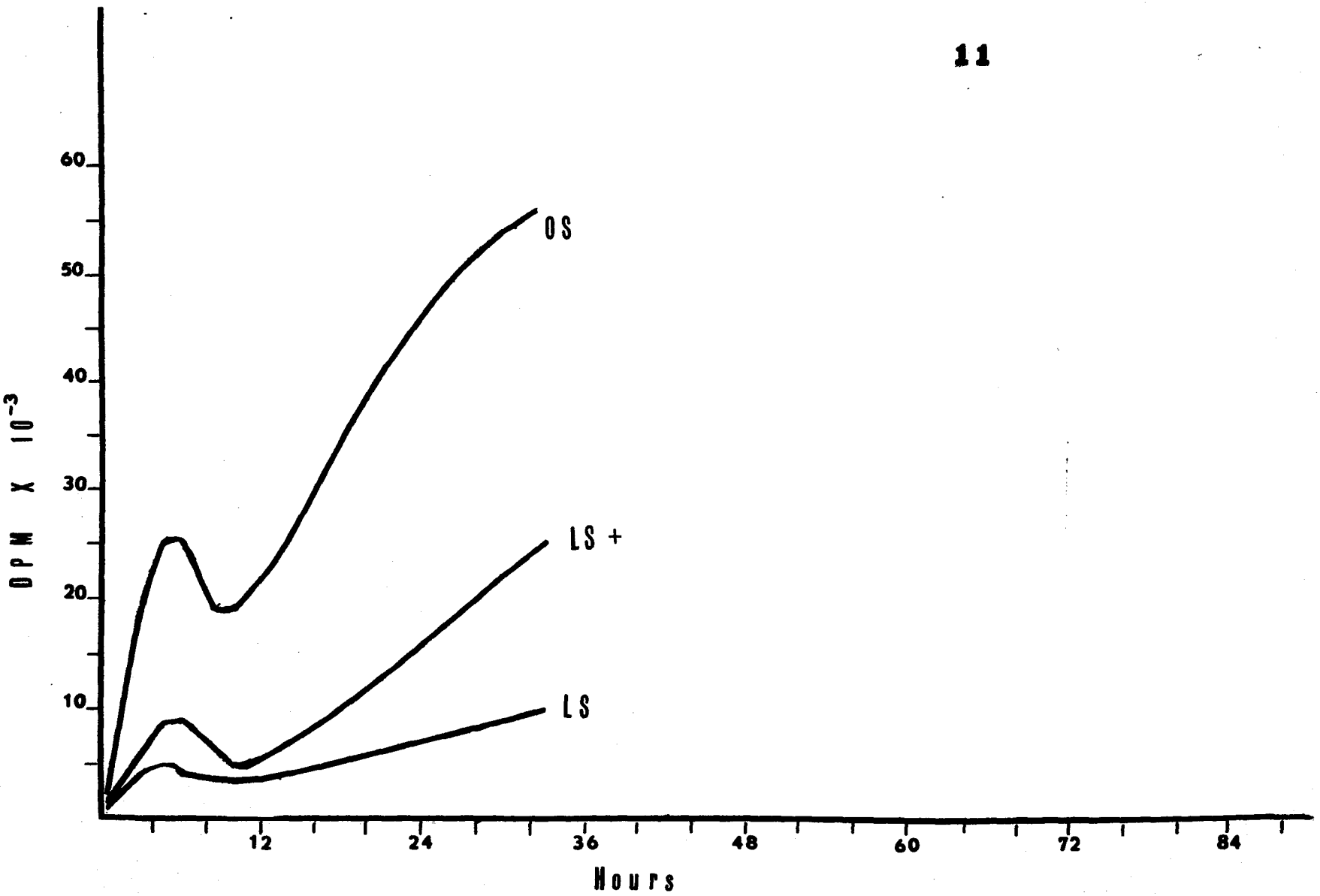


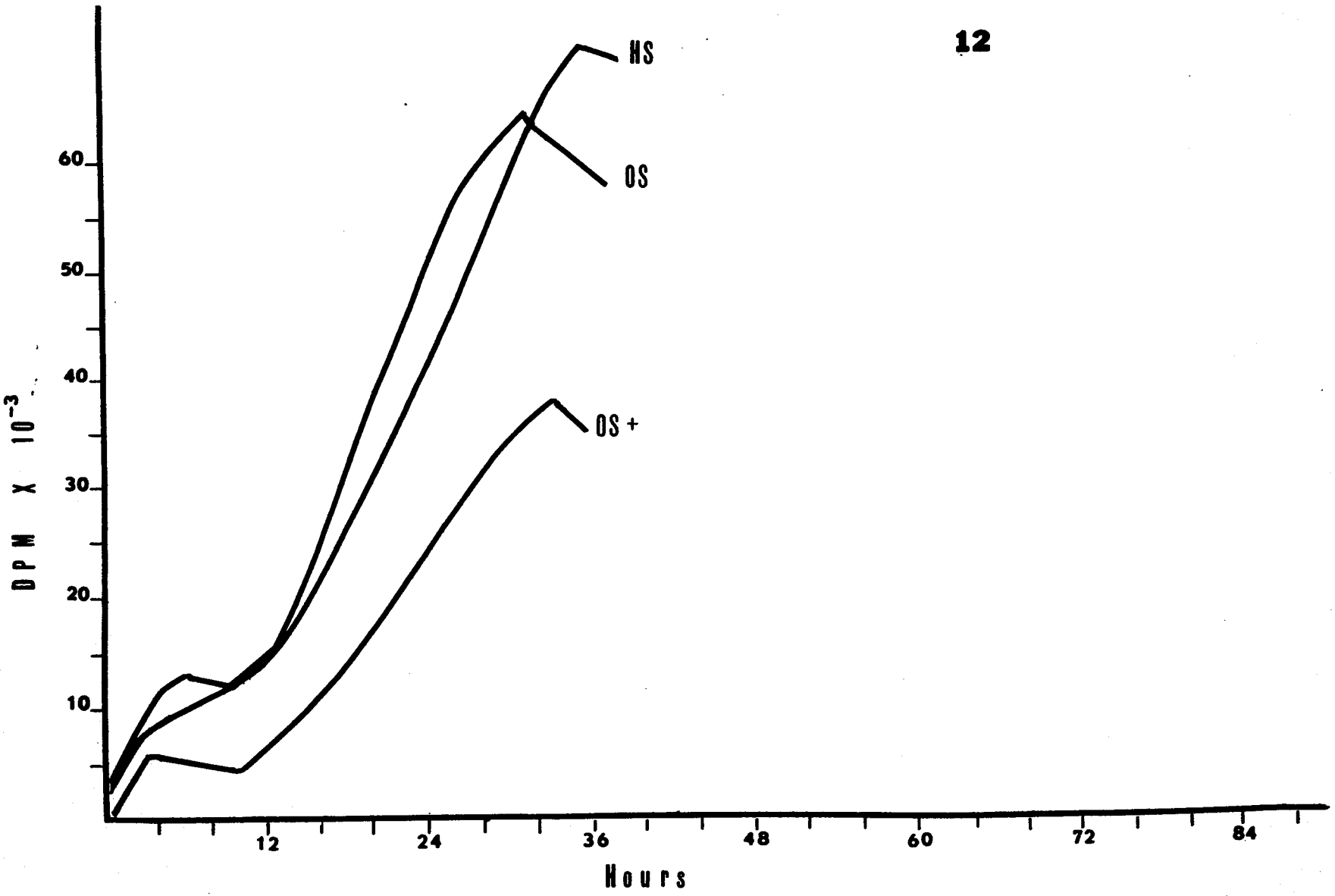
10 b



Figures 11 and 12

The effects of a mannitol osmoticum on the pattern of incorporation of C-14 valine. Figure 11 shows that the general pattern and level of protein synthesis is the same in LS and LS with an osmoticum (LS+) which simulates OS. Incorporation in OS is given for comparison. Figure 12 shows that addition of an osmoticum to OS (OS+) to bring the tonicity to the level of HS will not cause incorporation similar to HS. The time of the peak is slightly extended in OS plus osmoticum but the rate of incorporation appears depressed. The pattern of incorporation in the early stages in OS and OS plus osmoticum is similar.


60
50
40
30
20
10
DPM X 10^{-3}
OS
LS +
LS
12
24
36
48
60
72
84
Hours


HS
OS
OS +
60
50
40
30
20
10
DPM X 10^{-3}
12
24
36
48
60
72
84
Hours

Uptake and incorporation of protein and RNA precursors in SF, LS, OS, and HS

While the general pattern of synthesis shown above was the same at each Na^+ concentration, there was a difference in the rate at which morphogenesis and the correlated synthetic events occurred. Since it was possible that the rate of uptake might limit the rate of development, the effect of Na^+ on uptake was studied. The amount of each externally applied labelled compound was monitored in the water soluble pool, the cold TCA extract and in the cold TCA precipitate. The measurements were made on cells at three different stages of development and in three different Na^+ concentrations. Figure 13 show the distribution of label from valine, uridine, glutamate, glucose, and thymidine at various levels of sodium. These cells were all grown to the 32 hour stage in OS and placed in the test medium 30 minutes prior to beginning the experiment. Table 8 shows how the rate of valine incoroation changes with Na^+ concentration and stage of development.

To determine whether the observed changes in uptake and incorporation of these small molecules were being effected solely by the changes in the osmotic pressure of the medium, mannitol osmoticum was used to replace the NaCl. Figure 14 shows that SF medium brought to the same osmolarity as OS with mannitol would incorporate little

valine (TCA precipitate) and that valine uptake into the TCA soluble pool was decreased. Uridine uptake was not so effected but there was a slight drop in incorporation. The pattern is similar at both the 24 hour stage and the 32 hour stage.

It may be inferred from these results that, not only does the cell require sodium for the uptake of some molecules but that the concentration of sodium may effect the rate of uptake. In addition, it is shown that the function of Na^+ in this system is not entirely osmotic.

Studies of uptake and incorporation of valine in OS and SF demonstrated that in the absence of sodium, little (3.75% of OS value) labelled valine moved into the cold TCA soluble pool (Figure 13). However, there was little difference in the amount of label entering the water extractable pool (Figure 13). This indicated that while diffusion across the membrane was not affected by the presence of Na^+, the movement into cold TCA extractable pool was affected. In the presence of Na^+ the level of label in the cold TCA soluble pool was about 2 times higher than in the cold TCA extractable pool if Na^+ was absent (Figure 14). This could mean that Na^+ was involved in active accumulation of the amino acid.

When protein synthesis is inhibited with puromycin (Table 9) there is a drop in uptake in OS (control), but upon an upshift or downshift in Na^+ concentration more precursors enter the pool. This may indicate 1) a need

for protein synthesis to facilitate adjustment to the new environment and , 2) a membrane alteration permitting the increased entry of amino acid. In Table 10 a similar effect is shown with D-Actinomycin which inhibits RNA synthesis.

Figure 13. Uptake and incorporation at various levels of Na^+. The uptake and incorporation of valine and uridine is shown as a percent of maximum. Incorporation of glutamate, glucose, and thymidine in OS and in SF is also shown. Cells from 32 hours stage.

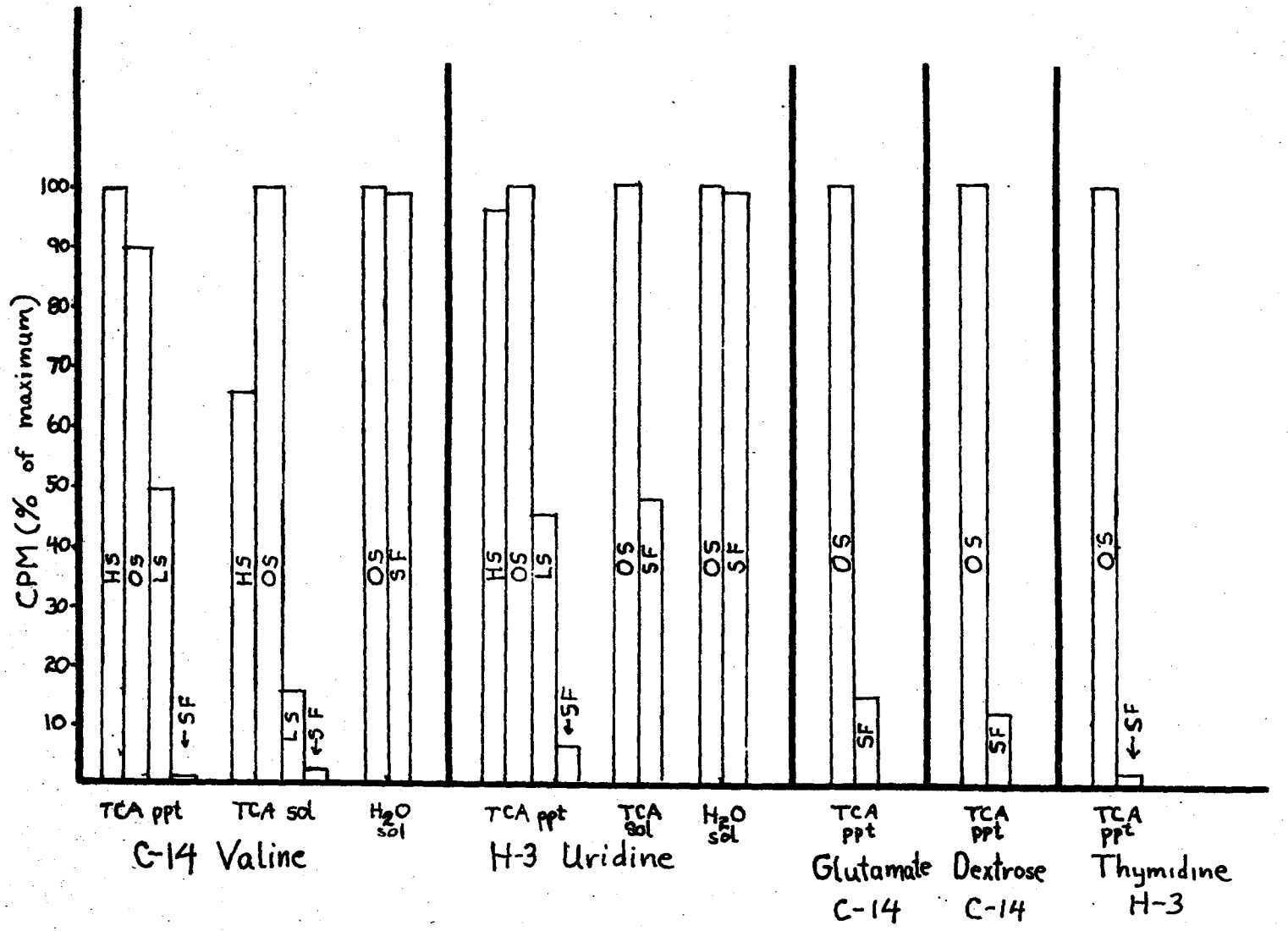

CPM (% of maximum)
100
90
80
70
60
50
40
30
20
10
HS
OS
LS
SF
TCA ppt
TCA sol
H2O sol
C-14 Valine
H-3 Uridine
Glutamate C-14
Dextrose C-14
Thymidine H-3

Figure 14. Effect of an osmoticum in SF on uptake and incorporation at 24 and 32 hours. Shows that there is little difference in the water extractable pools but that a considerable decrease of uptake and incorporation into the TCA soluble and TCA precipitable pools occurs regardless of the osmotic pressure. "SF+" indicates the sodium free medium with mannitol.

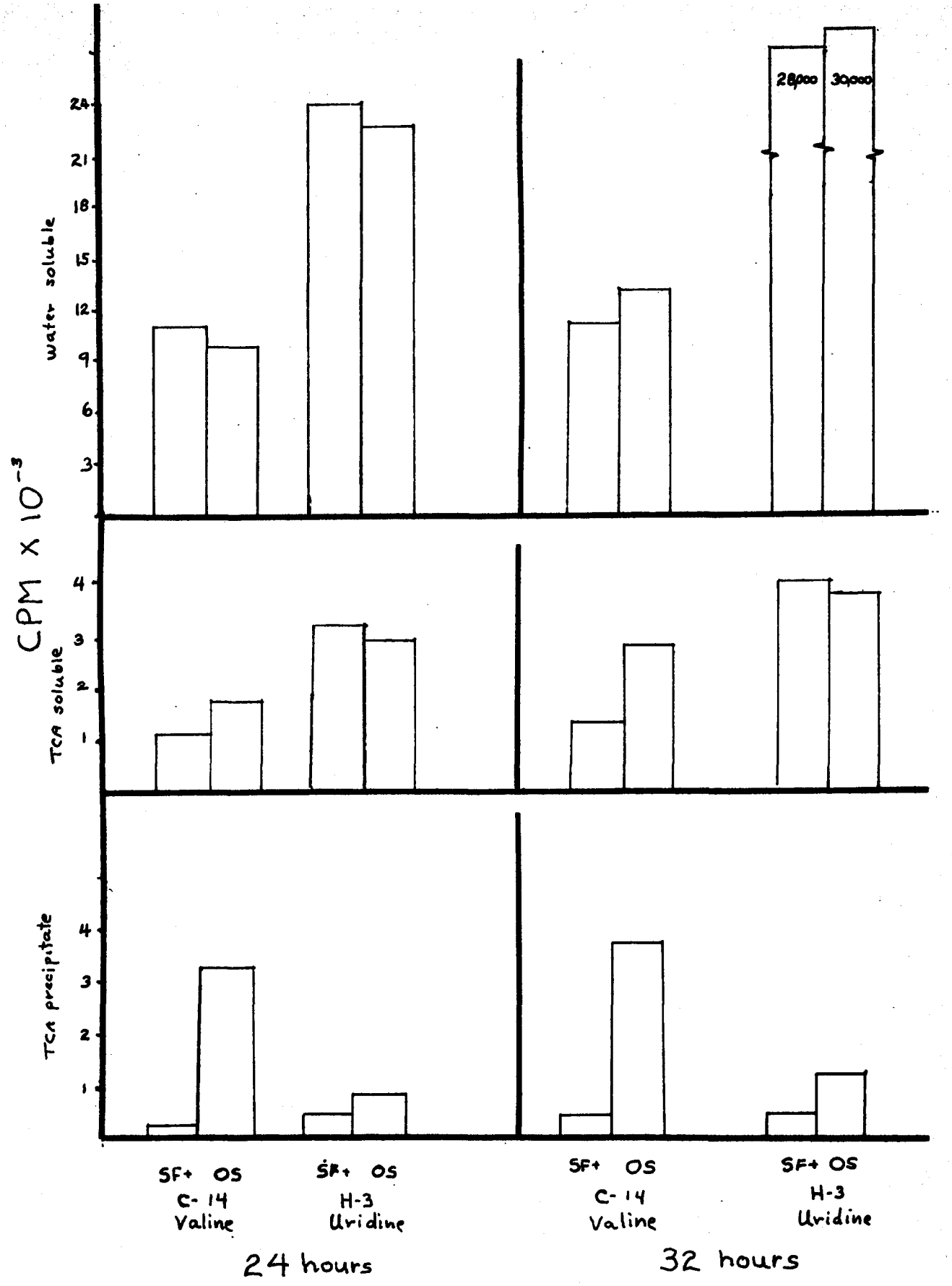

CPM x 10^{-3}
water soluble
TCA soluble
TCA precipitate
28,000
30,000
SF+ OS
C-14
Valine
SF+ OS
H-3
Uridine
SF+ OS
C-14
Valine
SF+ OS
H-3
Uridine
24 hours
32 hours

TABLE 8

Effect of external Na^+ concentration on rate of uptake and incorporation of valine and uridine at three stages of development in CPM-each column individual experiment.

Valine/Uridine

Molar Na^+ concentration	Cyst/germling	+15 hrs.	+32 hrs.
0.0	180/900	200/500	300/320
0.0216	400/900	1230/1000	10,000/10,000
0.237	13,340/4,700	2000/1400	18,500/19,000
0.517	9,970/6,300	920/950	20,000/20,000

TABLE 9

Effect of the inhibitor puromycin on uptake of C-14 valine upon changes to four different levels of Na^+ . Cells initially in OS and are 32 hours old.

	CPM of valine uptake to TCA soluble pool		
Na^+ Concentration of new medium mM	inhibited	non-inhibited	difference
0	13,500	9,500	+ 4,000
21.6	45,000	40,000	+ 5,000
237 (control)	116,000	236,000	- 120,000
517	167,000	127,000	+ 40,000

TABLE 10

Effect of D-Actinomycin on uptake and incorporation after upshift or downshift of Na^+

From OS

Expressed as CPM C-14 valine

	New Na^+ level	No D-Actinomycin	Ratio to control	D-Actinomycin	Ratio to Control
Incorporation	LS	7035	0.29	8920	0.39
	OS (control)	24200	1.00	22500	1.00
	HS	39500	1.63	27500	1.22
Uptake	LS	18500	0.15	22000	0.21
	OS (control)	12600	1.00	104000	1.00
	HS	166000	1.32	170000	1.63

Synthesis during the zoospore and cyst stage

Labelling studies consistently showed that the level of label incorporation into the cell material was very low at the new cyst stage. Studies using actinomycin-D and puromycin to inhibit RNA and protein synthesis showed that the initiation of germ tube development was independent of protein or RNA synthesis. However, the inability of subsequent stages to develop in the presence of either inhibitor indicated synthesis was required from that point on. Likewise, there was a sharp drop in labelling near the time zoospores began to be released from the cells. Apparently little synthesis was taking place since these inhibitors showed no effect on the zoospores or their ability to encyst.

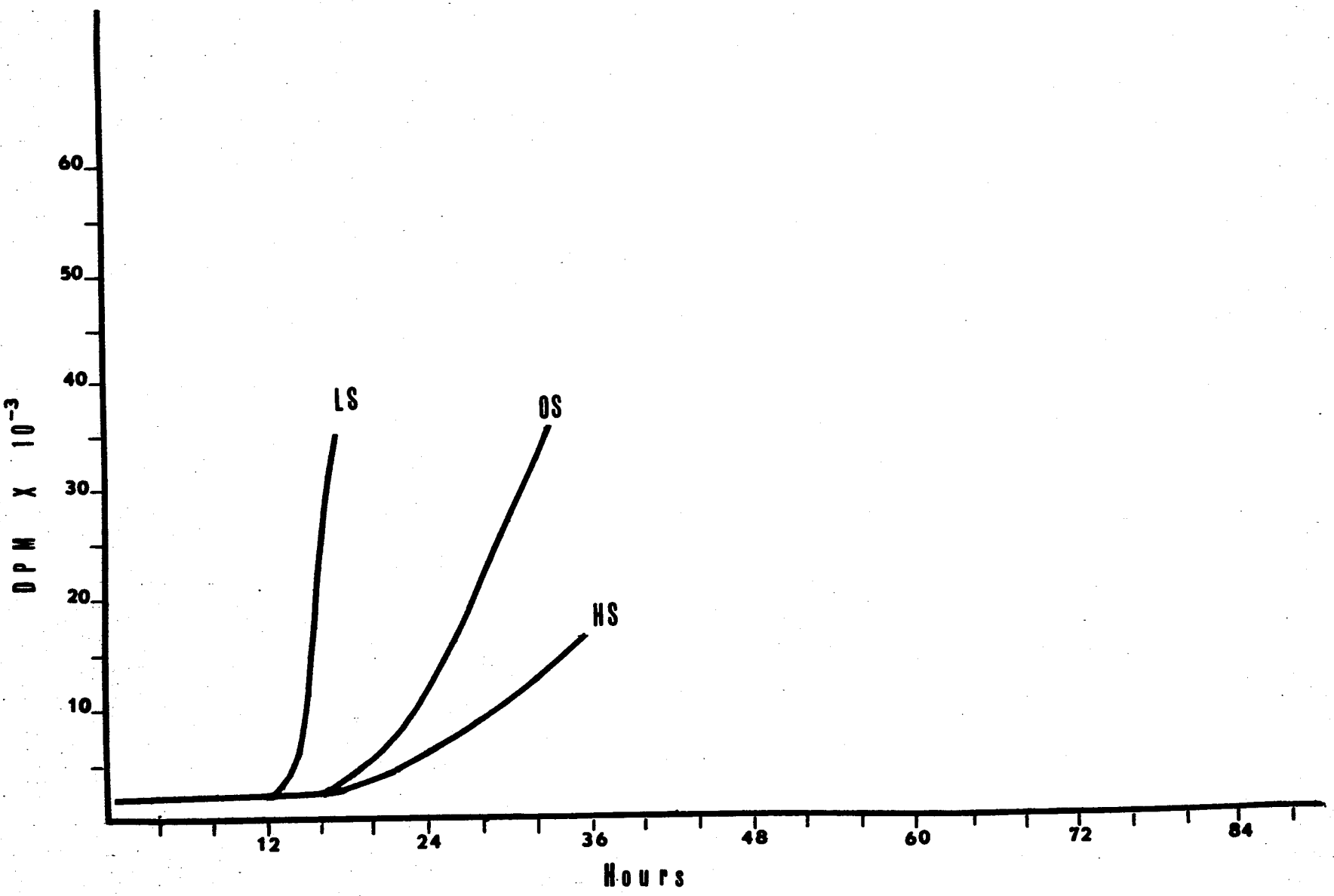
DNA synthesis during development at different Na^+ concentrations.

DNA synthesis was measured by H-3 thymidine incorporation (Figure 15). The nuclear cycle of division was confirmed using a chrom alum-hematoxylin method to stain the nuclei (Amon, 1968) and by observation with phase contrast microscopy. The stain showed nuclei only after the first division occurred so the appearance of two nuclei was used as an indication of the first division. Prior to the first division no nuclei could be indentified with this method, thus the actual time of onset of DNA synthesis was not precisely documented but a pattern was described.

In LS nuclear divisions and thymidine incorporation began at about 11 hours and proceeded rapidly, whereas, in OS, it began at about 13 hours and did not proceed as rapidly as in LS. The same was true for HS except that the divisions began at about 16 hours. These times indicated that the initiation of DNA synthesis could correspond to the time thallus enlargement and rhizoid proliferation was begun. It was not determined if synthesis continued beyond the time when the cytoplasmic granules moved basally. The initiation of rapid DNA synthesis began earliest in the lower concentrations of Na^+ which may be an indication that this developmental event could be under the same control as protein and RNA synthesis which are effected in a similar way by the Na^+ concentration.

Figure 15. DNA Synthesis

The incorporation of H-3 thymidine into TCA precipitable material at three concentrations of Na^+. The figure shows that the onset of incorporation begins later as the Na^+ concentration rises. Chrom-alum-hematoxylin stains confirm initiations of cell divisions at 11 hours in 21.6 m$\underline{M}$ Na^+, 13 hours in 237 m$\underline{M}$ Na^+, and 16 hours in 517 m$\underline{M}$ Na^+.


60
50
40
30
20
10
DPM X 10^{-3}
LS
OS
HS
12
24
36
48
60
72
84
Hours

Evidence for an alteration in the transport system upon transfer to a different concentration of Na^+.

If cells were heavily labelled with C-14 valine for a short period of time and then exposed to a media containing no sodium there was a quick loss of label from both the water and TCA extractable pools (Table 11). This would indicate that the membrane or other barrier to this exchange had been altered in the absence of Na^+. It was possible that exposure to different levels of sodium may have obligated the cell to "repair" this alteration. By using inhibitors of RNA and protein synthesis it could be demonstrated that full operation of the synthetic mechanism had an effect on the subsequent ability of the cell to take up and incorporate valine into protein after exposure to high and low levels of Na^+. Table 10 shows that in Actinomycin-D the ability of the cell to produce and take up its precursor was increased with the exception that in HS protein production was reduced. Table 9 shows that in the presence of puromycin, valine uptake was increased in cells transferred to higher and lower levels of Na^+. While this was somewhat unexpected, since in the controls uptake decreased, it does show that some alteration had occurred which was caused by an inhibitor of protein synthesis.

In another experiment cells were grown in OS for 32 hours then, placed in LS or HS. One group was labelled

with C-14 valine after only 10 minutes adjustment and a second group was labelled after 3.5 hours (+35.5 hours) adjustment to the new medium. Uptake and incorporation of the amino acid was compared to the control which was transferred to a fresh OS medium. After 10 minutes, uptake in LS was 11% of OS and in HS it was 40% greater than OS, but after 3.5 hours the differences from the controls were less. In LS the uptake was 40% of the control and in HS there was only a 2% increase over the control. The same trend was shown for the TCA precipitate. In LS at 10 minutes, incorporation was 30% of the control but at 3.5 hours it was 60% of the control. In HS protein synthesis was 89% of OS at 10 minutes and at 3.5 hours, 101% of OS. Apparently the 3.5 hours period allowed the cells to adjust their uptake and incorpoation mechanisms to the new levels of Na^+. This third experiment showed that the uptake and incorporation was similar to the first experiment and confirmed that the patterns of labelling were due to the period of adjustment (10 min. or 3.5 hrs.) and not due to the difference in stage of development.

Another somewhat different alteration in the permeability of the membrane occurred on a Na^+ downshift (OS → SF). Glutamate uptake into the water extractable pool increased in the absence of Na^+ (Table 12). The only explanations that seemed appropriate were that 1) sodium slowed diffusion across the membrane or 2) that

the cell in some way was selectively made more permeable to glutamate in the absence of Na^+. The latter could have been due to osmotic shock. This data also provided useful information on the availability of nutrient to the cell in Na^+ free media. Under Na^+ free conditions, glutamate apparently entered the cell more easily than when Na^+ was present (OS), but the incorporation into protein was about 15 to 30% of that observed in OS medium; therefore, nutrients might actually be available if only through diffusion.

TABLE 11

Rapid loss of valine from cellular pools when transferred from OS to SF (CPM after 10 minutes exposure).

	in SF	in OS
H_2O souble pool (lost to medium)	30,000	1,500
Remaining in TCA soluble pool	91,000	140,000

TABLE 12

Glutamate Uptake

Glutamate in H_2O extractable pool when Na^+ is present and in Na^+ free media

	CPM Glutamate C-14
Na^+ = 237 mM	5325
" "	7100
Na^+ free	34800
" "	38000

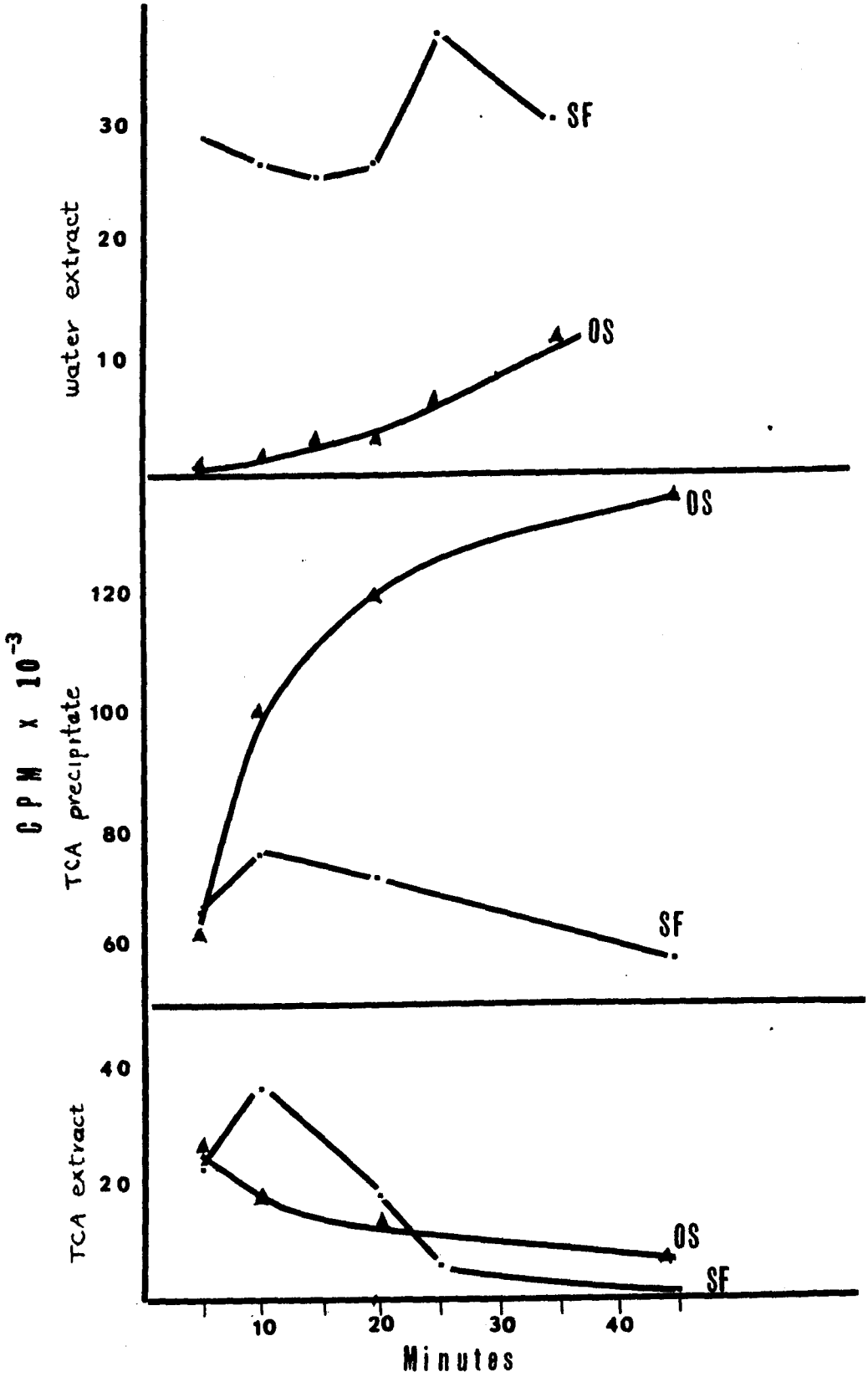
An attempt to measure endogenous protein synthesis.

An experiment was performed to assess the effect of lowered Na^+ content of the extracellular environment on the rate of endogenous incorporation of valine. Cells at the 32 hour stage were starved one hour in the 0.237M Na^+ salts (OS without dextrose or glutamate) to reduce endogenous reserves of amino acids or other protein precursors and the heavily labeled with C-14 valine (10^{-7}mM/ml) for 15 minutes after which the cells were washed free of label and placed in Na^+ free salts (SF without dextrose or glutamate). Samples were taken at intervals of 5, 10, 20, 25, and 45 minutes to see if there would be a decrease in protein synthesized in cells which were losing Na^+. Controls were in 0.237M Na^+ salts. At the same intervals cold TCA soluble pools were measured and labelled material diffusing from the cells was measured (similar to water soluble pool). Increased CPM in the TCA precipitate indicated continued synthesis of new protein. Cells in Na^+ free salts stopped accumulation of label at 10 minutes, but the control continued to accumulate the label (Figure 16). It was possible that synthesis was no longer taking place, the label was being recycled, or it was respired faster than it was produced. The small size of the TCA soluble fraction indicated the label might be turned over in the cell and respired quickly in the Na^+ free salts. The valine in pools dropped but

little label appeared to be incorporated in the protein. There is a strong indication that much amino acid is lost from the endogenous water soluble pool immediately on exposure to SF (Table 11) and it is also indicated that the amount of material in the cold soluble TCA extract fell to a low level soon after the transfer. This could have a large effect on protein synthesis observed. Therefore, while there may be reason to suspect a decrease in endogenous synthesis it is not yet clear if it does decrease at lowered endogenous sodium levels. In the first hour of exposure to SF, sodium level fell to a level similar to that which was normally found in cells grown in LS (See Section IV).

Figure 16 Endogenous Synthesis

The change in incorporation of C-14 valine in OS and SF salts from endogenous pools. The change in those pools is also plotted.


water extract
30
20
10
SF
OS
CPM x 10⁻³
TCA precipitate
120
100
80
60
OS
SF
TCA extract
40
20
OS
SF
10
20
30
40
Minutes

Synthesis of cell material at different Na^+ concentrations.

In LS the cells grew slowly and at maturity were only 1/3 the weight of cells grown in the higher concentrations of sodium. In the early stages of growth the cells in HS grew more slowly than in OS, but they achieved nearly the same weight since their cycle was longer. In a given time the cells grew fastest in OS, in terms of biomass, but in terms of life cycle time they completed a cycle most quickly at lower sodium concentrations (Table 13).

If a 15 hour old inoculum of cells grown in OS was placed in a sodium free medium, the cells continue to grow, but a somewhat reduced biomass is produced (Table 14). Cells at this stage treated with 12.5μg/ml puromycin or 5μg/ml D-Actinomycin cease growing immediately, an indication that protein and RNA synthesis were not obviously influenced by the lack of external sodium. It was probable that nutrients diffused across the membrane at a rate sufficient to support growth since levels of nutrient in the medium were quite high. Also some protein inside the cell may have been recycled to provide new source material for developmental changes (Timberlake, et al., 1973) In section IV it is shown that the sodium levels in the cell dropped during the first hour when the cell was placed in the sodium free medium, but then was maintained at an intermediate level for quite some time. The apparent sharp break in weight gain may indicate the internal Na^+ concentration had become limiting.

TABLE 13

Synthesis of cell material at different Na^+ concentrations

Expressed as mg increase in dry weight

	Na^+ concentration in mM		
Hours	21.6	237	517
5	2.0	4.9	5.3
7	1.6	4.1	3.1
16	4.2	8.2	5.4
21	9.2	12.3	9.1
31	11.5	16.3	10.9
42	20.6	38.4	25.9
56	30.4	64.0	31.0
63	35.5	84.0	37.0
73		101.0	57.9
80			76.6
90			91.7

TABLE 14

Mg increase in dry weight in Na^+ free medium

Changed from OS at + 15 hours

Hours	Control 237 mM Na^+	Na^+ free
5	3	3
10	2	2
15	5	5
26	16	13
61	45	38
73	60	41

Effect of DNP and Ouabain on entry and incorporation of C-14 valine

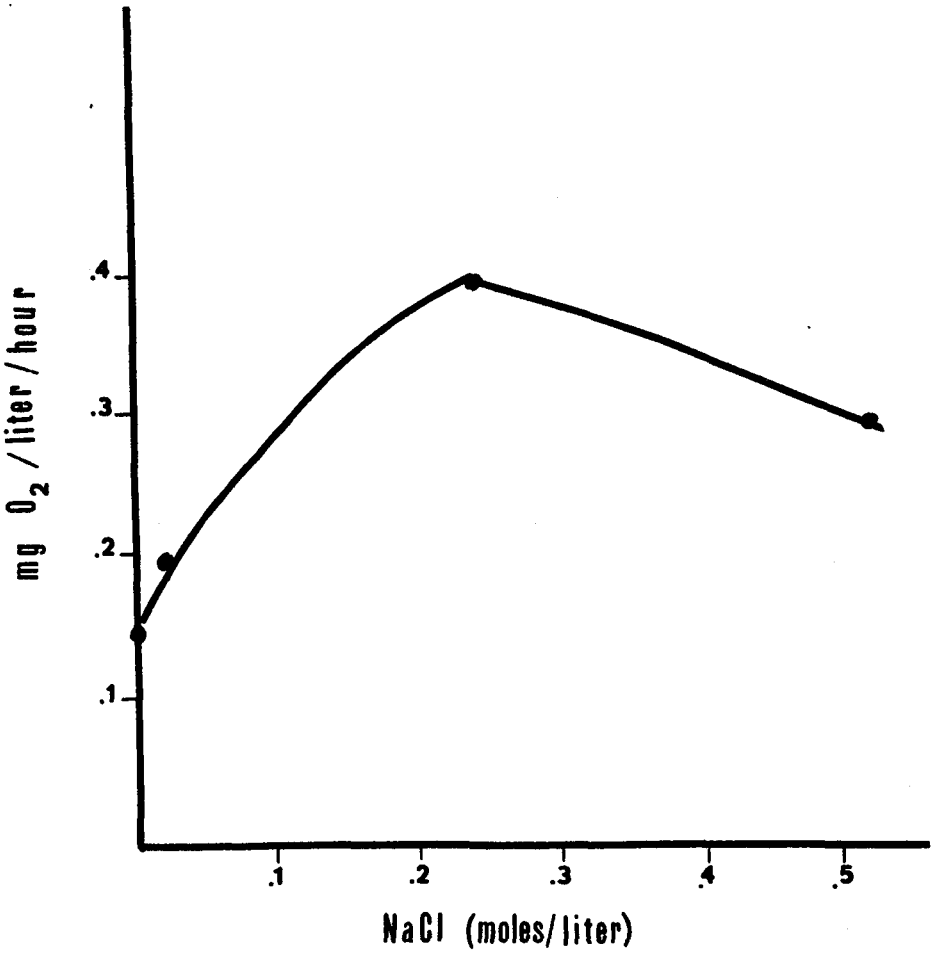
At a concentration of $5x10^{-5}$M 2,4-dinitophenol caused a six fold decrease in the uptake and incorporation of C-14 valine into the TCA precipitate, thus indicating that ATP may be necessary to drive the uptake mechanism. Further study showed that $1.4x10^{-4}$ to $1.4x10^{-3}$M ouabain, an inhibitor of K^+-Na^+ mediated ATPase, reduced uptake by 34 to 41%. This evidence supports the concept of an ATP-dependent uptake system which is mediated by Na^+ and may account for the decrease in incorporation in sodium free media.

The effect of Na^+ concentration on the rate of respiration.

The respiration rate was measured on cells grown for 34 hours in OS and equilibrated at the Na^+ concentration of the test medium one hour prior to the experiment. Each experimental vessel contained an equal inoculum of about $5x10^6$ cells in 120 ml of medium. The results are shown in Figure 17. The respiration rate was greatest in OS and declined at either higher or lower Na^+ concentrations. The curve produced here is similar to the curve showing the effect of Na^+ on the rate of protein synthesis (Section I). This could mean that the rate of protein synthesis is linked in some way to the effect of Na^+ on the respiratory rate.

Figure 17 Respiration and Na^+ Concentration

The change in the rate of O_2 utilization at four levels of Na^+. 32 hour cells initially in OS.


mg O_2 / liter / hour
.4
.3
.2
.1
.1
.2
.3
.4
.5
NaCl (moles/liter)

DISCUSSION

Uptake

The active uptake of amino acids and some other small molecules appeared to require the presence of Na^+ in the growth medium. The rate of uptake was related to the concentration of Na^+. The requirement for Na^+ in uptake could not be replaced by an osmoticum and changes in the osmotic pressure of the growth medium did not strongly affect the rate of uptake. The entry of substrate into the water souble pool, apparently by diffusion, was not dependent on Na^+. It was uptake into the TCA souble pool, but not the H_2O soluble pool, that seemed to influence susequent incorporation of the compound into macromolecules, an observation in agreement with those of Halvorson and Cowie (1961) on yeast.

There was also evidence that a change in the level of extracellular Na^+ had effects on the transport system which may have altered its proerties. The repair of that damage may require protein and RNA synthesis. A new transport system to facilitate uptake might be needed at different levels of Na^+. Epstein (1973) and Nissen (1974) showed that in barley roots there are apparently different uptake systems which function at different concentrations of Na^+ and K^+. If present in Phlyctochytrium such

systems might not be constitutive and could be induced by the change of Na^+ concentration in the medium.

The uptake mechanism appeared to be indirectly related to the respiration rate since there was some relationship between the rate of uptake, incorporation, and respiration at 32 hours into development (Table 8, Figure 17). As in Thraustochytrium roseum (Siegenthaler, Belsky, & Goldstein, 1967), there was a considerable amount of respiration occuring when uptake fell to a very low level in SF. This does not mean that the two systems are unrelated. Sprott and MacLeod (1972) suggest that electron transport leading to the terminal utilization of O_2 is part of the Na^+ dependent uptake system in marine bacteria, but that kind of system is not supported by the above data, because respiration occurs when uptake is nearly zero.

Other evidence indicated that uptake in Phlyctochytrium may be similar to $PO_4^{\equiv}$ uptake described for Thraustochytrium roseum (Siegenthaler, Belsky, Goldstein, & Menna, 1967) and for sugar in other fungi as discussed by Jennings (1974). The uptake system is sensitive to DNP, as is Thraustochytrium, and it is also sensitive to ouabain so it apparently involves the use of ATP and has a Na^+ dependent ATPase step. Siegenthaler, Belsky, Goldstein, & Menna (1967) suggest that Na^+ is required for the increase of oxidizable substrates and thus increases electron flow, and leads to an increased ATP production which could then be used in uptake. They did not

use ouabain so it is not known if the system was sensitive. Jennings (1974) also presents arguments for a phosphorylation step in uptake in fungi and says that for some organisms the uptake is facilitated by Na^+.

A system which is consistant with that reviewed by Jennings(1974), with the available data from Siegenthaler, Belsky, Goldstein, & Menna (1967) and with my own findings is as follows. Diffusion across the membrane can take place but concentration of the substrate inside requires the presence of external Na^+. In order for that substrate to go against a gradient to the TCA soluble pool (and thence to the TCA precipitate), it must be accompanied by a Na^+ stimulated carrier into the cell. Here it combines with another entity which binds it into the TCA soluble pool. This activity may be mediated by ATP and may involve phosphorylation. The step then would still require the presence of Na^+ to facilitate the activity of the ATPase. Once the molecule is in the TCA soluble pool it may release the Na^+ to be pumped back out of the cell (or compartmentalized in cytoplasm) and in so doing the substance is dissociated from the carrier.

Synthesis

Although uptake is impaired by the lack of Na^+ at all stages, growth and differentiation can continue in cells which are greater than 15 hours old. This must mean

that synthesis is continuing. The elevated permeability of the cell to glutamate, one of the nutrients of the growth medium, in SF would show that growth can continue based on diffusion of nutrients which are in high concentration (2 g/liter). Under these conditions growth is reduced but the cell is capable of completing the life cycle. It is also possible that some of the nutritional needs of the cell can be met by recycling the internal protein (Timberlake, et al 1973). Since development is quickly stopped by application of inhibitors of RNA and protein synthesis at stages beyond the germling it is unlikely that the cell is differentiating without new synthesis. Except in the conversion of zoospores to germlings there is no evidence of differentiation without synthesis.

The rate of growth had been shown to respond to Na^+ or salinity in many of the marine microorganisms which have been studied so far (MacLeod, 1965; Vishniac, 1960; Griffiths & Morita, 1973). In this study, however, the use of synchronously developing cultures has provided evidence that the requirement for Na^+ is not constant throughout the life cycle. This information on Phlyctochytrium adds insight to the effect of sodium on synthesis. The unmentioned assumption has been that external Na^+ is capable of influencing the internal process of protein synthesis. This would mean that the cell would have to be relatively passive to the inward and outward movement of Na^+. This concept is not supported by the present research. Earlier evidence in the

literature on marine bacteria had previously suggested that Na^+ did pass freely (Thompson & MacLeod, 1971), but further evidence has shown that the internal concentration of Na^+ is only $^1/_3$ the external concentration (Thompson & MacLeod, 1973). The bacterial cell, therefore, must have the capacity to regulate Na^+ in its interior. If regulation of this sort was possible in the organism presently under study it could explain the capability of the cells to synthesize protein in the absence of "external" Na^+. Likewise, if the cell does require Na^+ for synthesis it must not be able to retain it in the Na^+ free medium during the first 15 hours of development. During this initial 15 hour period the entry of glutamate apparently cannot satisfy the needs of the cell for growth as it does later in the cycle. It is possible that it cannot be incorporated into protein without the aid of intracellular Na^+. All attempts to show incorporation of amino acids or uridine into the TCA precipitate at this early stage have been unsuccessful.

If the cell can retain sodium, the results showing changes in the rates of synthesis due to external concentrations of Na^+ must be interpreted carefully. Although similar patterns in the peaks of synthesis were produced at each level of Na^+ the absolute rates of synthesis cannot be compared easily. The rates of uptake and incorporation are shown to differ for different levels of external Na^+. The data in Figure 13 indicates that the amount of incorporation

at different Na^+ concentrations is not directly related to the size of amino acid pools or the rate of uptake and may be more a function of the Na^+ concentration and the stage of development. Furthermore, it is possible that Na^+ may influence the rate of protein synthesis indirectly through its effect on uptake and respiration. There is a similarity between the respiration rate and incorporation of amino acid at each Na^+ level (Table 8 & Figure 17).

The rate of O_2 consumption is also affected by Na^+ in a pattern similar to the way Na^+ affects the rate of protein synthesis. Such a pattern is described for Thraustochytrium roseum (Siegenthaler, Belsky, & Goldstein, 1967), another obligately marine fungus, and Pythium marinum (Kazama, 1969). Kazama (1969) demonstrated that the differences in respiration at different concentrations of Na^+ were not due to osmotic pressure differences but could be attributed to the concentration of NaCl alone. The organism in this study showed a decrease in O_2 consumption of more than twofold when in a Na^+ free medium, but in the marine fungus Lulworthia medusa (Davidson, 1974) change to freshwater did not decrease the rate of respiration. One could speculate that there may be a fundamental difference between the way in which Na^+ effects the respiration and possible the growth of these two fungi.

The problem of studying synthesis where uptake is

altered was discussed by Timberlake, et al (1973). They attempted to correlate the rate of protein synthesis to the specific activity of amino acid pools and were satisfied in showing such a relationship. In the present study I did not find that such a procedure would greatly change the interpretation of the results, nor did I feel it was appropriate since I was not able to quantitate the experimentally induced changes in intracellular Na^+ which could influence incorporation rates. The results indicated trends which can be more fully understood when there is more information on how Na^+ effects the entire system.

SECTION IV THE PRESENCE OF INTRACELLULAR SODIUM

INTRODUCTION

The finding that the fungus could survive in a sodium free medium after sufficient exposure to a medium containing sodium indicated that either it had changed its requirement for Na^+ or that sufficient Na^+ was present inside the cell to support its necessary functions. The ability of the cells to grow, and the effect of inhibitors in the Na^+ free medium indicated that protein synthesis must be continuing even though labelling studies could not quantitate it. Why young (0-15 hr.) cells could not grow in SF, but 15 hour and older cells could, was vague. One hypothesis was that although uptake of amino acids may have been somewhat limited at each stage, some quality of the older cells allowed the retention and incorporation of nutrients that was not achieved in the younger cells. If the requirement for Na^+ is assumed to be intracellular in its nature, it could be that the older cells had the ability to retain Na^+. This section examines that ability to retain sodium at different levels of external Na^+.

Sodium retention by cells has received little attention in the literature except with respect to marine and halophilic bacteria (Thompson & MacLeod, 1973; Holmes,

et al, 1965). Sodium levels of some halophytes have been studied and some workers are interested in transport of ions in crop plants (See reviews by Nissen, 1974; and Epstein, 1973). A few fungi have been examined to determine the inorganic content of the mycelium (Cochrane, 1958). Norkrans and Kylin (1969) have investigated Na^+ and K^+ fluxes in halophilic and non-halophilic yeast. They found that Debaryomyces hansenii, a halotolerant yeast, had a strong extrusion mechanism for Na^+ when compared to the non-marine Saccharomyces cerevesiae. This mechanism presumably allowed it to maintain the internal Na^+-K^+ ratio at a more suitable level. They also show that the salt levels in the cells were not sufficient to counteract the osmotic potential of the medium.

MATERIALS AND METHODS

Inocula were prepared as previously described unless otherwise stated. Cultures were grown in 125 or 250 ml Erlenmeyer flasks at 250 RPM on the G-2 shaker at 24° C. For experiments using cysts, 20 or more 100mm petri dishes of YPD made with 18°/oo S.W. were inoculated with just enough previously synchronized cysts to cover the surface without crowding at discharge (approximately 10^4 - 10^5/ml). Plates were flooded just prior to discharge with OS salts and the zoospores were collected within 10 minutes by aspiration into 2X thioglycolate broth made with 18 °/oo sea water. This was immediately centrifuged for one minute at 1,000 RPM to pellet the cysts. The cysts were immediately rinsed with two changes (1 min. centrifugation) of OS salts and checked for presence of other developmental stages. Experiments were initiated only if cysts were found to represent more than 99.9% of the pelleted cells. The only other stage present (0.1%) was the zoospore. Where stages other than cysts were needed cultures were initiated with cysts and grown to the desired stage in the appropriate medium.

Dry weights and wet weights were determined on all stages except the cyst and early germling stages. The small volume of these stages made removal of interstitial water nearly impossible and attempts to measure wet weights

resulted in water content estimates for the cell from 100% to 30%. The other stages, however, gave relatively consistent results showing 74-89% water in the cells. The procedure consisted of harvesting the cells on Whatman number 2 filter papers by gentle vacuum filtration, rinsing once quickly with a small volume of the appropriate solution, such as sodium free salts if the cells were to be used in sodium analysis. The cells were then carefully removed from the filter paper with a spatula and placed in washed, tared polystyrene weighing boats. Wet and dry weights were determined in a Mettler analytical balance. The polystyrene boats did not add measurable K^+ or Na^+ and the drying procedure did not change their weight. Drying was carried out at 96% in a drying oven for 18-24 hours at which time no more weight loss could be measured. Samples were cooled in a dessicator prior to obtaining dry weights.

To collect Na^+ and K^+ the cells were removed from the dishes and placed in pyrex glass petri dishes and dissolved in a minimal amount of concentrated HNO_3 digested overnight and then the volume was brought to 25 or 50 ml with deionized water. Atomic absorption spectrophotometry was used to measure Na^+ and K^+. The water used in the procedure was deionized by reverse osmosis procedure and was used as a Na^+ and K^+ free standard for analysis.

Glassware and weighing boats used in analysis of

Na^+ and K^+ were washed with Sparkleen (Fisher) detergent and subjected to five rinses in glass distilled water, one rinse in deionized water, acid wash in 50% HNO_3 in deionized water and a final 2X rinse in deionized water. To insure that the glassware did not add significant amounts of K^+ and Na^+, dishes (polystyrene) without cells, but an equal weight of deionized water, were subjected to the drying procedure and then any residue was extracted overnight in deionized water. No Na^+ or K^+ residue was detectable. The same procedure was used with glass dishes except that they were subjected to extraction with HNO_3. No detectable Na^+ or K^+ was added by glass dishes.

Methods for enzyme activity.

Amylase activity was measured by production of reducing sugar from starch. The method was similar to that of Pettersson and Porath (1966). The cells were grown in the medium for 60 hours and aliquots of the medium were used as a source of enzyme for the test. The buffer used was 0.02M Tricine (Sigma). TRIS buffer was found to be inhibitory.

LDH was assayed spectrophotometrically by a modification of the method of Kornberg (1955). A cell homogenate was clarified by centrifugation prior to the reaction and was used without further purification.

NADH oxidases were assayed by using the above reaction mixture without the pyruvate being present. This measurement was a control for the LDH measurement. NADH (reduced nicotinamide adenine dinucleotide) and potassium pyruvate were purchased from Sigma Chemical Company.

RESULTS

The intracellular concentrations of Na^+ and K^+ in cells grown at three concentrations of Na^+

Cells growing in LS, OS, or HS were harvested at various times during their life cycles and rinsed either with sodium free salts for sodium measurements or in deionized water for K^+ measurements. Figure 18 shows that sodium levels seemed to decrease throughout the cycles whereas potassium tended to increase. Potassium values varied approximately 2 to 3 fold and were about 10 times the external K^+ concentration (0.234mg/ml) assuming the water content of the cell evenly distributed the K^+. On the other hand intracellular Na^+ varied over a 4 to 8 fold range and the Na^+ content of the cell varied from nearly the same as the external concentrations of Na^+ to about 1/3 the external concentrations. Table 15 and 16 show these relationships in cells at the 32 hour stage.

To determine if viable cell systems were required to maintain these concentration gradients, cells at 32 hours were incubated for 30 minutes in OS with 0.04% osmium tetraoxide to kill the cells. The cells so treated had an approximate 2 fold increase of Na^+ in the cell and an approximate decrease of K^+ by two fold. These new levels of Na^+ and K^+ did not exactly reflect the external con-

centrations but the exposure was long enough to show that a gradient was actually being maintained by viable cells.

At each external Na^+ concentration there was a corresponding internal level of Na^+. There does not appear to be a direct proportionality of internal Na^+ to external concentrations, but the higher the external concentration the higher the internal appears to be. Since killing of the cell with OsO_4 leads to higher internal concentrations it must be concluded that the levels are not due to equal concentrations of Na^+ on either side of the membrane. On the contrary it suggests that Na^+ is removed from or prevented from entering the cell against a concentration gradient.

Figure 18 The change of Na^+ and K^+ concentrations over time in three levels of Na^+.

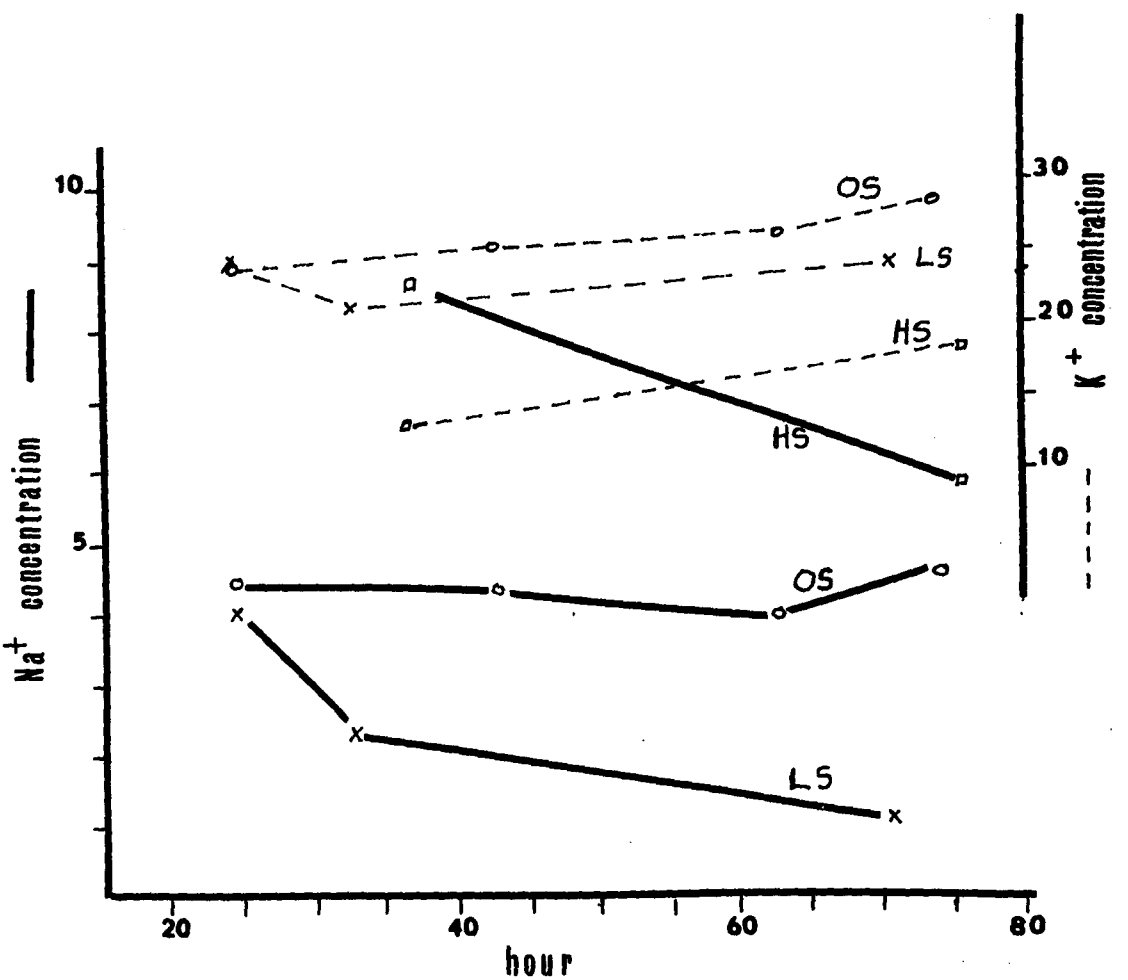

10
5
Na+ concentration
OS
LS
HS
HS
OS
LS
30
20
10
K+ concentration
20
40
60
80
hour

TABLE 15

Intracellular Na^+ Concentration and Extracellular Na^+ Concentration at 32-36 Hr Stage

	External Na^+ Concentration		Internal Na^+ Concentration		Ratio of mg/ml of Na^+ in medium to mg/g wet weight
	mM	$\frac{mg}{ml}$	$\frac{mg}{gram}$ dry wt.	$\frac{mg}{gram}$ wet wt.	
LS	21.6	0.496	2.27	0.454	1.1
OS	237	5.451	4.35	0.870	6.2
HS	517	11.891	8.59	1.718	6.9

Note: In presence of OsO_4 concentration goes up to 8.88 in 30 minutes.

Changes in intracellular concentrations of Na^+ and K^+ when external Na^+ is changed

Since cells can survive in Na^+ free salts after sufficient development in OS medium, it was possible that the ability to retain the Na^+ had increased. Conversely, cysts could not develop in the absence of Na^+, so experiments were run to determine the ability of each developmental stage to retain Na^+. Cells at the cyst stage were found to lose about 90% of their sodium content in the first 30 minutes exposure to SF salts* (Fig. 19). Likewise, if those cysts were exposed briefly to LS salts the Na^+ level fell to only 70% of the original concentration. These cells could then be exposed to HS salts and the concentration of Na^+ on the cells would rise to a value above the initial concentration when the cysts were in OS salts (Fig. 19). Internal concentrations of K^+ were not determined for this stage. These data seemed to support the hypothesis that cysts did not have the ability to concentrate Na^+ against a gradient and would account for the poor development if need for internal Na^+ could be proven. Cells at 15 hours had a different type of behavior. In SF initially the cells lost about 50% of the Na^+ but the decrease thereafter, on a concentration (mg/gram dry wt) basis was small (Fig. 20). The only way to know if the sodium is actually leaving the cell is to base the

*SF without glucose or glutamate. The same results can be obtained in a Na^+ free medium (SF).

measurements on the amount contained in a constant number of cells. Figure 21 shows that after the initial rapid loss the cell indeed retained nearly all of its sodium until late in its development. The loss later in the cycle may relect a degree of asynchrony in the cells. Cells reaching the point of zoospore production would release zoospores which could then encyst, and either one of these stages could lose Na^+ to the medium. Alternatively, the cells nearing the end of the developmental cycle could change their ability to retain Na^+.

Figure 20 shows that although Na^+ is retained as shown in Figure 21 there is a gradual dilution of the cytoplasmic concentration of Na^+. If a particular level of cytoplasmic Na^+ is required for optimal activity this dilution might result in decreased synthesis. A decrease in the final yield of cells placed in a Na^+ free medium at 15 hours has been observed (Table 14). However, if Na^+ is compartmentalized in some way this dilution may not antually take place. This decrease in cell yield could also be attributed to a decrease of uptake capacity as indicated by labelling studies (Section III).

The behavior of K^+ under these conditions is also plotted in Figures 20 and 21. While K^+ also drops (Figure 20) on a concentration basis, Figure 21 shows that in fact K^+ is taken into the cell during this portion of the cycle. This may mean that the cell has become more porous to K^+ but that uptake is still taking place.

Cells grown in OS and exposed to HS at 15 hours showed only a slight rise of Na^+ after one hour of exposure (4.40mg Na^+/gram dry weight to 5.92mg Na^+/gram dry weight). The K^+ concentration did not change.

At 32 hours cells grown in OS lost 67% of the Na^+ in one hour (4.35 to 1.40 mg Na^+/gram dry weight) if transferred to SF. Cells that were placed in HS at this stage increased the Na^+ concentration from 4.35 to 21.75mg Na^+ per gram dry weight, indicating that the cell may have become more permeable to Na^+. Here there was also a change of K^+ concentration from about 28mg K^+ per gram dry weight to 41mg K^+. This change could mean that the great increase in Na^+ under these circumstances caused a similar uptake of K^+ but the actual mechanism is unknown.

Effect of 2, 4 Dinitrophenol on retention of Na^+ (DPN)

When cells held in SF salts were exposed to $5x10^{-3}$M DPN, an uncoupler of oxidative phosphorylation, the cells lost two times as much Na^+ as controls without the inhibitor. On the other hand when cells grown in LS were transferred to OS with DNP, sodium was again lost to the medium when compared to the control (Table 17).

If the result was due to lack of adenosine triphosphate (ATP) production then ATP must be needed to retain Na^+ inside the cell. Another interpretation is possible. Since DNP is known to increase the respiration of cells it is possible that the increased respiration in each

case (more augmented in change of LS to OS) drives the sodium out of the cell through a respiratory or electron transport stimulated exit pump.

Figure 19 Change of Na^+ concentration in cysts when placed in Na^+ free medium from OS and from LS to HS.

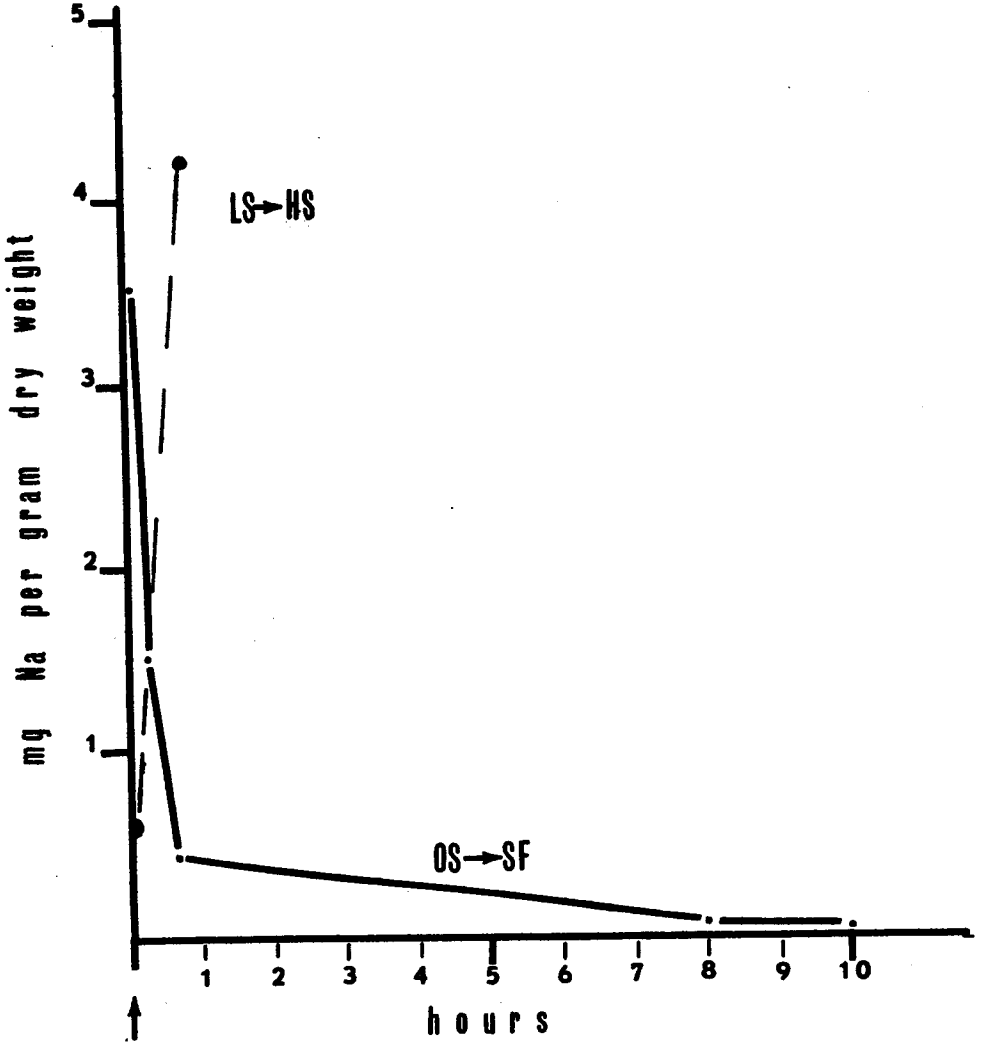

mg Na per gram dry weight
5
4
3
2
1
LS→HS
OS→SF
1
2
3
4
5
6
7
8
9
10
hours

Figure 20 Change in Na^+ and K^+ (mg ion/gm dry wt.) when cells were changed from OS to SF at 15 hours.

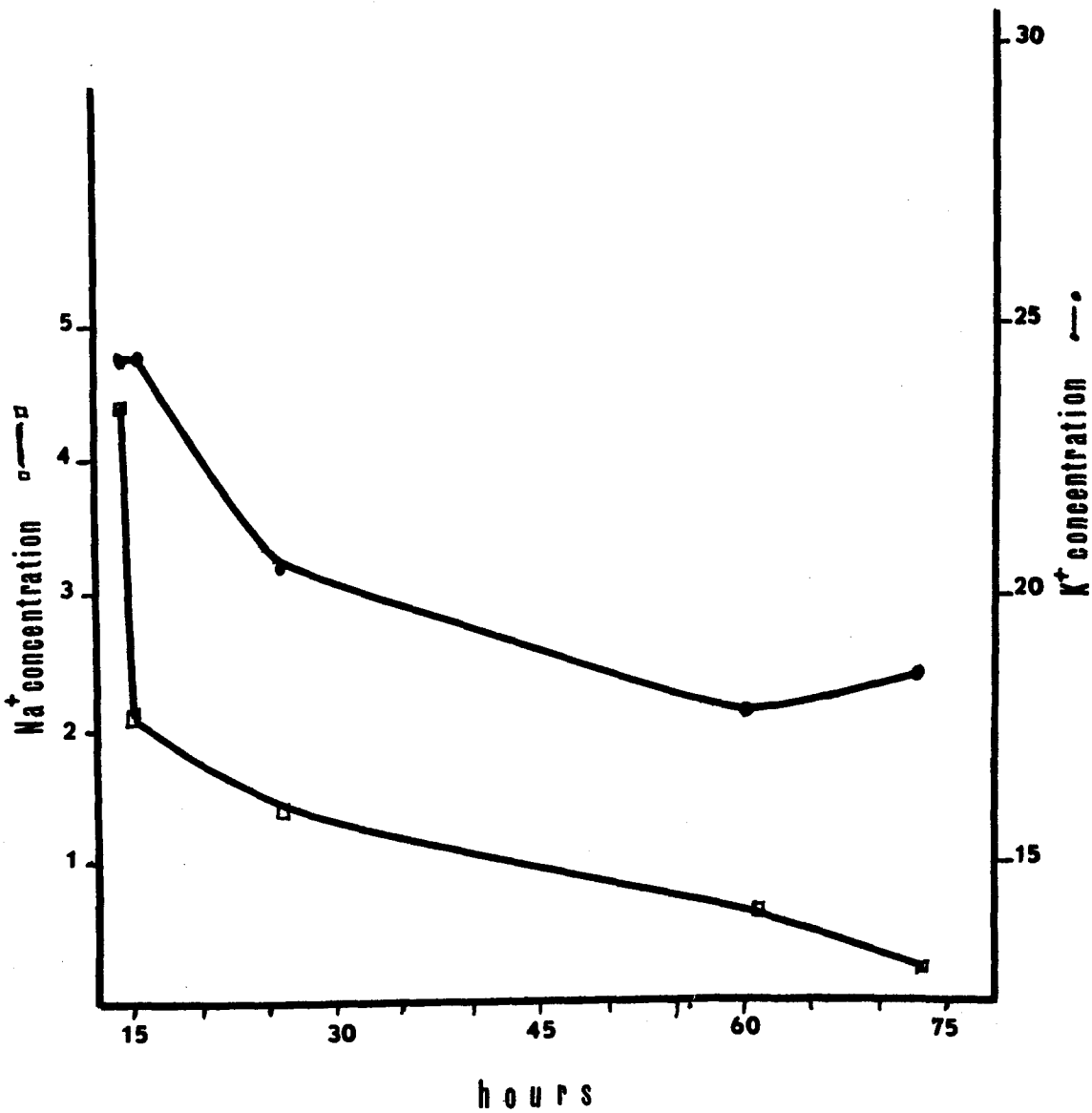

Na+ concentration
K+ concentration
5
4
3
2
1
30
25
20
15
15
30
45
60
75
hours

Figure 21 Change in mg Na^+ in a constant number of cells. Sample same as Figure 19 but replotted to reflect loss from cells without taking into account the increase in dry weight. Initial (15 hours) points are based on 1 gram dry weight so it will be comparable to Figure 20.

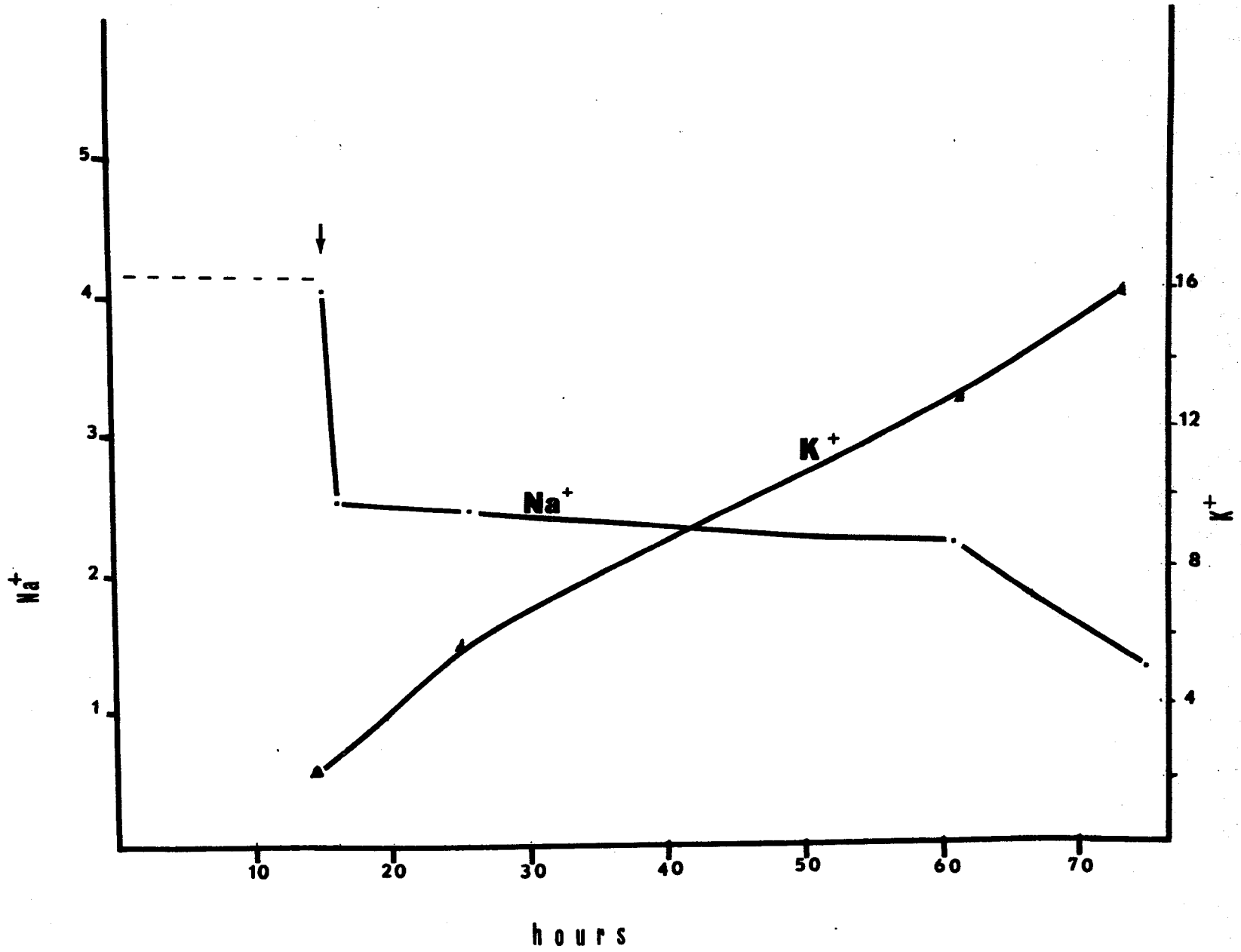

5
4
3
2
1
Na+
16
12
8
4
K+
K+
Na+
10
20
30
40
50
60
70
hours

TABLE 16

Intracellular K^+ vs. External Na^+ Concentration at 32-36 hrs.

	External Na^+	Internal K^+		Ratio Intracellular K^+/ Na^+
	mg/ml	mg/gram dry wt.	mg/gram wet wt.	
LS	0.496	21.03	4.21	9.26
OS	5.451	25.25*	5.05	5.80
HS	11.891	12.99	2.60	1.51

K^+ in all media is 0.006M (0.234 mg/ml)

*Falls to 12.11 in 30 min. in presence of OsO_4.

TABLE 17

Effect of DNP on Na^+ concentration in cells. Changes made from OS to SF and LS to OS with and without presence of DNP. Final concentration of Na^+ after one hour exposure is recorded. All cells at 32 hour stage and from OS medium. Normal level in OS is $\sim$4 mg Na^+/gram dry weight. In LS it is about 2.2 mg/gram dry weight.

Medium Change	Final Na^+ level (mg Na^+/gram dry weight)
OS → SF	1.42
OS → SF + DNP	0.77
LS → OS	2.58
LS → OS + DNP	1.67

Enzyme activity and Na^+

Amylase activity at a given pH is influenced by the Na^+ concentration. The level of Na^+ at which peak activity is observed is also influenced by pH. The enzyme may be adapted to pH regimes encountered at various salinities in nature. The pH generally increases in the estuary from 7.1 to 8.2 as the salinity increases from $1^o/oo$ to $30^o/oo$ (personal observation). The activity of amylase obtained from this fungus shows peaks of activity at high pH when Na^+ is high and at low pH when Na^+ is low. Therefore, it is apparently well adapted (Fig. 22).

Lactate dehydrogenase (LDH) activity is not appreciably affected by Na^+ except at the highest Na^+ level (Fig. 23). NADH oxidizase is on the other hand maximally active at the intermediate levels of Na^+ (Fig. 24). These last two systems are intracellular and should be compared to actual Na^+ inside the cell when that data becomes available.

Figure 22 Amylase activity as indicated by production of reducing sugars by DNS method at three different pH levels.

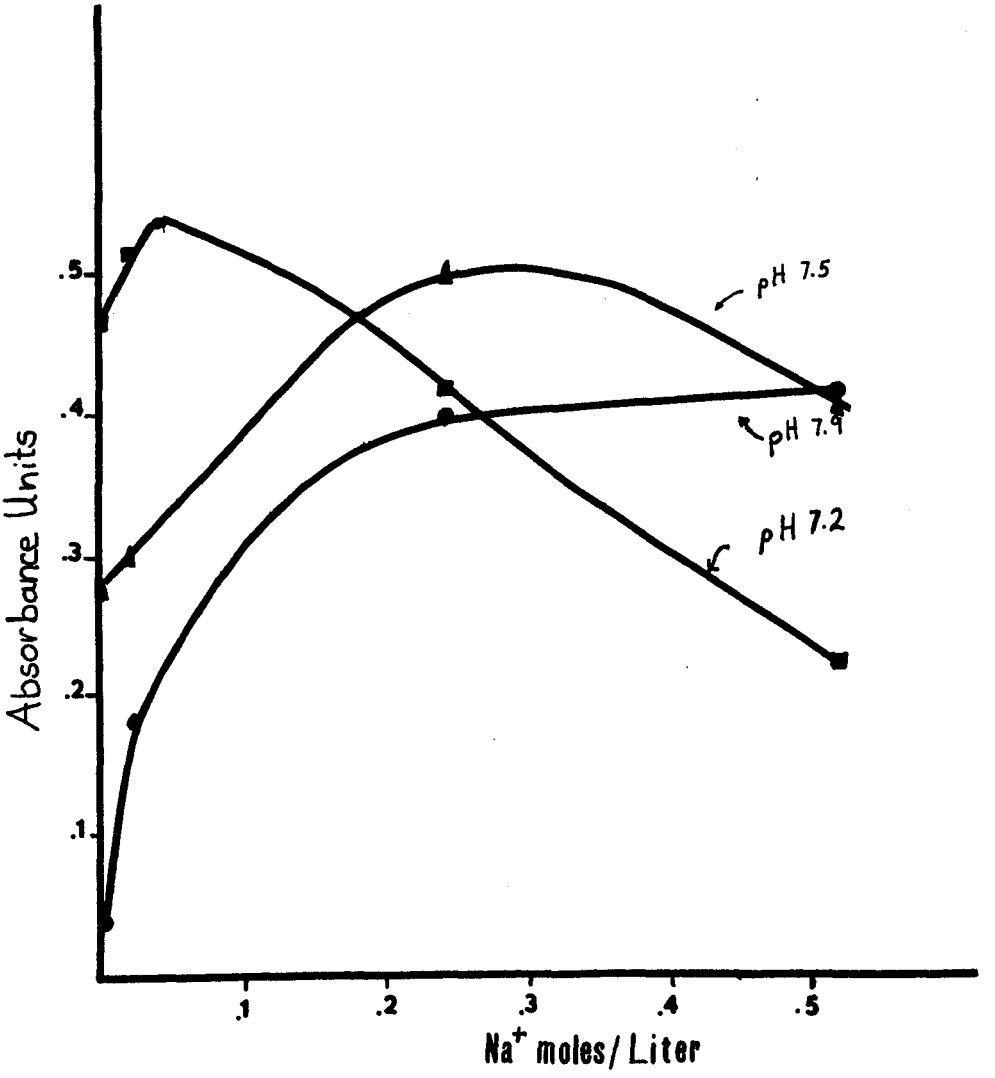

Absorbance Units
.5
.4
.3
.2
.1
.1
.2
.3
.4
.5
Na+ moles/Liter
pH 7.5
pH 7.9
pH 7.2

Figure 23. LDH activity at pH 7.85 by change in NADH absorbancy at 340 mμ.

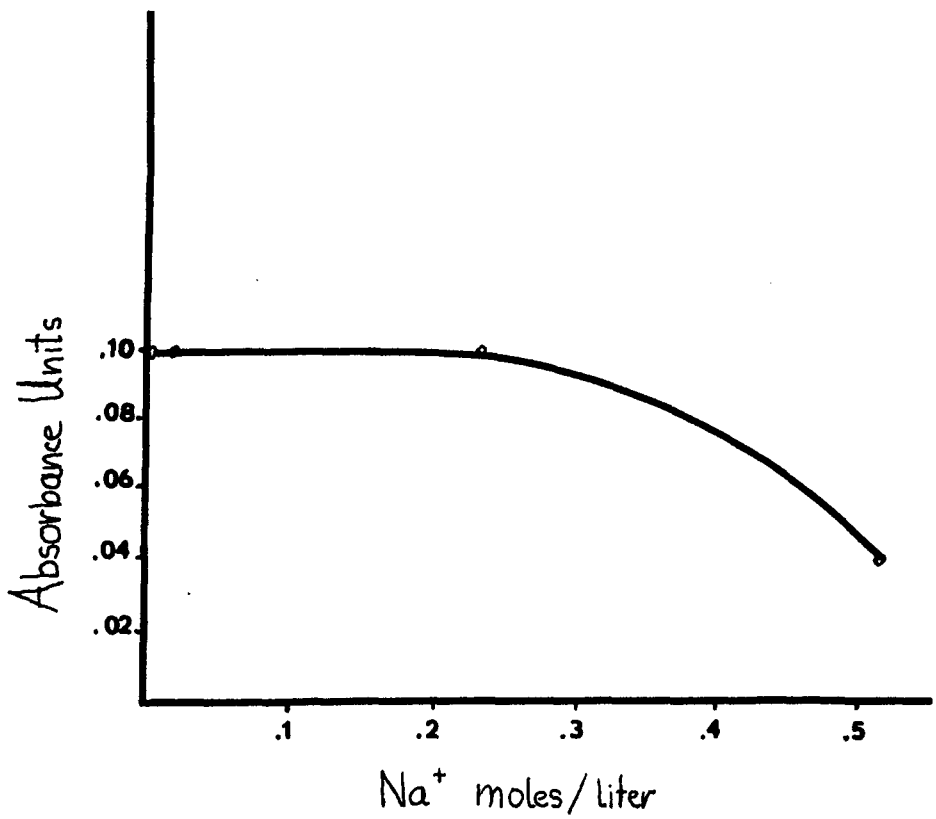
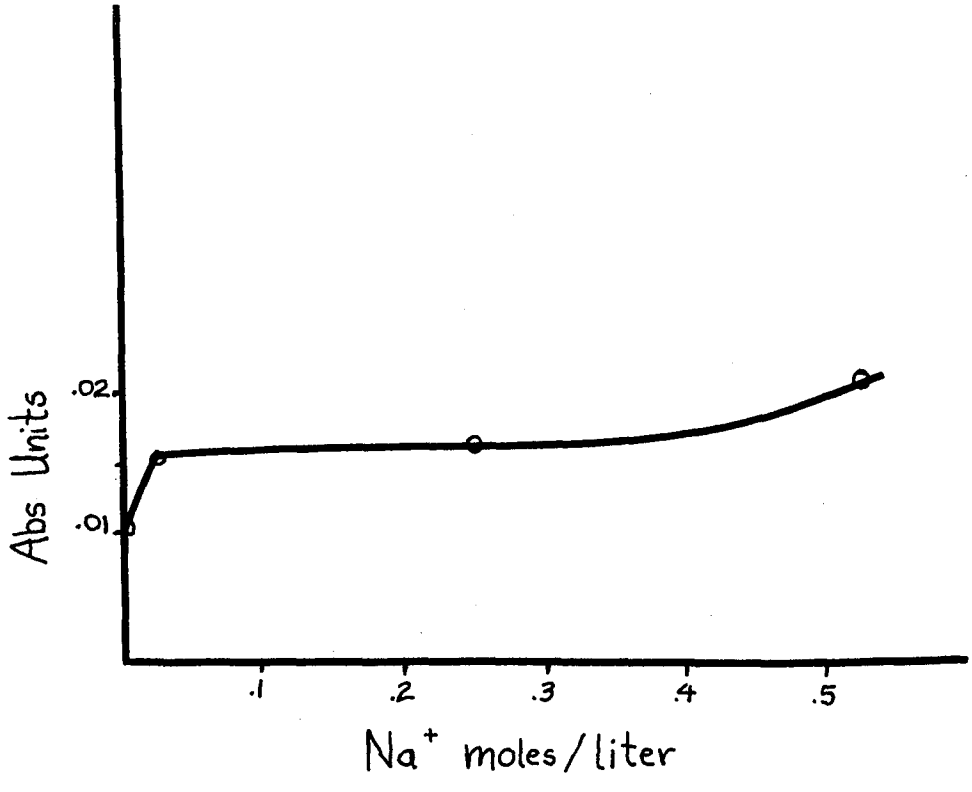

Absorbance Units
.10
.08
.06
.04
.02
.1
.2
.3
.4
.5
Na+ moles/liter

Figure 24. NADH oxidizing enzyme activity at pH 7.85 by change in absorbancy at 340 mμ.


Abs Units
.02
.01
.1
.2
.3
.4
.5
Na+ moles/liter

DISCUSSION

The major purpose of these experiments was to establish the ability of the cell to retain Na^+ internally at a particular developmental stage. It was shown that Phlyctochytrium is less capable of retaining Na^+ in a Na^+ free medium when the cell is at the cyst stage than when it is at 15 hours or later. There was also evidence that later in the cell cycle this ability to retain or regulate the Na^+ content of the cell might again decrease. As the cell developed beyond the cyst stage in a Na^+ enriched medium it acquired the ability to survive in Na^+ free media. Since the medium was difficient only in Na^+ and because increasing the osmotic pressure with mannitol did not increase the ability of cysts to survive in Na^+ free media it must be assumed that the Na^+ was in some way involved in growth and development. In the previous section it was shown that Na^+ could be related to uptake but that in the high nutrient levels of the SF medium development continued, presumably gaining sufficient nutrient by diffusion. Yet, the role of sodium was still undefined. The fact that sodium was retained in cells which were capable of development in a Na^+ free medium is further evidence that it is required for development of the cell. There

was a direct correlation between development in a Na^+ free medium, regardless of diffusable nutrients, or the effect of Na^+ on uptake and the ability of that cell to retain previously infused Na^+. However, it was not possible to conclusively demonstrate by labelling techniques that protein synthesis required Na^+ as a co-factor. One way to gain further information would be to demonstrate that a specific protein can be produced and that this mechanism can be turned on and off by manipulation of the Na^+ level of the cell. This has been done in a marine bacterium (Webb & Payne, 1971). Another approach would be to demonstrate a requirement for Na^+ in a cell free incorporation of amino acid into protein. The latter experiment has been carried out with leaves of the halophyte Suaeda maritima, but there it was demonstrated that Na^+ depressed the rate of incorporation (Hall & Flowers, 1973). Their conclusion was that the cell had the ability to compartmentalize the Na^+ into a vacuole and that the actual cytoplasmic concentration of Na^+ was very low. Whether this is actually the case in the present study is not known, but on the basis of the present evidence, I would believe, on the contrary that synthesis is stimulated by sodium. Marine bacteria and halophalic bacteria have some enzyme systems (Holmes, Dundas, and Halvorson, 1965) and protein synthesizing systems (Webb & Payne, 1971) which appear to be dependent on rather high levels of Na^+.

Pathways of glucose metabolism have been shown to be controlled by salinity in Vibrio marinus (Griffith & Morita, 1973).

If the other marine fungi have the ability to regulate the intracellular Na^+ concentration as this one apparently does, it may influence interpretation of several findings on their activities. The activity of the intracellular processes may be considerably limited by the Na^+ concentration in the cell and these processes may function well only in a narrow range. The present study presents evidence that external sodium influences the cell by 1) an influence on uptake and 2) by the way it affects the intracellular level of Na^+. Furthermore, there is some evidence that a portion of Na^+ in the cell is loosely bound. This is the portion which leaves the 32 hour cell during the initial exposure to a Na^+ free medium. The remaining sodium at this stage is evidently tightly bound because it does not leave the cell during an extended incubation period in a Na^+ free medium. Such strong retention of this normally soluble ion may indicate that it is part of the molecular structure of the cell. There is the possibility that Na^+ is compartmentalized in some way and this should be investigated. The claim by Siegenthaler, Belsky, & Goldstein that in Thraustochytrium "endogenous respiration occurred in the absence of any cations" must be revised to state "external cations", since there is now a clear distinction. An investigation into the

sodium content of Thraustochytrium is now needed to determine if it has a similar degree of control over intracellular Na^+. If Thraustochytrium has the ability to regulate intracellular Na^+, it might explain respiration in the absence of external Na^+.

Jennings (1974) discusses the possibility that fungi may require Na^+ to stimulate binding of substrate to carrier to aid in transport of sugars and amino acids across cell membranes. In doing this there would be an uptake of Na^+ and a Na^+ pump would eliminate excess Na^+ from the cell to stimulate the release of the substrate inside the cell. This system would not be entirely suitable to explain the function of the Phlyctochytrium system unless the Na^+ inside the cell, which may be similar to the external level in LS, is bound or compartmentalized in some fashion and not able to influence the equilibrium reaction Jennings shows. Of course, it is possible that a lack of sodium concentration differential would explain reduced growth in LS.

It will be necessary to look at the tightly bound and loosely bound fractions of Na^+ in the cell to completely interpret their functions. Loosely bound material could act as an osmoticum to the cell and the observed rapid flux out of the cell when transferred from OS to SF would agree with this function. Similar experiments on Na^+ retention in the presence of an osmoticum would be helpful. Also localization of Na^+ in the cell by techniques

which could identify the locus of Na^+ ultrastructurally may be useful if there is sufficient evidence that Na^+ does not move during preparation procedures.

One area of investigation which should be pursued is the possibility that vacuoles may compartmentalize Na^+ intracellularly. In Section II it was shown that at 15 to 24 hours vacuoles became recognizable with phase contrast microscopy, and it is conceivable that these structures may be related to the ability of the cell to retain sodium at that stage. Vacuoles have been implicated in compartmentalization of Na^+ in higher plants (Hall & Flowers, 1973). Additional studies on the mechanism of Na^+ transport are needed to determine how the external environment effects the intracellular concentrations of this ion.

It does not seem likely that rapid changes from OS to SF media would not alter the cell membrane in some way. Evidence has been presented that this change 1) shows an initial rapid loss of Na^+ - followed, at some stages, by a cessation of this loss, and 2) causes losses of amino acid initially. Furthermore, in a shift up to HS or a shift down to LS there is 1) a period of adjustment so that after three hours there appears to be an increased ability to take up and incorporate amino acid, and 2) a possibility of synthesis of new or more RNA required to make adjustment to a new Na^+ concentration. These factors may indicate stress but seem to be short term factors.

Once adjustment is made the cells develop at a rate similar to what should be expected in the new medium. The rapid flux of Na^+ out of the cell stops when it has reached a certain level as in Figure 21. It is not likely that all synthetic constituents of the cell would function well over a broad range of Na^+ concentrations as is indicated by various measurements of the internal content of the cells. There are differences in synthetic rates even after three hours adjustment to a new level of Na^+, and this may reflect the change in activity of these systems produced by the new Na^+ concentrations. Yet, the actual conditions inside the cell are unknown. It is also unclear if the varying concentrations of internal Na^+ cause the observed changes. It has been shown that Na^+ and K^+ change in a kind of relationship to one another and it is possible that the ratios of these ions or these actual levels in the cell may also effect the cellular activity (Norkrans & Kylin, 1969).

Studies on enzymes produced by Phlyctochytrium show that their activity can be affected by the Na^+ concentrations of the reaction mixture. While the extracellular enzyme amylase is affected by Na^+ concentrations this does not indicate how internal activities of the cell might be effected. However, there is the intriguing possibility that the cell's use of extracellular starch may be affected by salinity. LDH shows no change in activity at levels of Na^+ which would be expected in the

growing cell, unless there are concentrated compartment. There is some indication that NADH oxidizing enzymes may require elevated levels of Na^+ for maximum activity and this may account for the decreased activity of the cells when the Na^+ falls to very low levels. These experiments were preliminary in nature and need further investigation before conclusions are to be drawn.

Finally, it is possible that the ability of the cell to regulate sodium may be of specific value in the intertidal estuarine environment. In that kind of environment, salinity, and thus Na^+ concentration, can fluctuate greatly during the 6 hour period between high and low tides. A cell with the ability to regulate Na^+ could dampen the effects of changing external sodium concentrations and thus would be well suited to survival in that environment.

CONCLUSION AND SUMMARY

It is now possible to more clearly define what makes an organism marine. By studying how Na^+ can effect growth, morphogenesis, uptake, synthesis, and the cellular levels of Na^+ a better understanding of how the marine environment affects this organism has been gained. Based on the present study it may be said that a requirement for sodium for growth and development may characterize a truly marine fungus.

The generally accepted concept that Na^+ is a major limiting factor in marine fungi has again been supported, but a major modification has been made. By using synchronously developing cultures it was possible to demonstrate that during the major part of the cell cycle Na^+ was not required in the growth medium. For an organism which appears, from its inorganic nutritional requirements, to be well suited to an estuarine environment this may be an additional advantage. Estuaries, especially their intertidal portions, may have broad fluctuations in Na^+ content (salinity) and this organism is well adapted to withstand the fluctuations and even the ultimate absence of Na^+ in the external environment.

Since Na^+ appeared to be important to the function of the organism during the early portion of its cell cycle it was hypothesized that Na^+ might also have a role in the rest

of the cell cycle. The finding that the organism could retain a portion of the cellular sodium when placed in a sodium free medium during the later cycle made this idea even more attractive. The evidence available at the present time does not allow assignment of a functional role to sodium inside the cell, but it is clear that in cases when it is not present growth and development do not take place. Furthermore, the concentrations of Na^+ required for growth and development of this fungus far exceed that available in freshwater environments.

As in other marine microorganisms it has been possible to demonstrate that the uptake of exogenous substrates is related to the presence and concentration of sodium. Furthermore, based on the effects of inhibitors it is possible to say that the uptake mechanism involves ATP and a Na^+-K^+ dependent ATPase system, which means that the organism is more similar to the Thraustochtriaceae than to the marine bacterium B-16.

Although the Na^+ content of the medium cannot be related directly to cynthetic processes of the cell there does seem to be an effect on the development. The cell size, length of cycle, and the formation of exit papillae can be altered by manipulation of the Na^+ content of the medium. Since these activities are the result of the synthetic process Na^+ must in some way have an effect on this activity. The size of the exit papillae seems to be related to the osmotic pressure of the growth medium, so at least in that

instance Na^+ action may be a result of its influence as an osmoticum.

Even is the way in which Na^+ affects morphogenesis is not clear it is now evident that there is a great sensitivity of this organism to its inorganic environment. Soll (1970) and Soll and Sonneborne (1971) have shown that manipulation of the external ionic environment may be responsible for developmental changes. Based on the present research it is now possible to extend that statement to say that; changes in the external environment may affect the intracellular environment in ways that will affect development of the cell. Furthermore, the way in which those effects are expressed may depend to a great extent on the stage of the life cycle.

The alteration of morphology of this organism is highly important to the taxonomic understanding of the entire group of organisms. The findings here reiterate the contentions of several mycologists that the cells are so morphologically plastic that we may have to modify the taxonomic keys to include specific cultural conditions in order to use the morphological features upon which descriptions are based. This should include not only organic but inorganic nutritional conditions as well.

The finding that the organism can retain Na^+ during certain stages of development, that the cell may have a portion of the sodium bound in some way, and that the concentration of sodium inside varies in a complex way with the Na^+

outside, means that previous studies on the effects of sodium will need re-evaluation. Now any investigation into how the Na^+ concentration (or salinity) affects the organism should include an investigation on the internal presence and activity of Na^+ before conclusions are drawn as to how the external environment affects the biology of the organism.

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