EFFECTS OF ENVIRONMENTAL TEMPERATURE ON MEMBERS OF THE MUCORALES

by

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To my Parents and Family

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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SUMMARY

The following investigation characterises the interaction between temperature and growth in psychrophilic, mesophilic and thermophilic fungi in order to gain further insight into the physiological mechanisms underlying fungal growth at extreme temperatures. In the first part of the investigation, the effect of environmental temperature on the growth of vegetative mycelium and sporangiospore production and germination was considered in order to determine the cardinal temperatures of these activities in different thermal groups. Subsequent investigations of plasma membrane permeability suggested that plasma membrane structure and function may be significant in establishing both the upper and lower growth temperature limits characteristic of psychrophiles, mesophiles and thermophiles. Analysis of the plasma membrane fractions revealed significant differences in membrane phospholipid composition between these thermal groups and it is suggested that the differing cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi reflect the temperature ranges over which these organisms exhibit levels of plasma membrane fluidity sufficient to maintain membrane-associated growth processes. In contrast, the membrane protein components appear uniform in both character and thermostability and are therefore unlikely to contribute to this phenomenon.

Key Words:

Temperature, Fungal Growth, Plasma Membrane, Phospholipids.

PUBLICATIONS

ABSTRACTS

1) HAMMONDS, P. and SMITH, S.N. Physiological Responses to Extremes of Environmental Temperature by Fungi of the Order Mucorales.

2) HAMMONDS, P. and PUGH, G.J.F. Adaptation to Low Environmental Temperatures by an Antarctic Mesophilic Fungus.

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CHAPTER ONE

GENERAL INTRODUCTION

Temperature profoundly affects all the vital activities of microorganisms and is undoubtedly one of the most important environmental factors. The physiological responses of microorganisms to extremes of environmental temperature have been extensively studied (Cooney and Emerson, 1964; Rose, 1967; Crisan, 1973; Zuber, 1976; Heinrich, 1976; Brock, 1978; Kushner, 1978; Friedman, 1979; Zeikus, 1979; Zuber, 1979).

The temperature variations encountered in various aquatic and terrestrial environments are extreme enough to promote at least three distinct thermal groups of organisms. The vast majority of organisms thrive at moderate temperatures and are said to be mesophilic in their temperature requirements for growth. However, at the very low and high extremes of temperature, microorganisms and primitive plants predominate.

Our knowledge of the cold-loving or psychrophilic microorganisms is somewhat lacking, despite the fact that over 80% of the biosphere is permanently cold, that is less than 5C. The worlds' oceans occupy some 71% of the earths' surface, of which approximately 90% is less than 5C. In addition, the polar regions, including the continent of Antarctica and the permanently cold areas surrounding the Arctic Circle represent approximately 14% of the earths' surface. Cold habitats include seas, lakes, rivers, streams, soils, caves and man-made cold environments such as frozen and iced foods (Baross and Morita, 1978).

At the other end of the temperature scale, a wide variety of natural and man-made high temperature habitats exist, including active volcanoes, dry steam fumaroles, boiling or superheated springs, hot springs, sun-heated substrates such as soils, litter and rocks, self-heating, organic-rich materials such as compost piles and coal refuse piles, hot water domestic and industrial heaters, cooling waters from industrial processes, steam lines and many other habitats (Tansey and Brock, 1978).

In sharp contrast to psychrophiles, the biology of heat-loving thermophiles has been extensively studied. However, most investigations of the interaction between microbial growth and extreme temperatures relate to prokaryotes, such as bacteria. In comparison, the filamentous fungi have been little studied. Further, it is unfortunate that microbiologists have concentrated on one particular thermal group, instead of studying and comparing psychrophiles, mesophiles and thermophiles. If, indeed, the effect of temperature on all micro-organisms is a unified process, then it should prove possible to formulate a single hypothesis to explain thermal death in psychrophiles, mesophiles and thermophiles. The filamentous fungi occupying an intermediate position between the less organised prokaryotic bacteria and the more complex eukaryotic plants and animals, may be the ideal tools for investigating the physiological bases for growth at extreme temperatures (Crisan, 1973).

There are several problems posed by psychrophilic, mesophilic and thermophilic fungi, indeed by all such microorganisms. Of these, the ability of thermophilic fungi to grow at high temperatures lethal to both mesophiles and psychrophiles has received most attention. Much less studied is the inability of thermophiles to grow at the moderate temperatures at which mesophiles thrive. Conversely, psychrophiles face precisely the opposite problems to thermophiles. The ability of psychrophiles to grow optimally at low temperatures has received more attention than their inability to grow at even moderate temperatures.

The primary factor which determines the ultimate growth temperature limits of an organism is its genetic constitution (Crisan, 1973). Indeed, there is evidence that the ability to grow at high temperatures can be transmitted from thermophilic to mesophilic bacteria through genetic transformation (Sie, et al., 1961(a), 1961(b); McDonald and Matney, 1963). However, despite recent advances in genetic techniques, particularly in the field of recombinant DNA technology, our knowledge of fungal molecular genetics is not yet sufficiently advanced to make studies of the genetic basis of growth at extreme temperatures practical. Consequently, major emphasis has been placed on delineating the physiological bases of growth at extreme temperatures.

One approach to investigating mechanisms of growth at extreme temperatures is to use cold and heat-resistant mutant strains. Here, in theory at least, a physiological comparison between mutant and wild type should establish the mechanism allowing the mutant to grow at higher or lower growth temperatures compared with the wild type. However, the optimal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic organisms are based on a genetic constitution whereby the existence of fixed phenotypes permits optimal growth at low, moderate, or high temperatures respectively. In contrast, the thermal stability of an individual component or process may be a result of a genetic adaptability, whereby a limited phenotypic flexibility permits changes to meet temporary environmental fluctuations (Crisan, 1973). Mutant strains are likely to be characterised by such phenotypic flexibility, whereby changes in the thermal stability of an individual component or process allows growth at lower or higher temperatures compared with the wild type, but are unlikely to reflect long term changes in the genetic constitution of the wild type. It follows that the characterisation of temperature-sensitive mutant strains is of limited use in investigating mechanisms of growth at extreme temperatures.

In contrast, the variety of fungal species available for investigation includes numerous examples of psychrophiles, mesophiles and thermophiles. Further, there are often representatives of all three types to be

found in the same or related genera. Therefore, a comparison between closely related psychrophilic, mesophilic and thermophilic species from the same genus is more appropriate than using mutants to investigate mechanisms of growth at extreme temperatures.

Of the numerous hypotheses put forward to explain growth at high temperatures, Crisan (1973) considers only two to still be of major importance, namely, (i) macromolecular thermostability and (ii) ultrastructural thermostability.

The hypothesis of macromolecular thermostability suggests that thermophiles produce essential macromolecules, such as proteins (particularly enzymes) which exhibit an unusually high degree of thermostability. Therefore, as the growth temperature is increased, these macromolecules are still able to function and growth is maintained at temperatures in excess of the maximum limits of mesophiles and psychrophiles. Indeed, the enzymes (Koffler, 1957; Amelunxen and Lins, 1968; Farrell and Campbell, 1969; Amelunxen and Murdock, 1978) and certain structural proteins (Koffler et al., 1957; Smith and Koffler, 1971; Amelunxen and Murdock, 1978) of thermophilic bacteria demonstrate intrinsic thermostability. In contrast, the nucleic acids of thermophiles, including DNA and RNA, do not appear to be unusually thermostable (Mangiantini et al., 1965; Stenesh and Yang, 1967; Friedman, 1968; Farrell and Campbell, 1969).

The hypothesis of ultrastructural thermostability suggests that thermophiles contain ultrastructural elements or organelles which exhibit a greater degree of thermostability than their mesophilic and psychrophilic counterparts and is supported by considerable evidence (Haight and Morita, 1966; Iandolo and Ordal, 1966; Brock, 1967, 1969; Brock and Darland, 1970, 1972; Tansey and Brock, 1972). Tansey and Brock (1972) and Brock (1978) have suggested that it is the failure of eukaryotes, such as fungi, to produce cellular membrane systems that are both thermostable and functional that might explain the inability of such organisms to grow at the high temperatures characteristic of thermophilic prokaryotes.

Amongst the cellular membranes, the plasma membrane is of marked importance, in that not only does it serve to preserve structural integrity, but it also functions as a selective barrier in regulating nutrient uptake, removing toxic waste products and maintaining the correct ionic composition and water content of the cell. It may therefore be of considerable significance that changes in plasma membrane phospholipid composition, in response to changes in temperature have been implicated as determinants of both the upper and lower growth temperature limits (Esser and Souza, 1976; McElhaney, 1976). Further, proteins, the second major component of plasma membrane

lipoprotein have also been shown to exhibit a certain degree of thermostability in thermophiles and might, within the membrane, exert an additional effect on cellular thermostability (Crisan, 1973). Thermal death in microorganisms is a unified process, in that the deterioration of life at supramaximal temperatures is accompanied by certain physiological and structural changes which are similar, irrespective of an organism, being psychrophilic, mesophilic or thermophilic in its temperature requirements for growth (Crisan, 1973). Therefore, the above hypotheses should also apply to psychrophilic and mesophilic organisms in addition to the thermophiles.

The aim of this investigation, therefore, is to characterise the interaction between temperature and growth in psychrophilic, mesophilic and thermophilic fungi in relation to the hypotheses of macromolecular and ultrastructural thermostability. Closely related Mucoraceous species, representative of all three thermal types have been chosen for investigation. The first element of the investigation involves the characterisation of the temperature limits for growth and sporangiospore germination in these organisms. Subsequent work considers the interaction of growth temperature with plasma membrane structure and function, with both membrane lipids and proteins being considered. These factors are discussed in terms of potential effects on the differing

lower and upper growth temperature limits characteristic of psychrophilic, mesophilic and thermophilic fungi. In this way, it is hoped that further insight will be gained into the physiological mechanisms underlying microbial growth, particularly fungal growth, at extreme temperatures.

CHAPTER TWO

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE GROWTH OF VEGETATIVE MYCELIUM

INTRODUCTION

Depending on their cardinal growth temperatures, that is the minimum, optimum and maximum temperatures at which growth occurs, microorganisms are conveniently categorised as either psychrophiles, mesophiles or thermophiles. The following working definitions will be used in this investigation:-

Of the approximately 50,000 plus species of true fungi, the vast majority are mesophilic in their temperature requirements for growth. Such fungi grow optimally between 20-30C and cease to grow above 40C and below 5C.

A further 70 plus, taxonomically diverse species are thermophilic in their temperature requirements for growth (Tansey and Brock, 1978). These fungi have a maximum growth temperature at or above 50C and a minimum growth temperature at or above 20C (Cooney and Emerson, 1964).

A few fungi grow best at low temperatures and are termed psychrophilic. According to Morita (1975) the growth range of psychrophiles is restricted from 0C or less, to 20C or less, with an optimum growth temperature of 15C or less.

Baross and Morita (1978) have pointed out that there is undoubtedly a continuum of upper and lower growth temperatures in the microbial world and that

the categorisation of microorganisms based only on their temperature limits for growth is tenuous, at best. Unfortunately microbiologists, in attempting to make such categories more precise by fixing the temperature ranges over which growth is affected for psychrophiles, mesophiles and thermophiles, have produced a plethora of terms whose meanings may not be clear to individual microbiologists (Farrell and Rose, 1967). This may be illustrated by considering the following examples.

Amongst other terms used to describe microorganisms that grow at low temperatures, but do not fit the definition of Morita (1975) may be included facultative psychrophile and psychrotroph (Hucker, 1954; Eddy, 1960). The term psychrophile implies that these organisms are capable of growth within the temperature regime defined by Morita (1975), but may also exceed the upper temperature limit of true psychrophiles. Psychrotroph (Eddy, 1960) on the other hand, refers to organisms capable of growth at 5C, or below, regardless of their optimum or upper growth temperatures.

However, it is in the definition of thermophilism that most disagreements occur among microbiologists. Many prokaryotic microorganisms are capable of growth at very high temperatures, even in boiling water (Brock, 1978). In contrast, the upper temperature limit for growth of eukaryotic microorganisms is somewhat lower, approximately 60-62C, at which only a few species of

fungi can grow (Tansey and Brock, 1972). Therefore, at temperatures where fungi are considered to be thermophilic, many bacteria are considered mesophilic! Further, bacteria growing at high temperatures in particular, have been classified with a variety of potentially confusing terms including caldoactive (Heinen and Heinen, 1972), obligate or facultative thermophiles (Farrell and Campbell, 1969; Williams, 1975) and thermotolerant (Farrell and Campbell, 1969).

Mycologists have adopted some of this specific terminology to describe the temperature relations of fungi living at high temperatures. The definition of thermophilism (given earlier and used in this investigation) due to Cooney and Emerson (1964) however, is comparatively widely accepted among mycologists. In addition, Cooney and Emerson (1964) define a group known as thermotolerant fungi as those with maxima near 50C, but minima well below 20C.

GROWTH KINETICS AND ASSESSMENT OF GROWTH

Despite certain exceptions, mycelial growth kinetics are essentially the same in submerged liquid batch culture and on agar surfaces (Righelato, 1978). In both systems hyphal elongation is linear, but branching and cell division create new growing points, resulting in true exponential growth until both nutritional and physical factors become limiting (Griffin, 1981).

Subsequent to the introduction of a suitable inoculum, several phases of growth are apparent: the lag, exponential (or log), deceleratory, stationary and decline phases respectively.

The lag phase is generally thought to be a period of adaptation to the environment. In submerged liquid culture, it probably reflects the time taken for a spore inoculum to germinate and subsequently mobilise mycelial growth processes.

The exponential phase, often referred to as the logarithmic or log phase, is characterised by exponential growth due to an excess of substrates and absence of inhibitors. Thus, the increase in fungal biomass observed in this phase is exponential. Several growth parameters can be calculated in this phase and used to compare fungal growth rates under different environmental conditions. Two such parameters will be considered here.

(1) SPECIFIC GROWTH RATE μ

This represents the amount of organism produced by a unit amount of organism in a unit time. It is a constant characteristic of the environment and strain:

Rate of Growth = Constant X Organism Concentration

$$\text{i.e} \quad \frac{dx}{dt} = \mu \cdot x \qquad (1)$$

where μ = specific growth rate

x = mycelial dry weight

t = time

$\frac{dx}{dt}$ = rate of increase of x (growth)

Integration of (1) enables the concentration of organism at any time (t) to be calculated:

Thus: $$x_t = x_o \, e^{\mu t} \qquad (2)$$

and $$\ln x_t = \mu t + \ln x_o \qquad (3)$$

Simple manipulation of equation (3) enables the specific growth rate to be calculated:

Since $$\ln x_t = \mu t + \ln x_o \qquad (3)$$

Then $$\mu = \frac{(\ln x_t - \ln x_o)}{t_2 - t_1} \qquad (4)$$

where t_1 = time at beginning of log phase

t_2 = time at end of log phase

(2) MOLAR GROWTH YIELD

The specific growth rate observed in unrestricted growth is the maximum an organism is capable of under the particular environmental conditions. It is therefore useful in delineating the optimum temperature for growth. However, since it is essentially a measure of the rate of reaction, it gives no indication of growth yield in terms of carbon and nitrogen sources in the medium.

In the defined medium used in this investigation glucose is the major carbon source. Thus, the molar growth yield for glucose may be calculated as follows:

$$\text{Molar Growth Yield (Y)} = \frac{\text{maximum dry weight (g)}}{\text{moles glucose}}$$

Eventually, the rate of growth will slow down and reach a constant level in the deceleratory and stationary phases respectively. This is due to a depletion of nutrients concomitant with a build up of toxic waste products. In the decline or autocatalytic phase of the culture dry weight falls.

The most commonly used measures of fungal growth are colony diameter and dry weight. The relative ease with which fungi can be grown on an agar surface, coupled with linear growth of a colony on such a surface, has led to the widespread use of this technique for investigating the effects of numerous factors on fungal growth rates (Trinci, 1970; Trinci and Gull, 1970; Okanishi and Gregory, 1970). However, this technique cannot be used to accurately determine the specific growth rate of a colony without measuring the peripheral growth zone of that colony since, as a hypha increases in length and branches, the diffusion of nutrients to the centre of the colony becomes increasingly limited and only the outer or peripheral zone grows exponentially.

The peripheral growth zone may be measured by cutting selected chords into the colony and, using such methods, Trinci (1971) calculated the specific growth rate of nine species of fungi and found that the values obtained were close to the specific growth rate in submerged culture on the same medium. The nature of growth on agar surfaces in which spores are often produced plus the fact that once mycelium has been cut it is of no further use, makes the use of agar plates impractical for physiological investigations other than in scanning the effects of certain factors on a large number of species or strains (Righelato, 1978).

In this investigation, colony radial growth measurements were made initially to determine the range of temperatures for growth of the test species and to give an approximation of optimum growth temperature. Subsequently, a more accurate measurement of growth was made using colony dry weight measurements as the criterion for growth in order to calculate specific growth rates.

The relationship between temperature and biological activity (including growth and enzyme activity, for example) may be expressed by the Arrhenius equation:

$$k = Ae^{-E/RT} \qquad (1)$$

where k = rate constant

T = temperature (K)

R = gas constant

E = energy of activation

Application of the equation to the growth of micro-organisms results in k being considered as the specific growth rate (μ) and the activation energy is sometimes referred to as the temperature characteristic (Inniss and Ingraham, 1978).

Taking logs (1) becomes:

$$\ln \mu = \ln \frac{A - E}{R} \times \frac{1}{T}$$

This is the equation of a straight line, which may be obtained by plotting ln μ against 1/T, the so called Arrhenius plot. The slope of the line equals - E/R and allows the determination of the activation energy. The Arrhenius plot is useful for comparing the effect of temperature on different biological processes. Therefore, growth was expressed in terms of an Arrhenius plot in order to compare the effect of temperature on growth with other physiological processes, particularly enzyme activities, considered later in the investigation.

The work outlined below establishes the cardinal growth temperatures of the test species in order to determine their temperature requirements for growth. The effect of growth temperature on the specific growth rate and molar growth yield (and the effect of growth medium on these parameters) is also considered.

MATERIALS AND METHODS

(1) SPECIES

Mucor hiemalis Wehmer and *Mucor pusillus* Lindt (*M. pusillus* "Aston") were obtained from the mycological culture collection, Aston University. *Mucor psychrophilus* Milko was obtained from the Centraalbureau Voor Schimmelcultures, Netherlands. *Mucor pusillus* Lindt var Lacey (*M.pusillus* "Lacey") was obtained from the Commonwealth Mycological Institute, England. *Mucor hiemalis* Wehmer F. hiemalis was isolated on South Georgia, South Atlantic, during a British Antarctic Survey visit in 1980 and was kindly donated by Dr J.L. Hurst. Stock cultures were maintained on malt extract agar and glucose-asparagine agar (Appendix 2:1) and were subcultured at regular intervals.

(2) LINEAR EXTENSION

8 cm petri plates, each containing approximately 20ml of cool sterile malt extract agar (MEA - Appendix 2:1) were inoculated with mycelial discs of each species cut from actively growing mycelia on MEA. The plates were then incubated at temperatures over the range 5 to 60C. Three replicate plates per temperature per species were used. Colony diameters were measured at suitable time intervals and were taken as the mean of two diameters at right angles, measured by the use of "cross-hairs" drawn on the base of each plate. Growth rates were

calculated during the exponential growth phase. The experiment was repeated using glucose-asparagine medium (GAE - Appendix 2:1) and data were analysed using two-way analysis of variance. A key to statistical notation is given in Appendix 2:1.

(3) DRY WEIGHT

100ml Erlenmeyer flasks, each containing 25ml sterile GAE medium (Appendix 2:1) were inoculated with a spore suspension of each species. For each strain, flasks were incubated in a Gallenkamp orbital incubator at 100 r.p.m., over the range of temperatures suitable for growth, as determined previously by linear extension on agar.

For dry weight analysis, flasks were periodically removed and the mycelia harvested by suction filtration onto pre-weighed, dried and cooled Whatman No. 1 filter papers. For each temperature ten samples were taken, with two replicate flasks per sample. After drying to constant weight at 80C, the mycelia plus filter papers were cooled in a dessicator and re-weighed (dry weight was obtained by subtracting the weight of filter paper alone).

For each strain, at each temperature, two replicate curves of mycelial dry weight against time were plotted. From these, the specific growth rate and molar growth yield were calculated as described previously and data were analysed using two-way analysis of variance.

RESULTS

(1) LINEAR EXTENSION

Growth rates are shown in Figure 2:1 (Appendix 2:2) and the results from each strain considered in turn below.

M. PUSILLUS "ASTON"

This strain grew from 25-50C, with an optimum growth temperature of 40C on both media. However, growth rates on MEA were significantly higher compared with those on GAE at all temperatures suitable for growth. The optimal growth rate of 49.00 mm day^{-1} at 40C on MEA was the highest growth rate recorded for any of the strains investigated.

M. PUSILLUS "LACEY"

The response of this strain was similar to that of M. pusillus "Aston". Again, the temperature range for growth was 25-50C, with an optimum growth temperature of 40C on both media. Similarly, growth rates on MEA were significantly higher than those on GAE. Growth rates on both media were similar to those observed for M. pusillus "Aston" at all temperatures suitable for growth.

FIGURE 2.1

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE (i) LINEAR EXTENSION

FIGURE 2:1

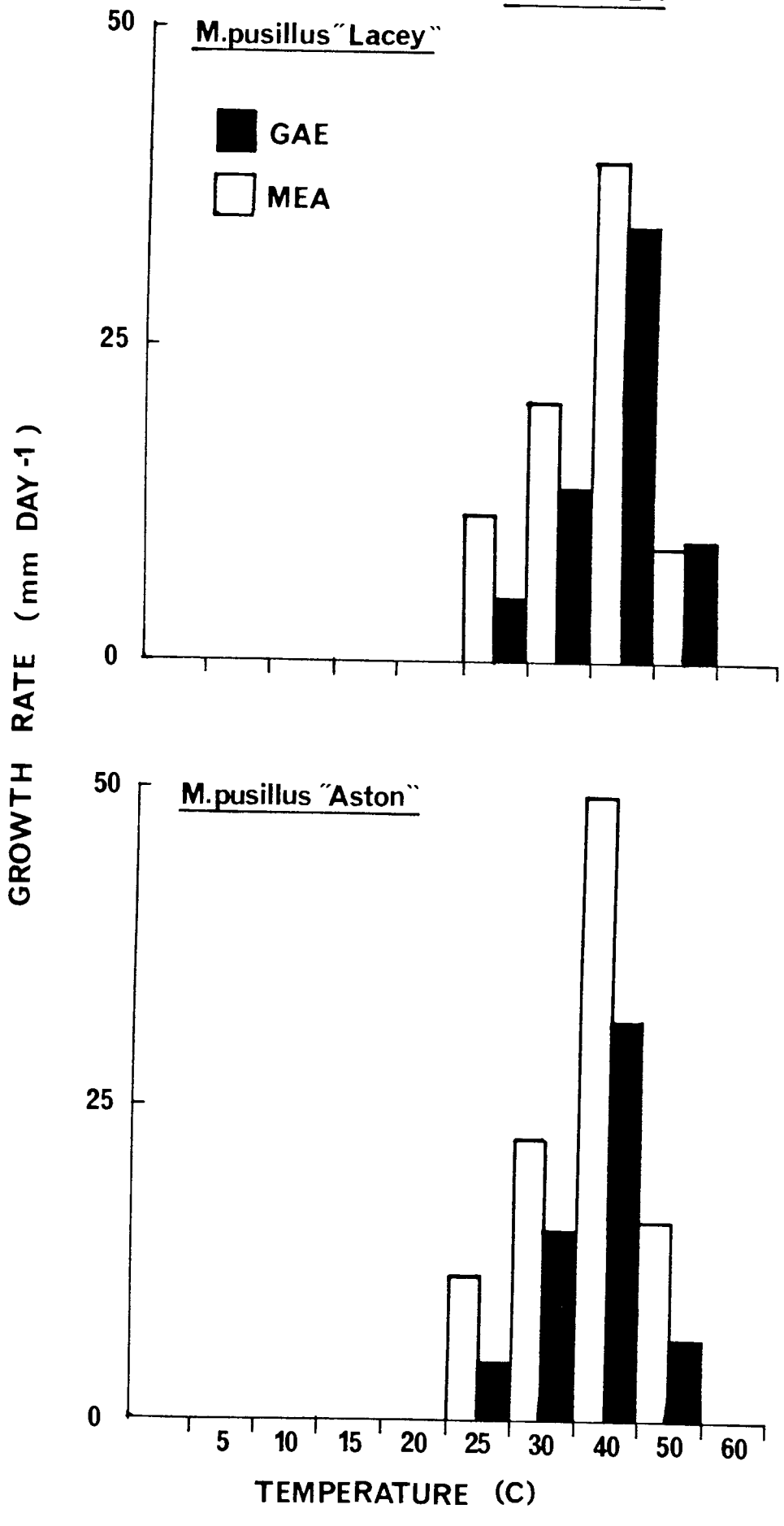

M.pusillus "Lacey"
GAE
MEA
50
25
0
M.pusillus "Aston"
50
25
0
GROWTH RATE (mm DAY-1)
5
10
15
20
25
30
40
50
60
TEMPERATURE (C)

FIGURE 2:1

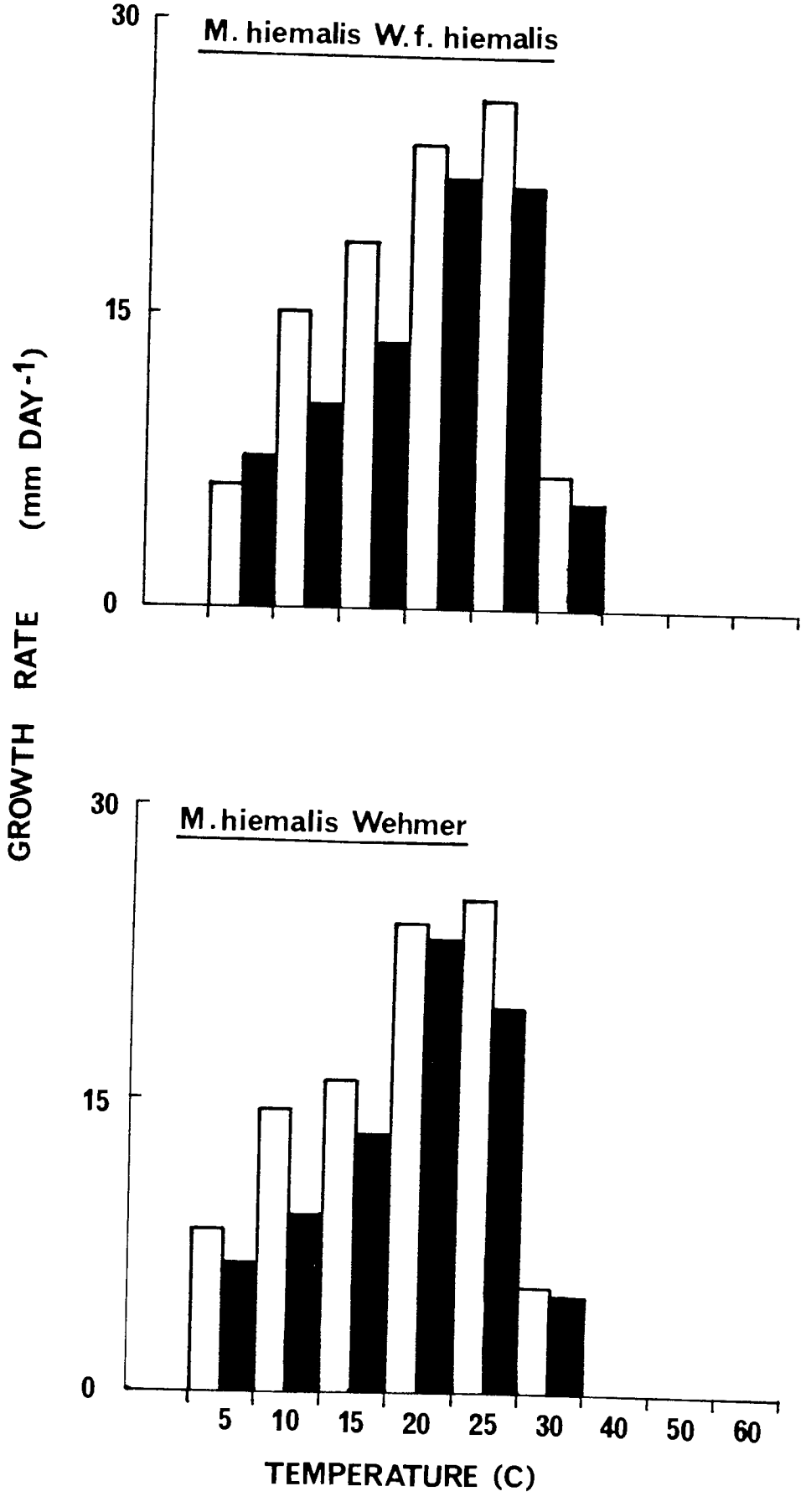
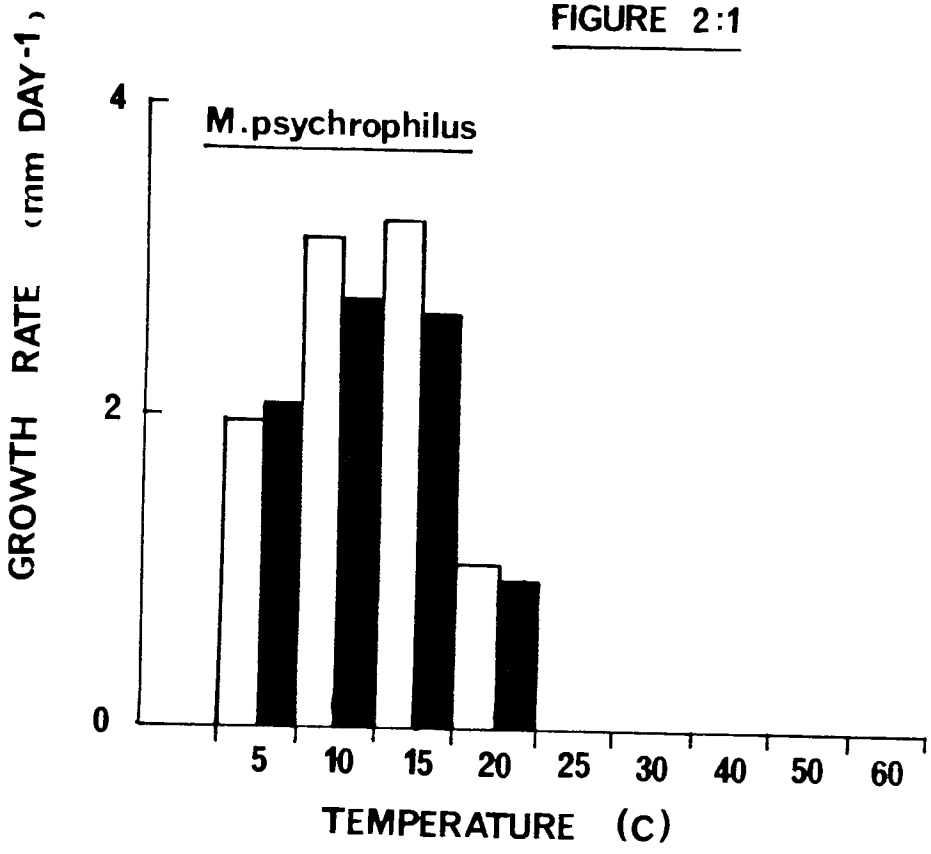

M. hiemalis W.f. hiemalis
30
15
0
M.hiemalis Wehmer
30
15
0
GROWTH RATE (mm DAY-1)
5
10
15
20
25
30
40
50
60
TEMPERATURE (C)

FIGURE 2:1


GROWTH RATE (mm DAY-1)
4
2
0
M.psychrophilus
5
10
15
20
25
30
40
50
60
TEMPERATURE (C)

M. HIEMALIS WEHMER

This strain grew from 5-30C, with an optimum growth temperature of 20-25C on both media. Like M. pusillus, growth rates on MEA were significantly higher compared with those on GAE. The optimal growth rate of 23.80 - 25.25 mm day^{-1} at 20-25C on MEA was approximately half of that observed for M. pusillus "Aston" at its optimum growth temperature of 40C.

M. HIEMALIS WEHMER F HIEMALIS

The Antarctic strain of M. hiemalis showed a similar response to the temperate strain of the same species. The temperature range for growth was 5-30C, with optimal growth between 20-25C, in common with the temperate strain. Similarly, growth rates on MEA were again significantly higher than those observed on GAE, with an optimal growth rate of 23.56 - 25.89 mm day^{-1} at 20-25C on MEA again approximately half of that observed for M. pusillus "Aston" at its optimum growth temperature of 40C.

M. PSYCHROPHILUS

This species grew from 5-20C, with an optimum growth temperature of 10-15C. Growth rates were significantly higher on MEA compared with GAE at all temperatures suitable for growth. This species was characterised by very low growth rates at all growth temperatures compared with M. hiemalis and M. pusillus.

(2) DRY WEIGHT AND MOLAR GROWTH YIELD

Results are summarised in Figure 2:2 (Appendix 2:3) and Figure 2:3 (appendix 2:4) respectively. Each strain is considered in turn below.

M. PUSILLUS "ASTON"

In common with growth on agar, this strain grew from 25-50C, with an optimum growth temperature of 40C (Figure 2:2). The molar growth yield ranged from 69.60 - 79.85 mg dry weight mol^{-1} glucose, with maximum yield at the optimum growth temperature of 40C (Figure 2:3).

M. PUSILLUS "LACEY"

This strain showed a similar response to M. pusillus "Aston" in terms of growth, with a temperature range for growth of 25-50C and an optimum growth temperature of 40C. M. pusillus "Lacey" showed a higher rate of growth than M. pusillus "Aston" at 40C, with a specific growth rate of 0.0397 h^{-1} compared with 0.0328 h^{-1} for M. pusillus "Aston" (Figure 2:2). The molar growth yield ranged from 63.39 - 79.52 mg dry weight mol^{-1} glucose (Figure 2:3). However, in contrast to M.pusillus "Aston", maximum molar growth yield was observed at the maximum growth temperature, and not the optimum growth temperature.

FIGURE 2.2

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE (ii) ARRHENIUS PLOT FOR GROWTH (DRY WEIGHT)

KEY:

- □ M. PUSILLUS "ASTON"
- ■ M. PUSILLUS "LACEY"
- ○ M. HIEMALIS WEHMER
- ● M. HIEMALIS WEHMER F. HIEMALIS
- ✪ M. PSYCHROPHILUS

FIGURE 2:2

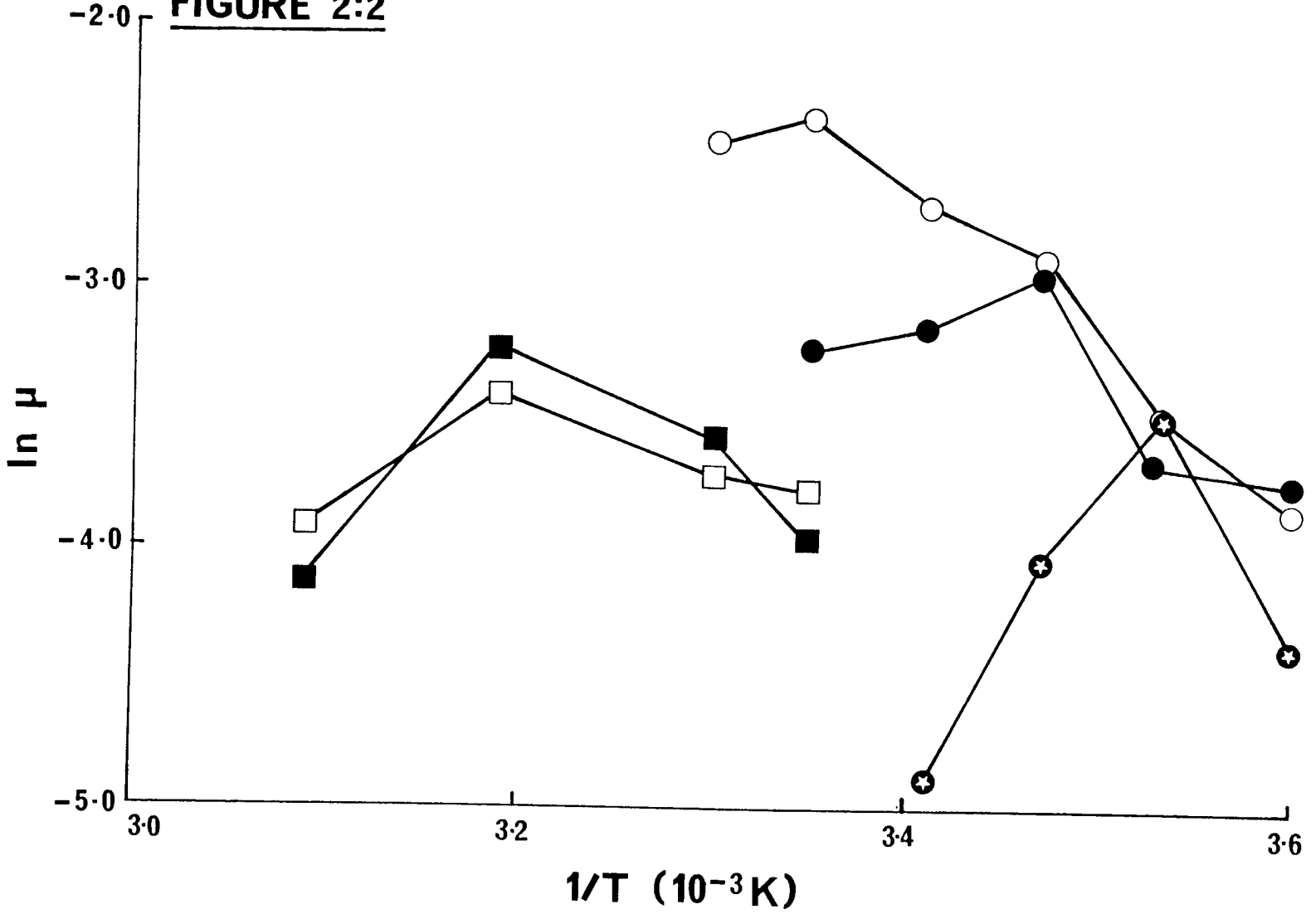


FIGURE 2.3

THE EFFECT OF GROWTH TEMPERATURE

ON MOLAR GROWTH YIELD

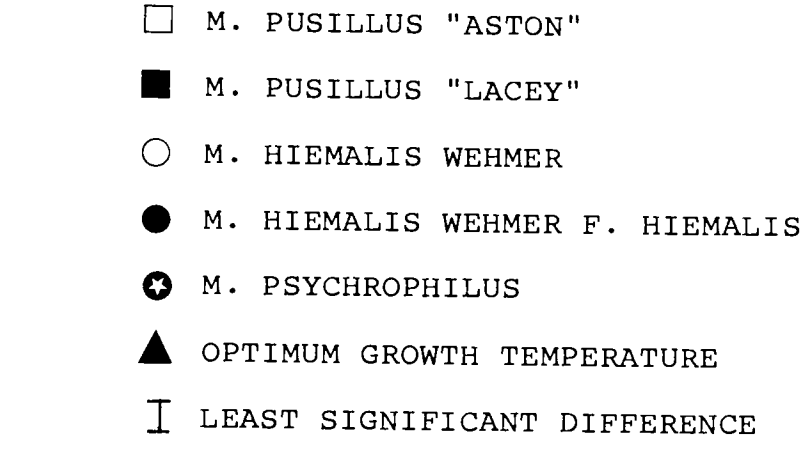
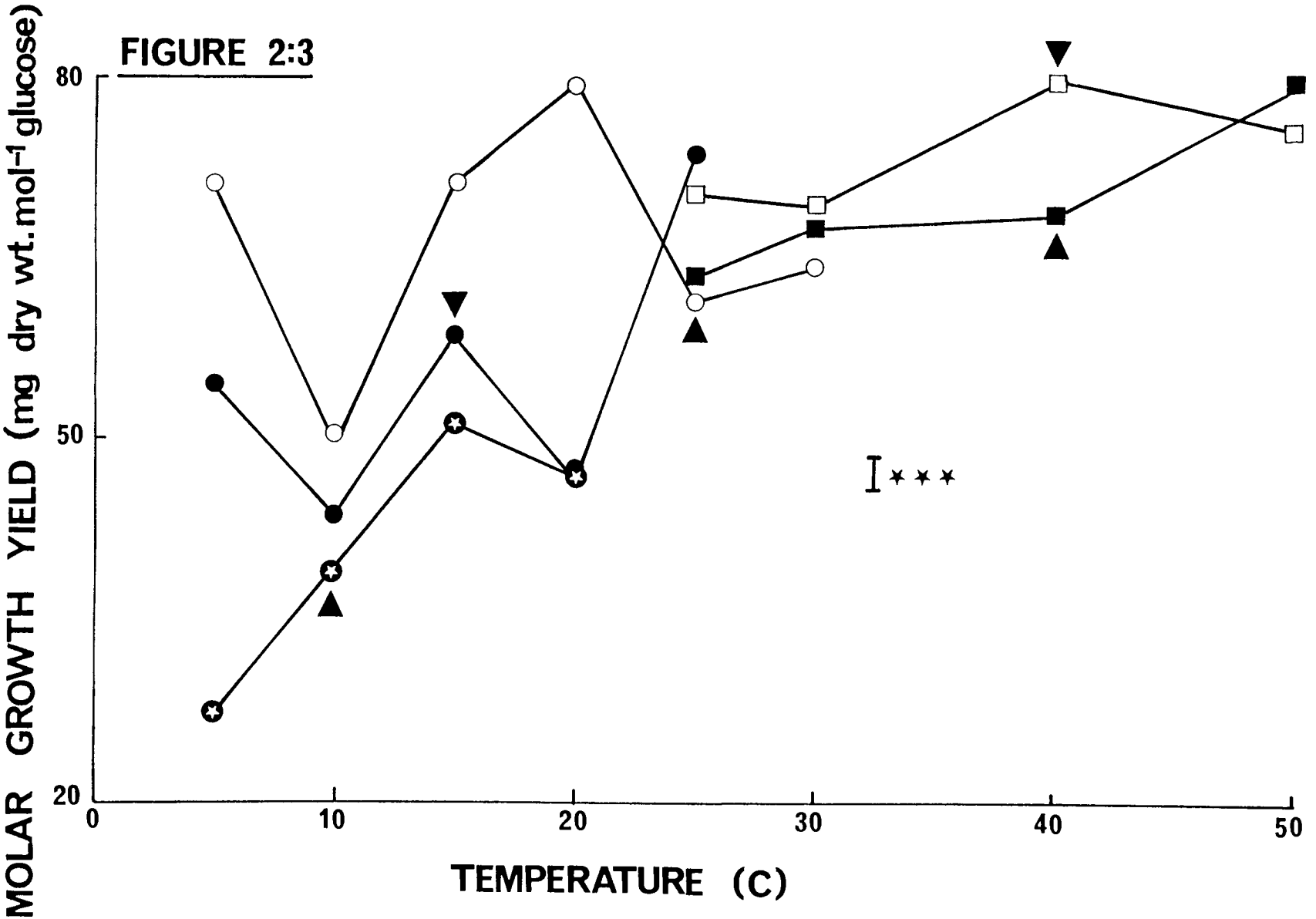


FIGURE 2:3


MOLAR GROWTH YIELD (mg dry wt.mol⁻¹ glucose)
80
50
20
0
10
20
30
40
50
TEMPERATURE (C)

M. HIEMALIS WEHMER

In common with growth on agar, this strain grew from 5-30C. Optimal growth occurred at 25C in contrast to 20-25C on agar media. Also, in contrast to growth on agar, the growth rates observed for this strain were significantly higher than those observed for both M. pusillus strains. Thus, at optimum growth temperatures, M. hiemalis Wehmer grew at a specific growth rate of 0.0953 h^{-1} compared with 0.0397 h^{-1} for M. pusillus "Aston" and 0.0328 h^{-1} for M. pusillus "Lacey" (Figure 2:2). Molar growth yield ranged from 49.92 - 79.73 mg dry weight mol^{-1} glucose with maximum yield at 20C, rather than at the optimum growth temperature of 25C (Figure 2:3).

M. HIEMALIS WEHMER F. HIEMALIS

The Antarctic strain showed a significant reduction in both temperature range for growth and optimum growth temperature compared with growth on agar. Thus, growth was restricted to 5-25C, compared with 5-30C on agar media. Further, there was a significant reduction in optimum growth temperature from 25C to 15C (Figure 2:2). Molar growth yield ranged from 43.77 - 72.17 mg dry weight mol^{-1} glucose. Maximum yield was observed at the maximum growth temperature, rather than at the optimum growth temperature (Figure 2:3).

Although growth rates were slightly lower than those observed for M. hiemalis Wehmer, both strains of M. hiemalis showed higher specific growth rates than the two M. pusillus strains at temperatures suitable for growth.

M. PSYCHROPHILUS

In common with growth on agar, M. psychrophilus grew from 5-20C. However, the optimum growth temperature was 10C compared with 10-15C on agar. Again, specific growth rates were significantly lower than those observed for M. hiemalis and M. pusillus, at all temperatures suitable for growth, with a specific growth rate of only 0.0304 at optimum growth temperature (Figure 2:2). The molar growth yield was also significantly lower than M. hiemalis and M. pusillus at temperatures suitable for growth, ranging from 20.73 - 51.37 mg dry weight mol^{-1} glucose. Maximum molar growth yield was at 15C, and not at optimum growth temperature.

DISCUSSION

Despite the inconsistencies in growth rates and temperature optima apparent between the two methods of measuring growth, the test organisms may be classified according to their responses to environmental temperature.

Both strains of M. pusillus grew from 25-50C, with an optimum growth temperature of 40C in both systems. These strains may therefore be considered thermophilic in their temperature requirements for growth.

At the other end of the temperature scale, M. psychrophilus grew from 5-20C with an optimum temperature for growth of 10C. Therefore, this species is psychrophilic in its temperature requirements for growth.

Between these two extremes, the two strains of M. hiemalis may be considered as mesophilic in their temperature requirements for growth. On agar, both the temperate and the Antarctic strain grew from 5-30C, with an optimum growth temperature of 20-25C. However, in liquid medium, the growth range of the Antarctic strain was restricted from 5-25C, with an optimum growth temperature of 15C. In contrast, the temperate strain showed a similar response to that observed on agar. Psychrophilic microorganisms characteristically show significantly lower growth rates compared with mesophiles and thermophiles. The Antarctic strain of

M. hiemalis showed similar growth rates to its temperate counterpart, but significantly higher growth rates than the psychrophile, M. psychrophilus. This strain may therefore be considered as a cold-adapted mesophile rather than a facultative psychrophile or psychrotroph.

The linear extension method proved useful in determining the temperature range for growth and in giving an approximation of optimum growth temperature. This method also demonstrated the effect different media may have on fungal growth rates since growth rates on MEA were consistently higher than those observed on GAE in all of the Mucoraceous fungi investigated. MEA is a more complex medium than GAE and complex media usually support higher growth rates than do simple media (Righelato, 1975). However, linear extension methods are of limited value, particularly in physiological investigations. In contrast, liquid shake cultures can be used for accurate growth rate experiments, colony dry weight being used as the criterion for measuring growth (Solomons, 1975). Originally, growth was measured in liquid ME as well as in liquid GAE. However, autoclaving of liquid ME produced a precipitate of variable dry weight. Since this would have resulted in erroneous estimates of mycelial dry weight, particularly during the early stages of growth when mycelial biomass is low, liquid ME was abandoned in favour of liquid GAE medium, which does not generate a precipitate after

autoclaving. Further, the use of a simple, defined medium facilitated the calculation of molar growth yield.

The Arrhenius plot for growth (Figure 2:2) shows that the response to environmental temperature was similar, in terms of the trend in specific growth rate with temperature, irrespective of a strain being psychrophilic, mesophilic or thermophilic. Thus, each strain showed a linear increase in specific growth rate with temperature, indicating the obedience of specific growth rate to temperature on the Arrhenius principle regarding a chemical reaction. However, from optimum to maximum growth temperatures, each strain showed a significant reduction in specific growth rate, manifest in deviations from linearity in the Arrhenius plot (Figure 2:2). Non-linearity in Arrhenius plots has been attributed to a number of factors. These include changes in membrane function as a result of changes in membrane lipid phase changes (Wilson et al, 1970; Wilson and Fox, 1971) or changes in enzyme activity in response to temperature (Brandts, 1967; Farrell and Rose, 1967). These factors are considered in detail in subsequent parts of this investigation.

Although all of the Mucoraceous fungi investigated showed similar Arrhenius plots for growth in that each plot comprised linear and non-linear components, there were significant differences in specific growth rates

between the thermal groups. In particular, the mesophiles and thermophiles showed significantly higher specific growth rates than the psychrophilic species. Further, when calculated as specific growth rates, the mesophiles showed significantly higher growth rates than the thermophiles, although the reverse was true on the equivalent agar medium. It would be unwise to attach any significance to this apparent contradiction since growth rates on agar (linear growth) do not allow the accurate determination of the specific growth rate unless the size of the peripheral growth zone of a colony is measured (Trinci, 1971). When such measurements have been made, values obtained for the specific growth zone on agar are close to the specific growth rate in liquid medium (Trinci, 1971). Therefore, the trend in specific growth rates between the thermal groups is mesophile > thermophile > psychrophile in order of decreasing specific growth rates.

Detailed information on fungal growth rates adequate for comparison is extremely difficult to obtain since a standard format is not used, many authors referring only to a certain weight of organism produced after so many hours of batch growth. However, Trinci (1969) recorded a specific growth rate of 0.099 h^{-1} at 25C for M. hiemalis, a similar value to that of the temperate M. hiemalis strain used in this investigation, which had a specific growth rate of 0.0953 h^{-1} at

the same temperature. Trinci (1969) also showed the effect of temperature on the growth of Aspergillus nidulans, recording specific growth rates of 0.148 h^{-1}, 0.215 h^{-1} and 0.360 h^{-1} at 25, 30 and 37C respectively.

However, in contrast to growth and temperature, Figure 2:3 shows that there was no apparent correlation between maximum molar growth yield and optimum temperature. With glucose as carbon source, the molar growth yields of the psychrophile were significantly lower than both the mesophilic and thermophilic species. However, the thermophiles produced marginally more biomass than the mesophiles in response to temperature. Therefore, high specific growth rates do not necessarily result in high molar growth yields. This may be explained by considering the factors which determine these parameters. The specific growth rate is a measure of the rate of a reaction, that of microbial growth. It reflects the rate at which a unit amount of organism is reproduced during exponential growth and is, therefore, independent of organism concentration. In contrast, the molar growth yield is a measure of the amount of organism produced in terms of carbon and nitrogen sources in the growth medium. Since it is independent of specific growth rate, which itself is independent of organism concentration, there are, therefore, no grounds for suggesting a positive correlation between specific growth rate and molar growth yield in response to temperature. Indeed, only the thermophile, M. pusillus "Aston" showed maximum

molar growth yield at optimum growth temperature, the remaining species investigated showing no such correlation between maximum growth yield and optimum growth temperature.

Microbial growth involves an interrelated sequence of physiological processes which are influenced by temperature. A comparison of some of these processes in psychrophilic, mesophilic and thermophilic fungi is of interest in order to gain an insight into the interaction of growth and temperature. However, the response of microorganisms is not restricted to growth alone. The successful colonisation of fresh nutrient material also relies on successful and adequate propagule production. The effect of temperature on sporangiospore production and germination are therefore worthy of consideration prior to an investigation into the physiological processes that delineate the different cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi.

CHAPTER THREE

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON SPORANGIOSPORE PRODUCTION AND GERMINATION

INTRODUCTION

Within the developmental cycle of filamentous fungi the asexual spore can claim a unique duality of function, being integrally involved both at the beginning and at the end of the cycle (Smith, 1978). Gregory (1966) described the fungal spore as follows : "the fungus spore, in contrast to the vegetative mycelium, is a nucleate portion delimited from the thallus, characterised by cessation of cytoplasmic movement, small water content and low metabolism, lack of vacuoles and specialised for dispersal, reproduction and survival."

Investigations into the effect of environmental temperature on both spore formation and germination have been extensive. Both of these processes can be induced or activated by high and low temperatures (Burnett, 1976; Anderson, 1978; Smith, 1978; Furch, 1981). In many cases, striking differences between the temperature optima for growth and germination have been reported (Sussman and Halvorson, 1966; Peberdy 1980 (a)). In some fungi, the condition known as microcycle conidiation is induced by temperatures generally higher than those required for normal germination (Anderson, 1978; Smith, 1978; Peberdy, 1980(b)).

ASSESSMENT OF GERMINATION

Numerous criteria have been used for assessing spore germination (Manners, 1966; Sussman and Halvorson, 1966).

Here, a spore was considered to have germinated when the emerging germ tube was as long as it was broad.

Spore germination has frequently been expressed as a percentage after a given period of time. Whilst this may be satisfactory in certain instances, in the case of temperature it may not be so. This is because conditions which are not far removed from the optimum may reduce the rate of germination, without affecting the ultimate germination percentage appreciably (Manners, 1966). In order to overcome this problem, the rate of germination was determined at a given temperature and expressed as the latent period of germination (LP_{50}). This is the time taken, in hours, for 50% of spores to germinate. The optimum temperature for germination is now defined as that temperature which results in the minimum latent period of germination (Manners, 1966).

Although of vital importance for successful propagation, germination is not the sole area in which temperature may influence propagules. A further understanding of the interaction of temperature with spores can only be obtained by a concomitant investigation of the effect of temperature on sporangiospore production (sporangiosporogenesis).

MATERIALS AND METHODS

1) SPECIES

As Chapter 2.

2) THE EFFECT OF TEMPERATURE ON SPORANGIOSPORE PRODUCTION

Sporangiospores of each strain were suspended in sterile distilled water and adjusted to a concentration of at least 5 x 10^6 spores per ml using a haemocytometer. Several drops of spore suspension were then placed on sterile GAE agar (Appendix 2:1) in 8 cm petri plates and spread evenly over the surface using a sterile glass rod. Plates were incubated at temperatures from 5-50C and were examined by light microscope for sporulation at suitable time intervals.

At temperatures where sporulation occurred, the time taken for at least 70% of sporangiophores to mature was recorded. A sporangiophore was considered to have matured when it had produced sporangiospores. However, in the case of M. psychrophilus this method of assessment proved impractical since both sporangiophore production and maturity were low. Therefore, in this species the effect of temperature on sporangiospore production was determined by comparing the percentage sporulation after 7 days incubation at a given temperature. Two replicate plates per temperature per strain were used, and in this way both the temperature range for

sporulation and the time taken for sporulation to occur at a given temperature within this range, were determined for each strain.

3) THE EFFECT OF TEMPERATURE ON SPORANGIOSPORE GERMINATION

Sporangiospores of each strain were suspended in sterile distilled water and adjusted to a concentration of at least 5 x 10^4 spores per ml using a haemocytometer. Five drops of suspension were then placed on sterile distilled water agar (Appendix 2:1) in 8 cm petri plates. Plates were incubated at temperatures from 5-50C and the percentage germination was assessed at suitable time intervals. At least 100 spores were counted for each assessment, under five different fields of view. Two replicate plates per temperature per strain were used. The time taken for 50% germination at a given temperature, the latent period of germination, was determined from a plot of percentage germination against time at that temperature. At certain temperatures, the rate of germination was so low in some strains that 50% germination was not reached. In such cases, the maximum percentage germination reached at that temperature was recorded instead.

RESULTS

The effects of temperature on sporulation and germination are demonstrated by Figure 3:1 (data are given in Appendices 3:1 and 3:2) and are contrasted with growth temperature in Table 3:1. Results from each species will be considered in turn.

M. PUSILLUS "ASTON"

The temperature range for sporulation of 25-40C was narrower than that for growth (25-50C), with no sporulation at the maximum growth temperature of 50C. Sporulation was fastest at the optimum growth temperature of 40C (24 hours) and slowest at the minimum growth temperature of 25C (72 hours). The germination data for this strain (Appendix 3:2) refer to sporangiospores produced at 40C. These germinated from 25-50C, with an optimum germination temperature of 40C. Germination was very low at 25C with a maximum germination percentage of only 5.2%. Although sporulation did not occur at the maximum growth temperature of 50C, sporangiospores produced at 40C germinated readily at 50C.

M. PUSILLUS "LACEY"

This strain showed a similar response to the other thermophilic strain of M.pusillus in both sporulation and germination. Thus, sporangiospores were produced from 25-40C, with no sporulation at the maximum growth

FIGURE 3.1

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON SPORANGIOSPOROGENESIS (SPORULATION) AND SPORANGIOSPORE GERMINATION

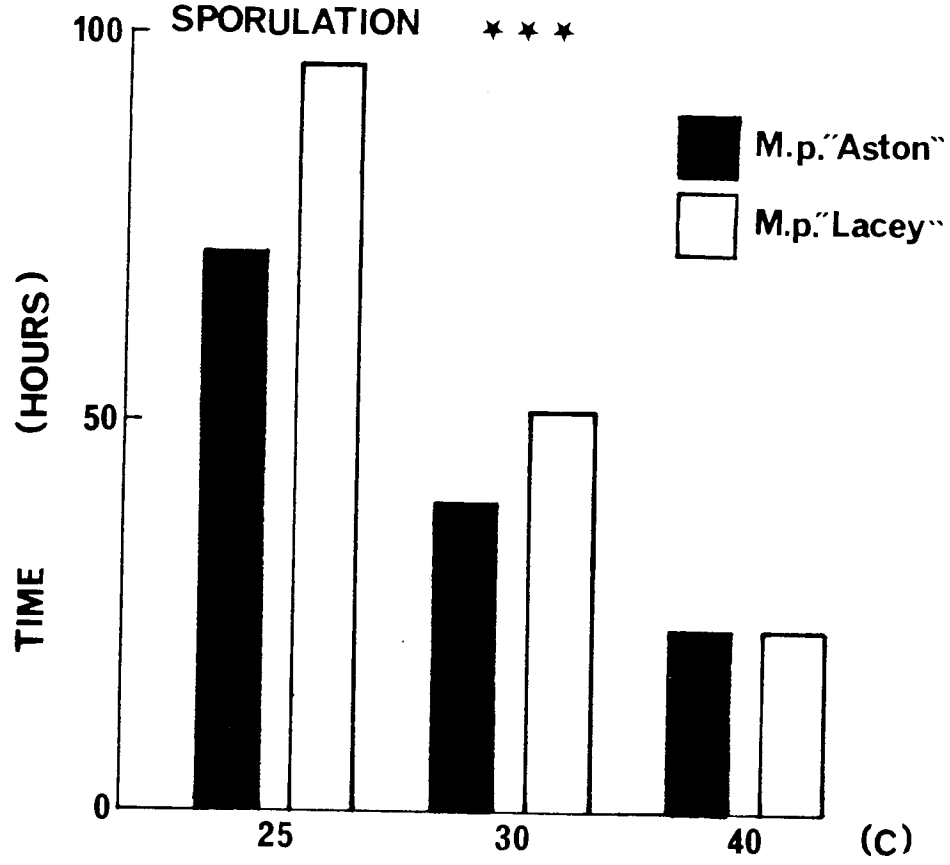
KEY:

▼ OPTIMUM GERMINATION TEMPERATURE

☆ MAXIMUM PERCENTAGE GERMINATION

FIGURE 3:1

M.pusillus



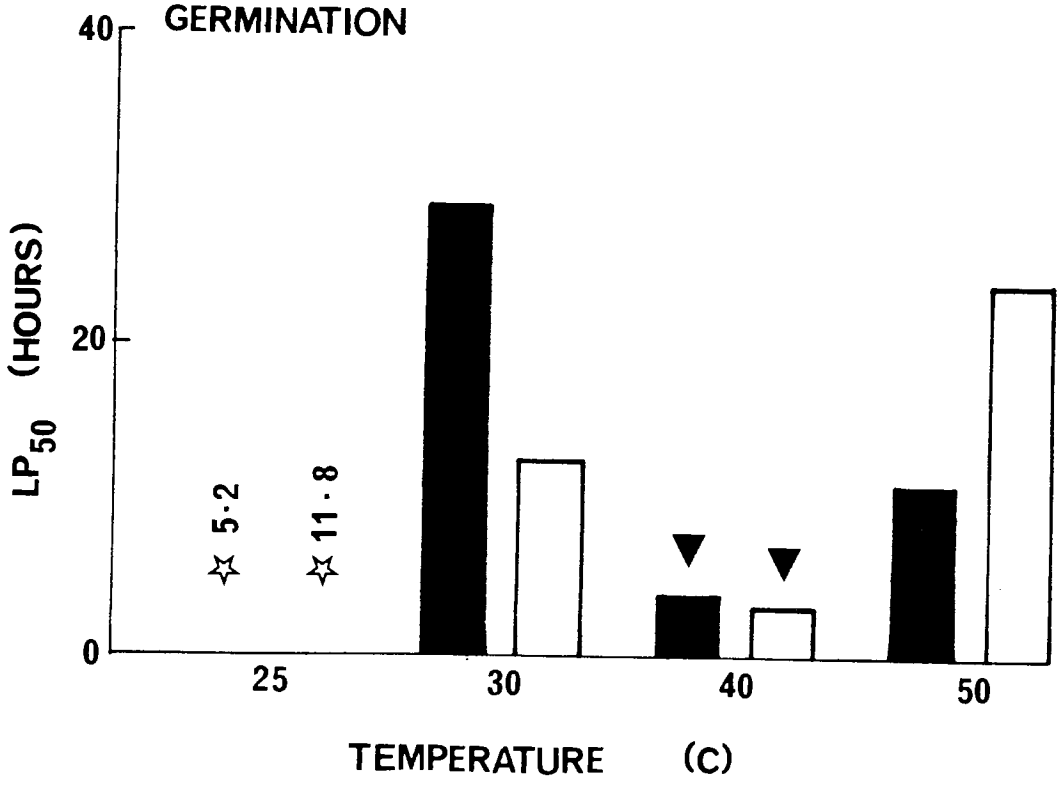
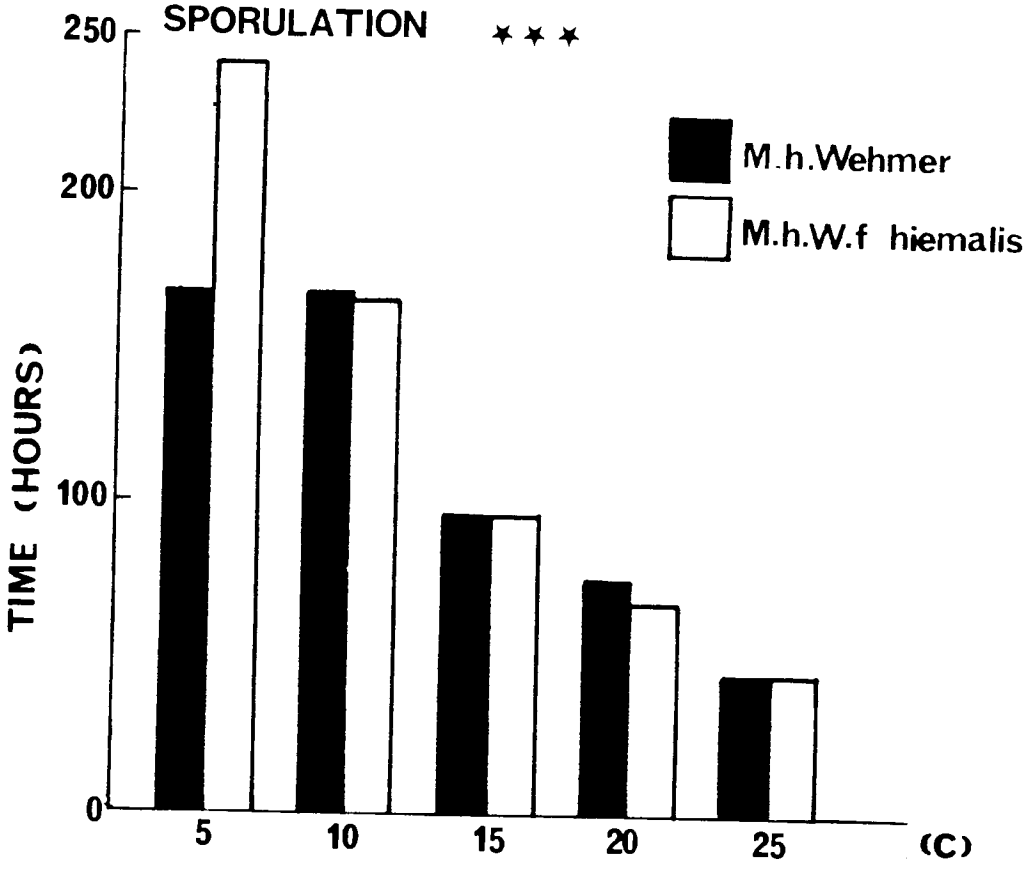


FIGURE 3:1

M.hiemalis



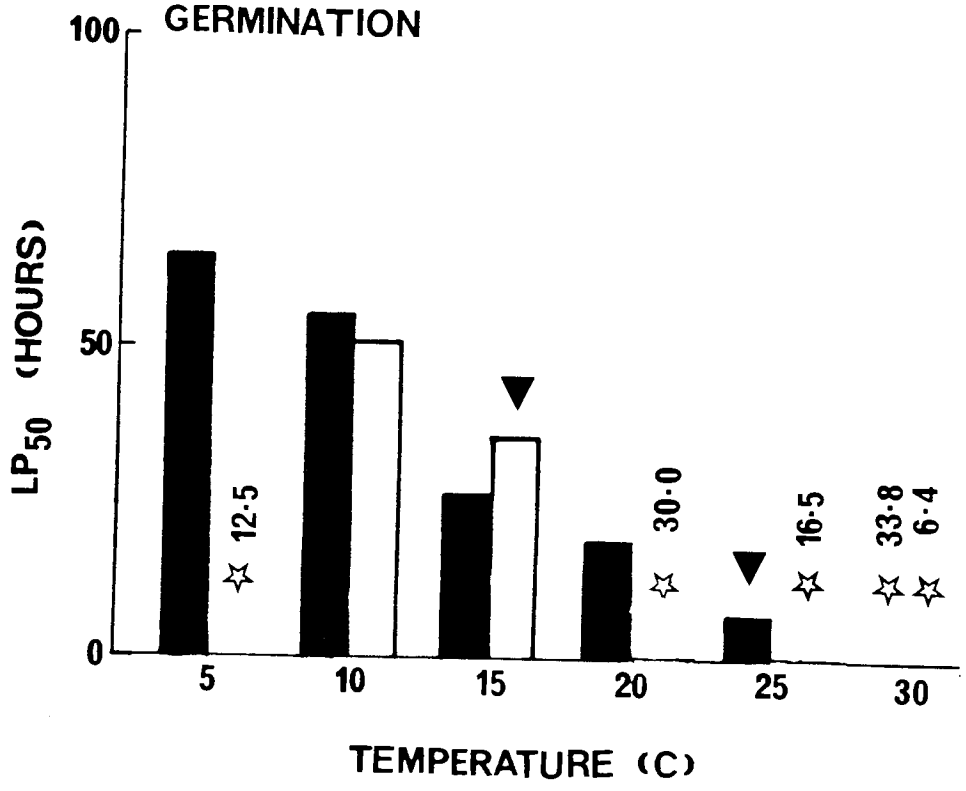
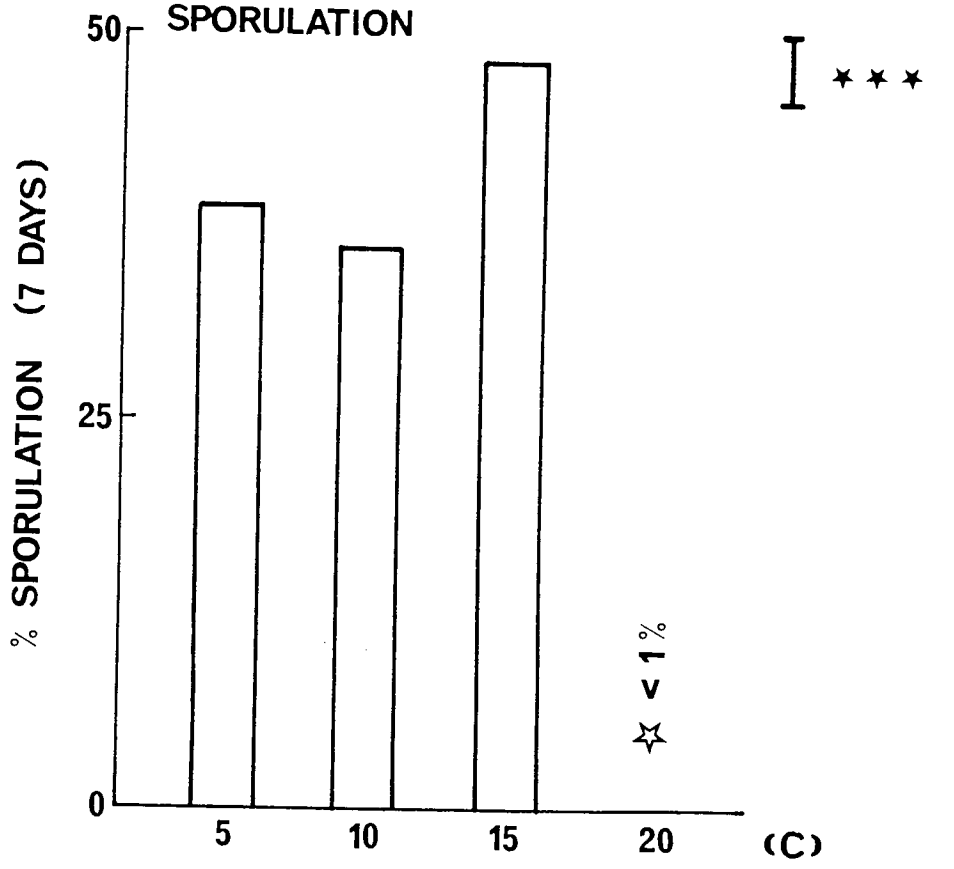
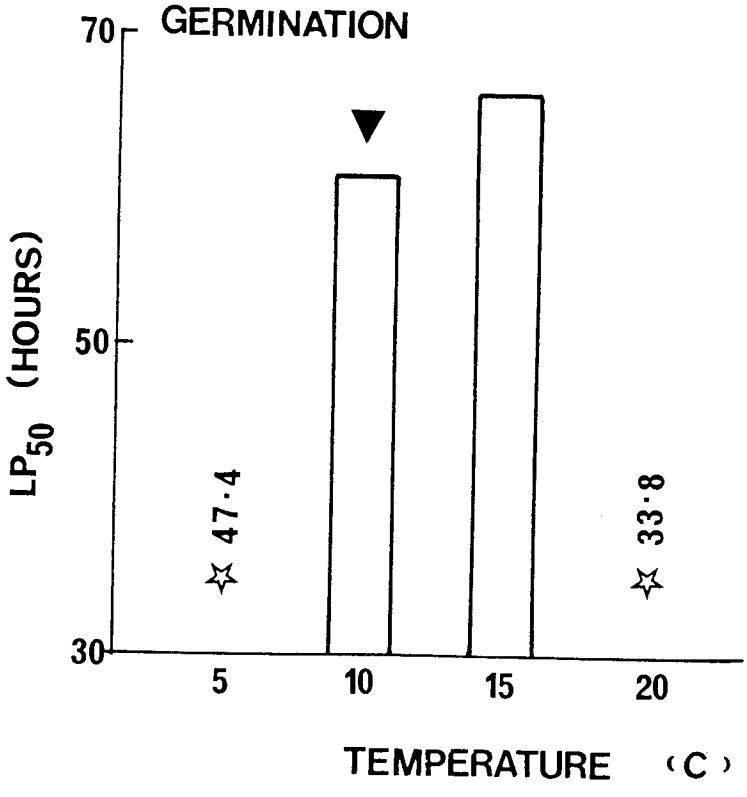


FIGURE 3:1

M.psychrophilus





temperature of 50C. Again, the rate of sporulation was lowest at 25C and highest at 40C. The germination data (Appendix 3:2) refer to sporangiospores produced at 40C. Like the other strain, sproangiospores germinated from 25-50C, with optimum germination at 40C. Similarly, germination was low at 25C with a maximum germination percentage of only 11.8%.

M. HIEMALIS WEHMER

The temperate mesophilic strain of M.hiemalis sporulated from 5-25C, with no sporulation occurring at the maximum growth temperature of 30C. The rate of sporulation was lowest at 5C and highest at 25C. The germination data (Appendix 3:2) refer to sporangiospores produced at 25C. The temperature range for germination was 5-30C, with 25C being the optimum germination temperature. The rate of germination increased from a minimum at 5C to a maximum of 25C, but fell significantly at 30C where a maximum germination of 44.2% was recorded.

M. HIEMALIS WEHMER F. HIEMALIS

The Antarctic strain showed a similar response to that of the temperate strain in terms of the temperature range for sporulation. Thus, sporangiospores were produced from 5-25C and again the rate of sporulation was lowest at 5C and highest at 25C. The germination data (Appendix 3:2) refer to sporangiospores produced

at 25C. The effect of temperature on sporangiospore germination in the Antarctic strain differed significantly from that of the temperate strain. Although germination occurred over the same temperature range of 5-30C, the optimum temperature for germination was 15C in the Antarctic strain compared with 25C in the temperate strain. In addition, the percentage germination of sporangiospores of the Antarctic strain was generally lower than that of the temperate strain in that 50% germination was only reached at 10C and 15C in the Antarctic strain. In addition, the Antarctic strain showed poor germination from 20-30C, falling from 30% maximum germination at 20C to 6.4% maximum germination at 30C.

M. PSYCHROPHILUS

The rate of and percentage sporulation were significantly lower in this psychrophilic species compared with the mesophilic and thermophilic species. Sporangiospores were produced from 5-20C, although less than 1% sporulation was recorded at the maximum growth temperature of 20C. Percentage sporulation after 7 days was highest at 15C and the germination data (Appendix 3:2) refer to sporangiospores produced at this temperature. Sporangiospores germinated from 5-20C, with an optimum germination temperature of 10C. Germination was low at the minimum growth temperature of 5C and also at the maximum growth temperature of 20C, with 47.4% maximum germination at 5C and 38.8% maximum germination at 20C.

TABLE 3:1 CARDINAL TEMPERATURES FOR GROWTH AND SPORANGIOSPORE PRODUCTION AND GERMINATION

SPECIES	TEMPERATURE RANGE FOR GROWTH (C)	OPTIMUM GROWTH TEMPERATURE (C)	TEMPERATURE RANGE FOR (i) SPORULATION (ii) GERMINATION (C)	OPTIMUM GERMINATION TEMPERATURE (C)
M. PUSILLUS "ASTON"	25-50	40	(i) 25-40 (ii) 25-50	40
M. PUSILLUS "LACEY"	25-50	40	(i) 25-40 (ii) 25-50	40
M. HIEMALIS WEHMER	5-30	25	(i) 5-25 (ii) 5-30	25
M. HIEMALIS W.F. HIEMALIS	5-25	15	(i) 5-25 (ii) 5-30	15
M. PSYCHROPHILUS	5-20	10	(i) 5-20 (ii) 5-20	10

DISCUSSION

Temperature clearly has a significant effect on the asexual reproductive phase of the life cycle in addition to that observed on the vegetative growth phase.

The cardinal temperatures for growth, sporangiospore production and germination of the psychrophilic, mesophilic and thermophilic Mucoraceous fungi investigated are summarised in Table 3:1. This shows that, within each thermal group, the optimal temperatures for growth and spore germination were identical, although differences in the cardinal temperatures for growth and sporulation were apparent.

In particular, the thermophilic strains of M.pusillus showed a narrower temperature range for sporulation compared with growth, with no sporulation occurring at the maximal growth temperature of 50C. However, the temperature ranges for growth and sporangiospore germination were identical, with a comparable optimum of 40C. It might be expected that the spores of thermophiles would require high temperatures for germination and, indeed, this is the case. However, as Jack and Tansey (1977) have shown, not all such organisms have such a requirement.

The differences in optimal growth temperatures between the temperate and Antarctic strains of the mesophile, M.hiemalis, were also apparent in the temperature optima for spore germination. Thus, although both strains sporulated over the range 5-25C, the Antarctic strain showed optimal germination at 15C compared with 25C for the temperate strain. Both strains produced spores capable of germination (albeit low) at 30C, although neither strain sporulated and only the temperate strain was capable of growth at this temperature.

The temperature range for sporulation in the psychrophile, M.psychrophilus, was effectively narrower than that for growth since although sporulation was observed over the temperature range 5-20C, sporulation was less than 1% even after 7 days incubation at 20C. The optimal temperature for both growth and sporangiospore germination was 10C.

In general, the rates of sporangiospore germination showed similar responses to changes in environmental temperature to those observed for growth rates on agar, with the rates of spore germination differing significantly between thermal groups. At their respective temperature optima for germination, the sporangiospores of the thermophiles showed higher germination rates than the mesophiles. In addition, both of these groups showed significantly higher germination rates than the psychrophilic species.

It is interesting to note that in the thermophiles, temperate mesophile and the psychrophile, sporulation did not occur at their respective maximal growth temperatures. This implies that temperature affects the process of sporangiospore production in a different manner to that for sporangiospore germination. In addition, sporangiospore production would appear to be more thermolabile than sporangiospore germination in the Mucoraceous species investigated.

In summary, the temperature ranges for the production of asexual sporangiospores of psychrophilic, mesophilic and thermophilic Mucoraceous fungi were narrower than the temperature ranges for vegetative growth of the same species. In contrast, the cardinal temperatures for sporangiospore germination and vegetative growth were identical in the same species.

CHAPTER FOUR

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON PLASMA MEMBRANE PERMEABILITY

INTRODUCTION

The nature of the life style of the saprophytic fungus demands efficient plasma membrane function. The secretion of extracellular enzymes and the resorption of their digestion products are mediated via the transport processes of the membrane. Any factor which causes an irreversible lesion in these membrane processes will inevitably cause reduced viability and maybe even cell death.

The leakage or efflux of metabolites out of a cell has been used as an indicator of membrane permeability and cell viability in both prokaryotes and eukaryotes. Moreoever, there is considerable evidence to suggest that this phenomenon is caused by membrane damage. Eilam (1965) used the rate of efflux in senescing plant tissue as an indicator of membrane permeability. Matthews and Bradnock (1967) observed that seeds demonstrating excessive efflux of metabolites were not only in a poor physical state, but were also more susceptible to pathogens. Spores of the fungus Botrytis cinerea have been shown to lose their viability due to efflux of endogenous metabolites required for germination following prolonged exposure to periods of leaching (Blakeman, 1973). Ismail B. Sahid, Smith and Lyon (1981) showed that bipyridyl herbicides such as paraquat, caused efflux of potassium and phosphate ions in Mucoraceous

fungi. Furthermore, Smith and Lyon (1977) showed that efflux induced by paraquat could be correlated with the rapid disruption of internal structures, including the plasma membrane. In addition, Hammond et al, (1974) showed that polyene antibiotics reduce viability by binding to sterol components of the plasma membrane. This can cause the efflux of up to 90% of non-bound potassium ions. These and similar investigations have shown that metabolite efflux is a reliable indicator of plasma membrane integrity (Simon, 1974). The effect of temperature on membrane integrity as a possible factor in determining the upper and lower temperature limits for growth clearly warrants investigation. A positive correlation between a decrease in the thermostability of the plasma membrane (shown by increasing metabolite efflux) and the increasing maximal growth temperatures of psychrophilic, mesophilic and thermophilic fungi would clearly be important in determining the physiological bases for growth at extreme temperatures. Indeed, there is evidence to suggest that the low maximum growth temperatures shown by psychrophilic microorganisms might be due, in part, to a thermolabile lesion in the plasma membrane.

Haight and Morita (1966) demonstrated efflux of amino acids and nucleic acids above the maximum growth temperature of the marine psychrophilic bacterium, Vibro marinus. In addition, Kenis and Morita (1968)

showed that heat-shocked cells of V. marinus also leaked nucleotides and respiratory enzymes at higher temperatures. However, efflux and lysis were shown to occur simultaneously after death, and loss of membrane function was not indicated in this case. Similarly, Alsobrook et al (1972) showed efflux of RNA, protein, carbohydrate and inorganic phosphate with increasing temperature in Bacillus psychrophilus. Again, though, death of this psychrophile at supramaximal temperatures was not attributed to membrane damage.

In contrast, work on fungi suggests that metabolite efflux may well be a significant factor in loss of viability at high temperatures. Langvad and Goksoyr (1967) showed that supraoptimal temperatures caused the degradation and efflux of nucleic acids in Merulius lacrymans. Nash and Sinclair (1968) showed that exposure to the supramaximal temperature of 35C resulted in a rapid loss of viability in the psychrophilic yeast, Candida nivalis. Heat-induced damage was shown by efflux of phosphate, amino acids and nucelotide monophosphate. Significantly, this was not due to cell lysis, indicating a breakdown in membrane permeability as the prime cause of the observed efflux. Ward (1971) demonstrated that a similar process occurred in a psychrophilic basidiomycete, with amino acid and carbohydrate efflux occurring at supramaximal temperatures.

The investigation outlined below examines the effect of temperature on plasma membrane permeability by comparing metabolite efflux in psychrophilic, mesophilic and thermophilic species of Mucor. A positive correlation between metabolite efflux and the cardinal growth temperatures of such fungi will clearly implicate plasma membrane structure and function as important factors in determining growth at extreme temperatures.

MATERIALS AND METHODS

1) SPECIES

As Chapter 2.

2) THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

Individual 100 ml Erlenmeyer flasks containing 25 ml sterile GAE medium (Appendix 2:1) were inoculated with a spore suspension of each species. The flasks were incubated in a Gallenkamp orbital incubator at 100 r.p.m., at the optimum growth temperature of the species under investigation and for sufficient time to produce log phase mycelia.

The resulting colonies were harvested by suction filtration onto Whatman No. 54 hardened filter paper and washed with an excess of sterile, osmotically-stabilised buffer composed of 20 mM TES (Tris-(hydroxymethyl)-methyl-2-aminoethanesulphuric acid) buffer, pH 7.0, with 0.6 M $MgSO_4$ as osmotic stabiliser. In order to avoid temperature-shock, the buffer-stabiliser was equilibrated at the optimum temperature of the species under investigation prior to use. Each colony was then transferred rapidly into an Erlenmeyer flask containing 25 ml sterile buffer-stabiliser. Flasks were subsequently incubated at a given temperature over the range 5-60C for an 18 hour period, during which the flasks were

gently shaken. Following incubation, colonies were again harvested by suction filtration. The filtrates were retained and analysed for phosphate ions and amino acids. The mycelial colonies were refluxed for 1 hour in 1N HCl, filtered onto pre-weighed and dried Whatman No. 1 filter paper and dried overnight at 80C, in order to determine the residual colony dry weight. The HCl filtrate was also assayed for phosphate ions and amino acids. In this way, both intracellular and extracellular levels of both metabolites were determined.

Three replicate flasks were used per species per temperature and the results analysed using two-way analysis of variance.

PHOSPHATE ASSAY

Aliquots of 1 ml filtrate were mixed with 1 ml colour reagent. The colour reagent was freshly prepared by mixing together 4 ml of 16% ammonium molybdate in 10N sulphuric acid with 36 ml distilled water and 2g ferrous sulphate. 1 ml of 0.5N trichloroacetic acid was added and the optical density of the resulting blue colour was estimated at 660 nm against a reagent blank on a Cecil spectrophotometer. The concentration of phosphate in each aliquot was determined by comparison with known values from a previously constructed standard curve (Appendix 4:1).

AMINO ACID ASSAY

Total concentration of amino acids was determined by the technique of Yemm and Cocking (1955). 0.5 ml of 4M sodium acetate buffer, pH 5.0, was mixed with a 1 ml aliquot of filtrate. Subsequently, 1 ml of ninhydrin working solution (freshly prepared by mixing 120 ml of 9.58 gL^{-1} ninhydrin in methoxyethanol with 40 mg of ascorbic acid in 4 ml distilled water) was added to each aliquot. Samples were then heated in a boiling water bath for 15 minutes, allowed to cool, and the optical density of the resulting blue colour was estimated at 520 nm on a Cecil spectrophotometer. The total amino acid concentration in each aliquot was determined by comparison with known values from a previously constructed standard curve (Appendix 4:2).

RESULTS

M. PSYCHROPHILUS

Phosphate loss is demonstrated by Figure 4:1 (data are given in Appendix 4:3) which shows that there was no efflux at all at 5C and 10C and a slight rise in efflux from 10-20C. Above 20C, the maximum growth temperature, there was a highly significant efflux of phosphate ions, reaching a peak at 40C and levelling off from 40-60C. Efflux of phosphate ions rose from 11.6 - 53.1% of total phosphate from 15-25C, and from 53.1 - 79.5% of total phosphate from 25-40C (Appendix 4:3). Concomitant with this efflux there was a significant decline in intracellular levels of phosphate.

A similar trend was apparent with respect to amino acid efflux. Again, there was little efflux from 5-15C, but highly significant efflux from 20-40C, with a levelling off, as with phosphate efflux from 40-60C. Efflux of amino acids rose from 17.0% to 72.3% of total amino acids from 15-40C. Concomitant with efflux of amino acids from the cell, intracellular levels of amino acids showed a significant reduction.

M. HIEMALIS WEHMER

The temperate strain of the mesophile, M. hiemalis, showed a similar response to the psychrophile,

FIGURE 4.1

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE EFFLUX OF PHOSPHATE IONS AND AMINO ACIDS

KEY:

i	INTRACELLULAR
e	EXTRACELLULAR
I	LEAST SIGNIFICANT DIFFERENCE

FIGURE 4:1

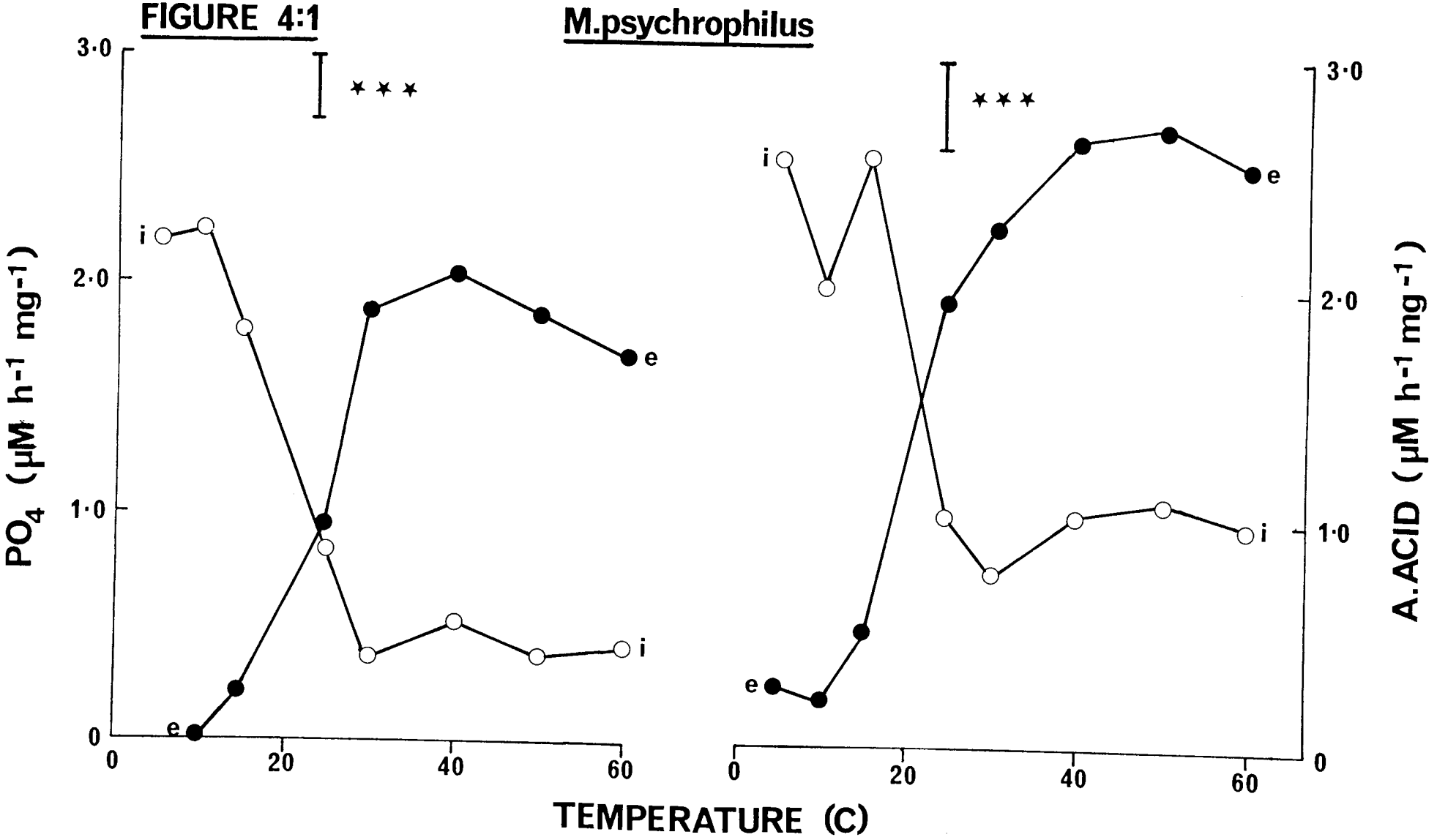

M.psychrophilus
3·0
2·0
1·0
0
PO_4 (μM h^{-1} mg^{-1})
A.ACID (μM h^{-1} mg^{-1})
i
e
0
20
40
60
TEMPERATURE (C)

FIGURE 4:1

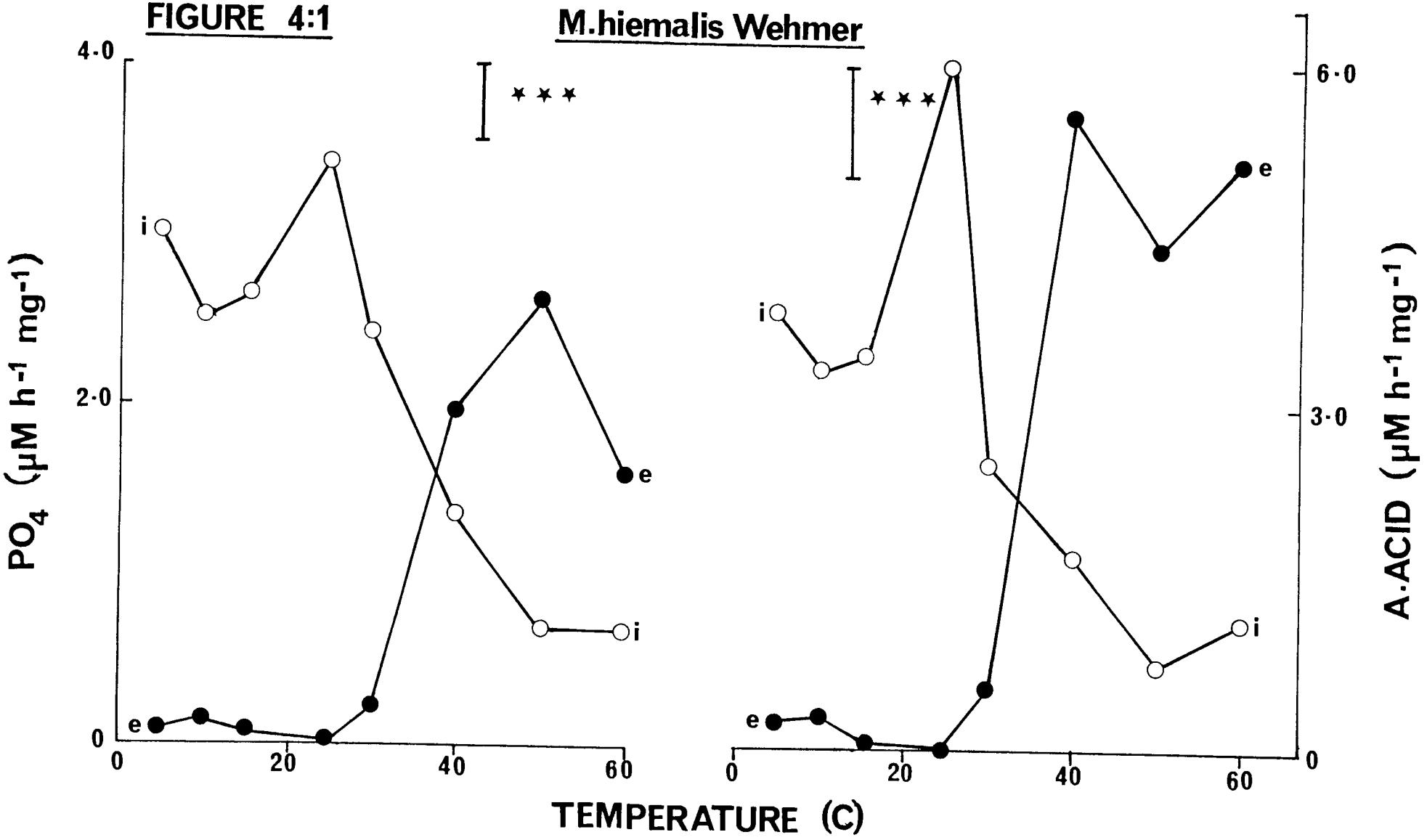

M.hiemalis Wehmer
4·0
2·0
0
PO_4 (µM h^{-1} mg^{-1})
i
e
i
e
0
20
40
60
6·0
3·0
0
A.ACID (µM h^{-1} mg^{-1})
i
e
i
e
0
20
40
60
TEMPERATURE (C)

FIGURE 4:1

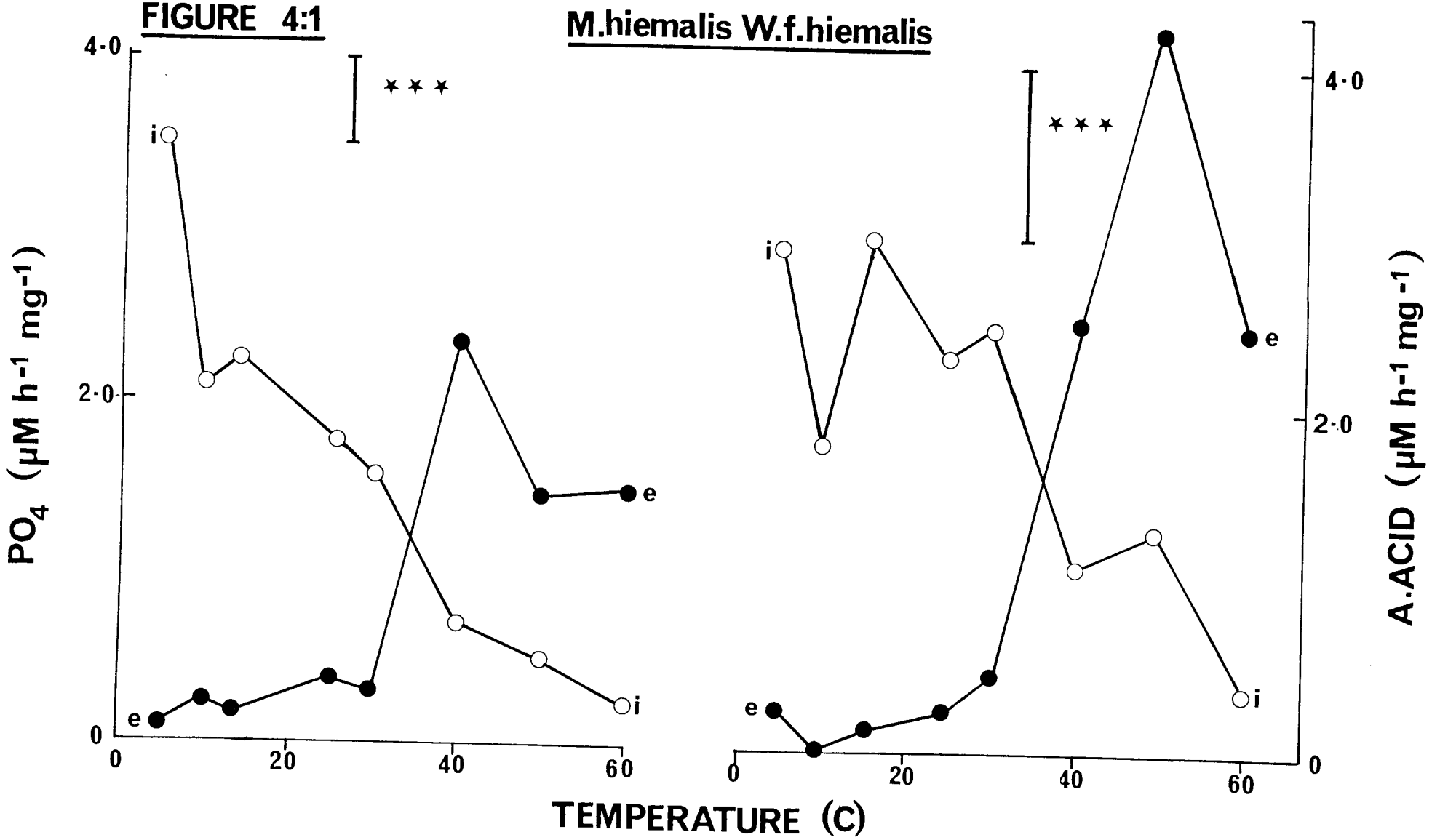

M.hiemalis W.f.hiemalis
4·0
2·0
0
i
e
***
PO_4 (µM h^{-1} mg^{-1})
A.ACID (µM h^{-1} mg^{-1})
0
20
40
60
TEMPERATURE (C)

FIGURE 4:1

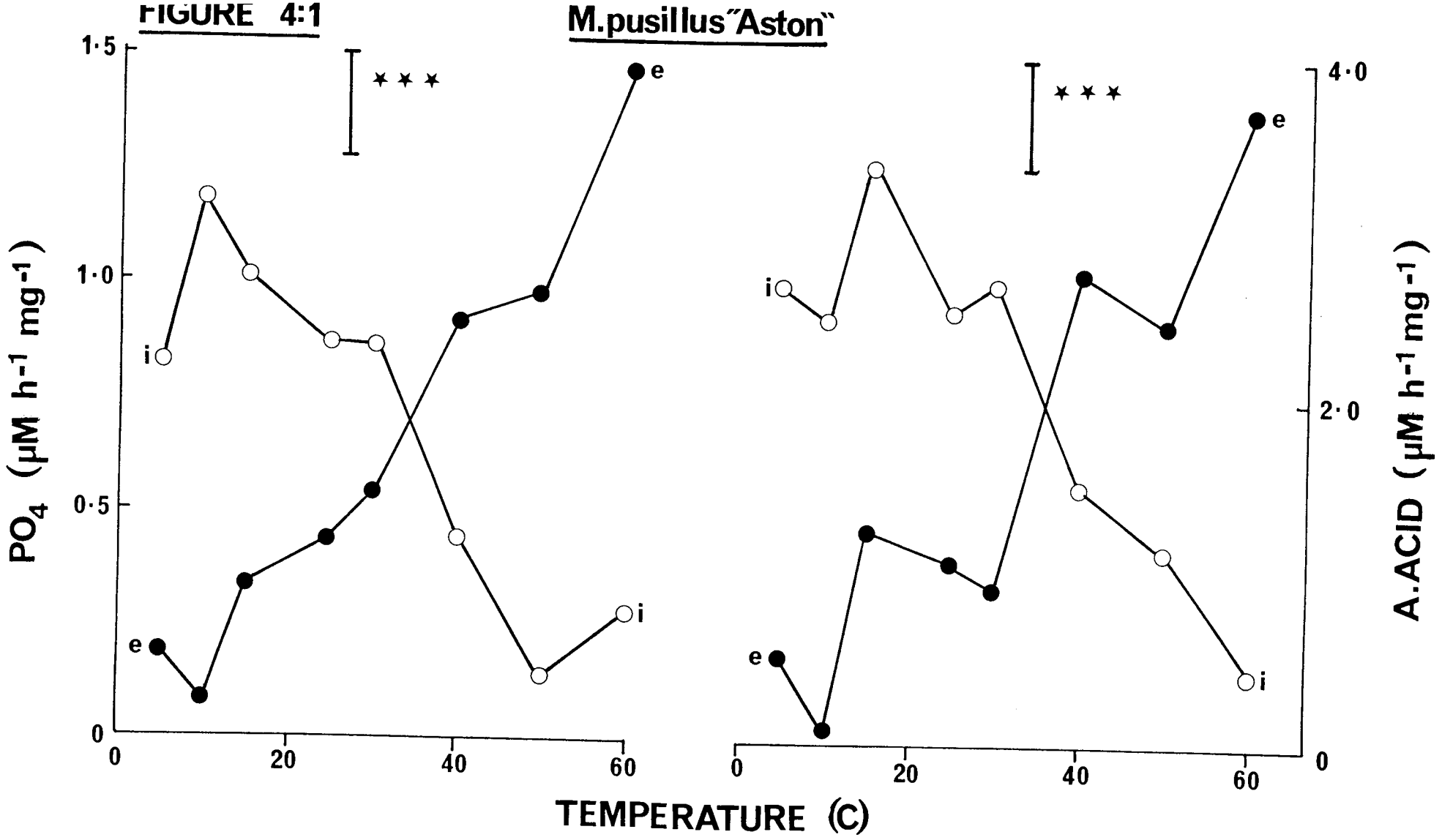
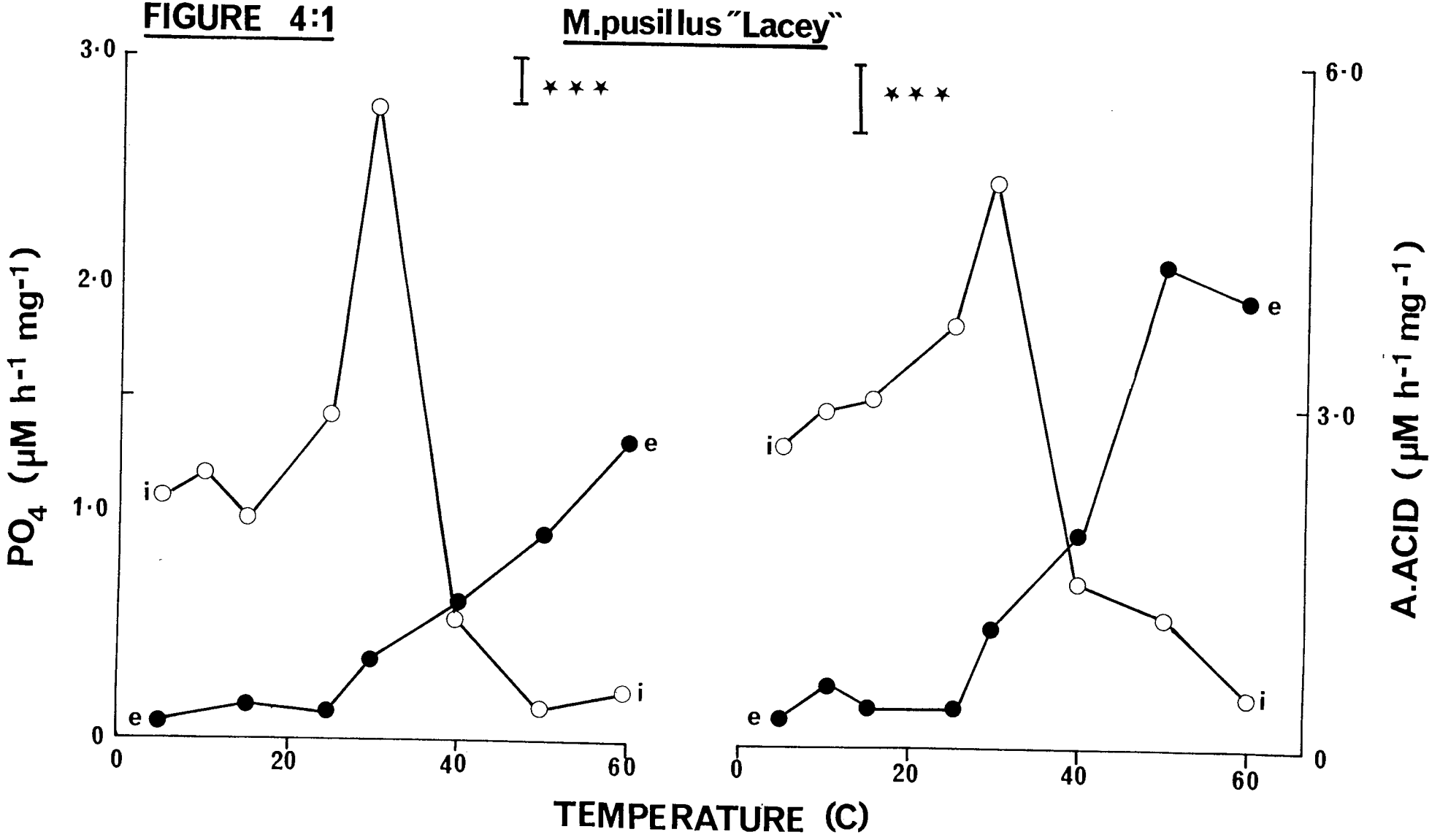

M.pusillus"Aston"
1·5
1·0
0·5
0
PO4 (µM h-1 mg-1)
***
e
i
0
20
40
60
4·0
2·0
A.ACID (µM h-1 mg-1)
TEMPERATURE (C)

FIGURE 4:1


M.pusillus "Lacey"
3·0
2·0
1·0
0
PO_4 (µM h^{-1} mg^{-1})
i
e
e
i
0
20
40
60
6·0
3·0
0
A.ACID (µM h^{-1} mg^{-1})
TEMPERATURE (C)

M. psychrophilus although the temperature at which efflux became significant was higher (figure 4:1). In the case of phosphate, there was little or no efflux from 5-30C. However, above 30C, the maximum growth temperature, there was a highly significant efflux of phosphate ions. From 30-40C, efflux of total phosphate rose from 18.3 - 50.4% (Appendix 4:3). This levelled off from 40-60C.

The efflux pattern for amino acids was, again, similar to that of phosphate ions. Thus, there was little efflux from 5-30C, but from 30-40C efflux of total amino acids rose from 24.7 - 61.4% (Appendix 4:3). Again, efflux levelled off from 40-60C.

M. HIEMALIS WEHMER F. HIEMALIS

The Antarctic strain of M. hiemalis showed a very similar response to that of the temperate strain with respect to both phosphate ion and amino acid efflux. Again, the temperature at which efflux of both metabolites became significant was 30C. Again, there was highly significant efflux of both phosphate ions and amino acids from 30-40C, with a levelling off from 40-60C. Phosphate ion efflux rose from 16.7 - 76.8% total phosphate from 30-40C, amino acid efflux rising from 14.9 to 69.7% over the same temperature range.

M. PUSILLUS "ASTON"

The thermophile M. pusillus "Aston" showed a slightly different response to the mesophilic and psychrophilic species in being more "leaky" in general at temperatures suitable for growth. However, over the supramaximal temperature range of 50-60C, there was highly significant efflux of both metabolites. Phosphate ion efflux rose from 67.4% at 40C to 84.% total phosphate at 60C, with total amino acid efflux rising from 63.7 - 89.9% over the same temperature range (Appendix 4:3). The minimum growth temperature of this strain is 25C and, below this temperature, efflux of both metabolites was similarly low.

M. PUSILLUS "LACEY"

This strain showed a similar response to the other thermophilic strain of M. pusillus. Thus, efflux of both phosphate ions and amino acids was significant over the supramaximal temperature range of 50-60C, with total phosphate ion efflux rising from 46.7 - 70.8% from 40-60C and total amino acid efflux rising from 48.3 - 68.5% over the same temperature range. Again, below 25C, the minimum growth temperature, there was little efflux of both metabolites.

DISCUSSION

Environmental temperature had a significant effect on the plasma membrane integrity of the psychrophilic, mesophilic and thermophilic Mucoraceous fungi investigated.

In summary, within each thermal group there was little marked change in metabolite efflux at temperatures suitable for growth. However, at supramaximal growth temperatures, there was highly significant efflux of both phosphate ions and amino acids. Thus, the psychrophile, M. psychrophilus, showed highly significant metabolite efflux at temperatures in excess of 20C, with the mesophile, M. hiemalis, showing this at temperatures in excess of 30C and the thermophile, M. pusillus showing significant efflux at the supramaximal growth temperature of 60C.

There is considerable evidence suggesting that both phosphate ions and amino acids are actively taken up from the growth medium where they form intracellular pools within the hypha (Burnett, 1976; Whitaker, 1976). Efflux of these and other essential metabolites will eventually be detrimental to fungal metabolism, manifest in reduced growth rates and finally cell death. Here, at supramaximal growth temperatures, all of the Mucoraceous strains investigated lost some 60-80% of total phosphate ions and amino acids. It would appear, therefore, that there maybe a thermolabile lesion in the plasma membrane

that delineates the upper temperature limit for growth at least. Further, the temperature at which this lesion occurs determines whether an organism is classified as psychrophilic, mesophilic or thermophilic.

It is worth noting that thermophilic strains of M. pusillus were consistently more "leaky" than both the mesophilic and psychrophilic species. Previous work has established that, although the mesophiles and thermophiles both showed significantly higher growth rates than the psychrophilic species, the mesophiles showed the highest growth rates compared with the other thermal groups. The fact that M. pusillus is more susceptible to metabolite efflux than the mesophile, M. hiemalis, even at optimum growth temperature, may be a determinant of the reduced growth rates shown by the thermophile compared with the mesophile.

The thermophilic strains of M. pusillus showed little efflux at temperatures below the minimum growth temperature, demonstrating that the plasma membrane was probably intact at these temperatures and suggesting that a breakdown in membrane integrity is unlikely to delineate the minimum growth temperature. However, this does not preclude a more subtle change in membrane function determining the minimum growth temperature.

An investigation of plasma membrane structure and function in psychrophilic, mesophilic and thermophilic fungi is therefore of some consequence. In particular,

the characterisation of lipid and protein components, together with an investigation of their thermostability may well determine if plasma membrane structure and function is significant in delineating the differing upper and lower growth temperatures characteristic of these microorganisms.

CHAPTER FIVE

THE EFFECT OF GROWTH TEMPERATURE ON TOTAL CELLULAR AND PLASMA MEMBRANE LIPID COMPOSITION

INTRODUCTION TO THE INTERACTION BETWEEN TEMPERATURE AND MEMBRANE LIPIDS

The observation that growth temperature plays an important role in determining the lipid composition of poikilothermic organisms has been well documented (McElhaney, 1976; Inniss and Ingraham, 1978).

Fungal lipids are distributed between two distinct regions of the cell. Cytoplasmic lipids comprise the major concentration, occurring as acylglycerols. A smaller, yet potentially more significant concentration of lipid is found in the membrane systems of the cell. The major components of these membrane lipids are the phospholipids which, in association with proteins, determine some of the critical properties of the membrane (Chapman, 1967).

Crisan (1973) observed that, although cytoplasmic lipids exhibit compositional changes as growth temperature varies, their prime function is that of energy storage and, as such, compositional changes in cytoplasmic lipids are probably of minimal importance with respect to the thermal stability of the cell. In contrast, temperature-induced compositional changes in the phospholipids of cellular membranes may contribute significantly to thermostability.

Amongst the cellular membranes, the plasma membrane is of marked importance in that not only does it serve to preserve structural integrity, but it also functions as a selective barrier in regulating uptake of nutrients,

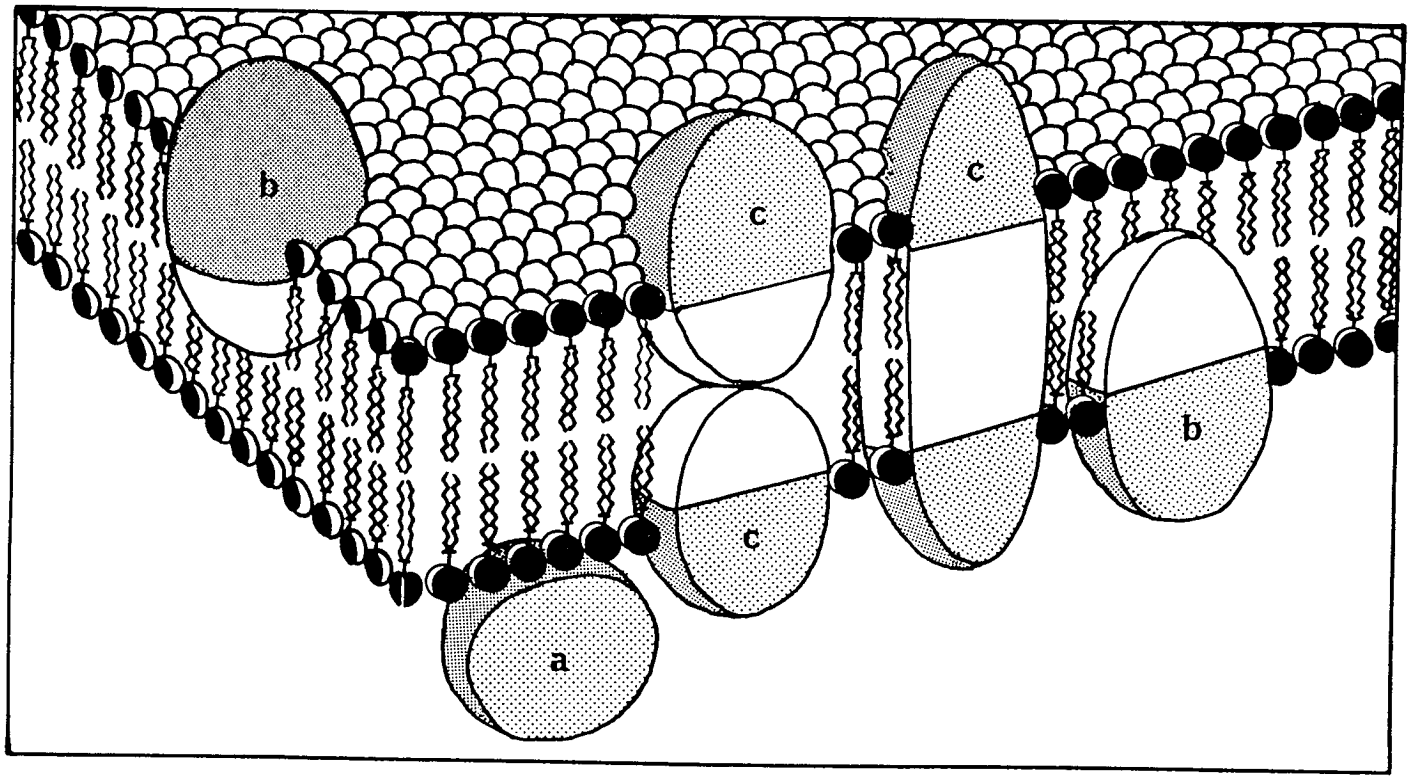
removing toxic waste products and maintaining the correct ionic composition and water content of the cell. Figure 5:1 (a) is a diagrammatic representation of a typical plasma membrane. This shows that the structural framework of the membrane is made up of a bilayer of phospholipids reflecting the amphipathic nature of the phospholipid molecule. Thus, the hydrophilic heads of the phospholipid molecules form both the outer and inner membrane surfaces. In contrast, the hydrophobic tails of the phospholipids face inwards, meeting at the centre of the membrane. The membrane proteins are classified as either extrinsic or intrinsic, depending on their location with respect to the lipid framework. Thus, extrinsic proteins are located at or near to either the outer or inner membrane surface ((a) and (b), in Figure 5:1(a)), while intrinsic proteins penetrate the membrane to varying depths ((c) in Figure 5:1 (a)).

In the fluid-mosaic model of plasma membrane structure (Singer and Nicolson, 1972), the plasma membrane is viewed as a dynamic structure in which the membrane proteins "float" in a fluid phospholipid bilayer. This concept of fluidity of phospholipids concerns the hydrocarbon chains, and it is here that temperature plays a crucial role. Phospholipids exhibit multiphasic properties and, in order to function correctly, membrane phospholipids must be at or near to the point of transition between a thermotropic gel phase and a liquid crystalline

FIGURE 5.1(a)

A TYPICAL PLASMA MEMBRANE

FIGURE 5:1(a) : A Typical Plasma Membrane



after Capaldi (1974)

FIGURE 5.1(b)

PHOSPHATIDYLCHOLINE : A TYPICAL PHOSPHOLIPID MOLECULE

FIGURE 5:1 (b)

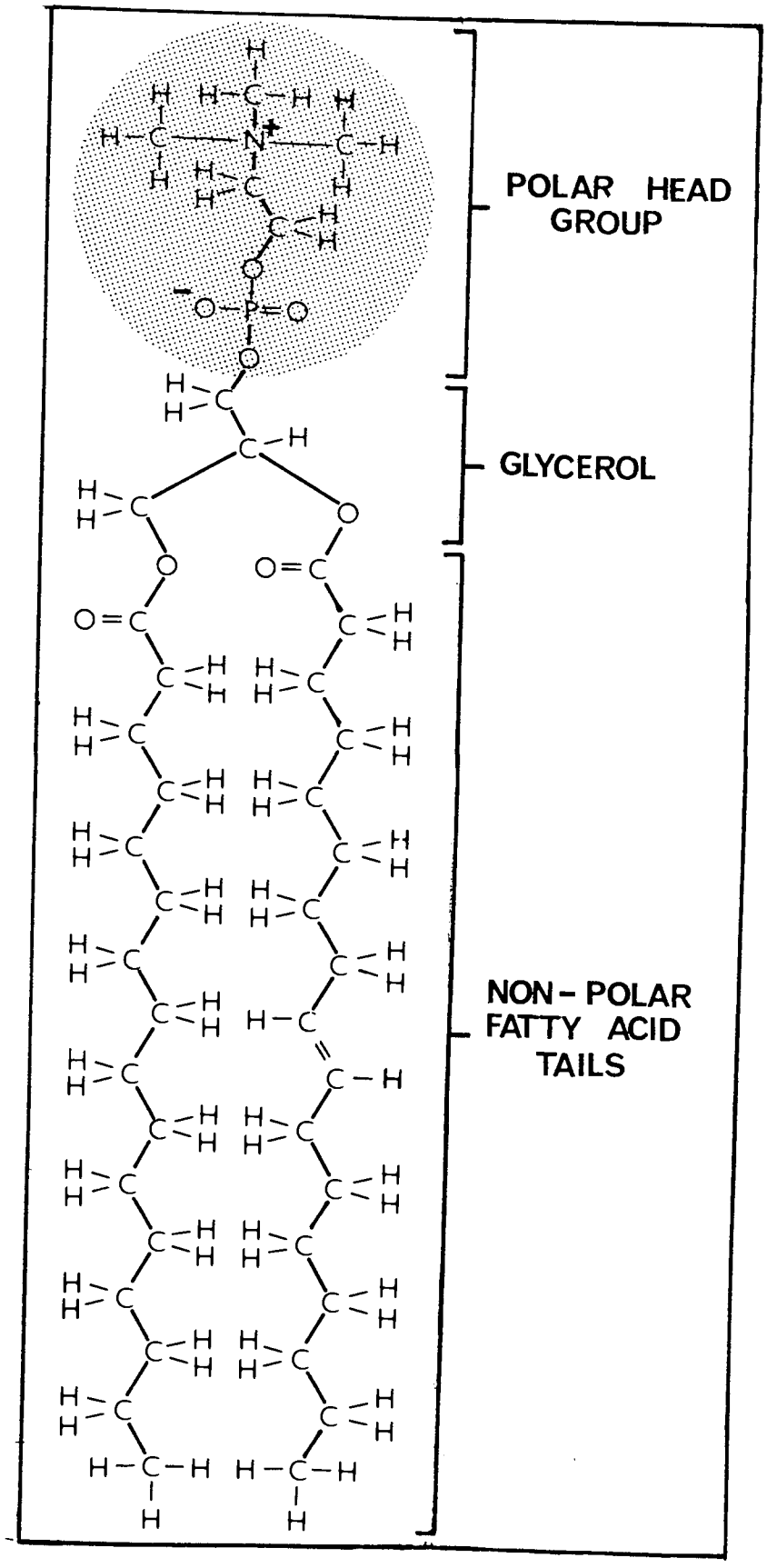
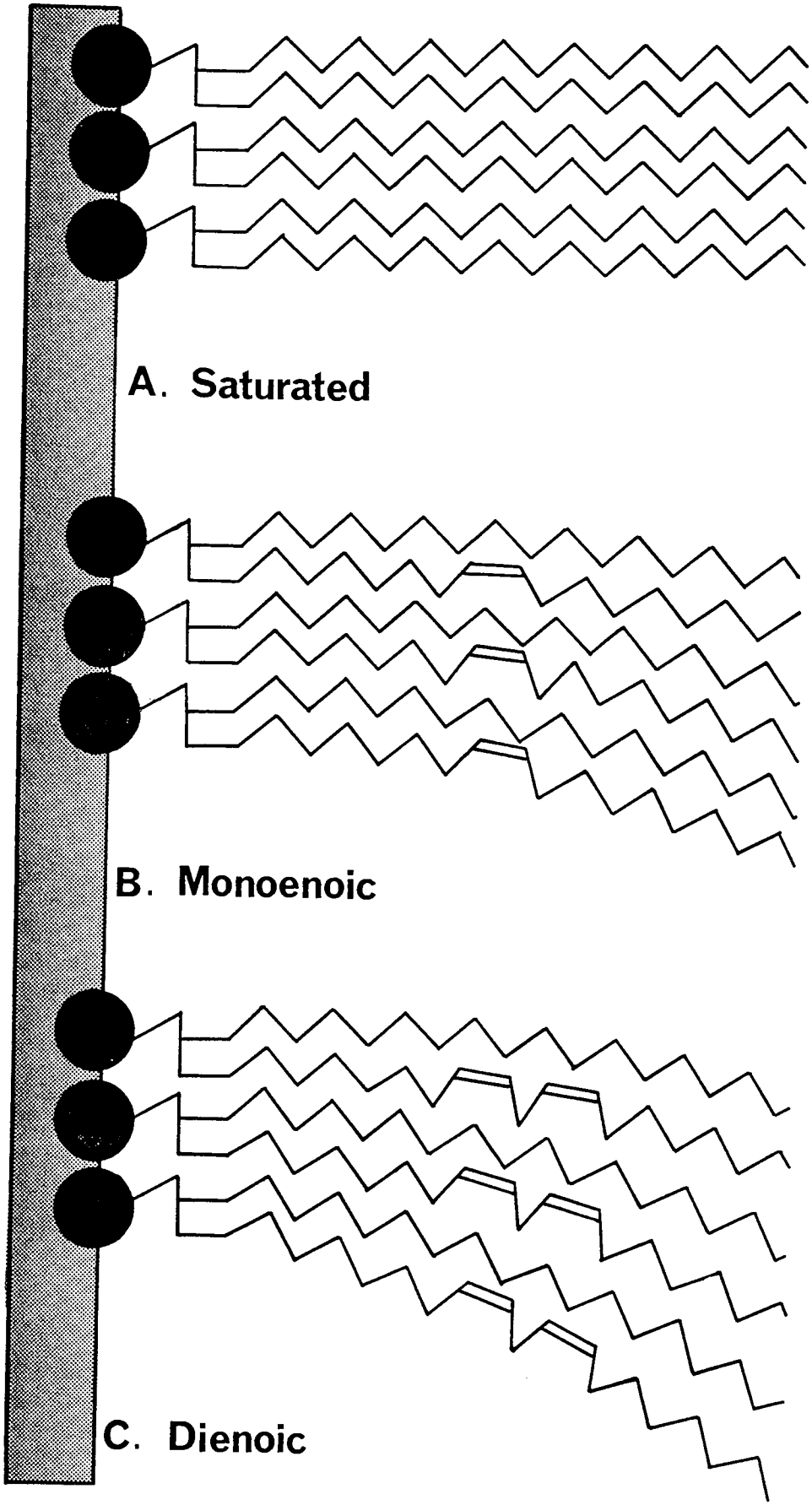


FIGURE 5.1(c)

VARIATIONS IN PHOSPHOLIPID FATTY ACID COMPOSITION WITH RESPECT TO THE DEGREE OF UNSATURATION

FIGURE 5:1(c)



phase (Byrne and Chapman, 1964; Chapman, 1969). Only at this transitional point does the membrane exhibit the necessary degree of flexibility needed to function correctly. Phase transition in a phospholipid is a function of the melting point of the lipid moiety, which is determined by the molecular characteristics of the hydrocarbon chains of its component fatty acids.

A typical phospholipid molecule is shown in Figure 5:1 (b), that of phosphatidylcholine (lecithin). This shows that the hydrophobic fatty acid tails may either be saturated, with a hydrogen atom linked to every carbon bond, or unsaturated with free carbons. The degree of unsaturation is an important determinant of the melting point of the phospholipid and also, therefore, the temperature at which the gel to liquid-crystalline phase transition occurs.

In general, as growth temperatures are increased, phospholipids tend to incorporate fatty acids that are more saturated, less branched, and that have longer chain lengths. Consequently, as growth temperature increases there is a concomitant increase in the point at which the gel to liquid-crystalline phase transition occurs (McElhaney, 1976).

A relatively high degree of unsaturation has been found in lipids of higher plants, certain animals and certain microorganisms grown at low temperatures (Weete, 1980). Figure 5:1(c) demonstrates how variations in the

degree of unsaturation may disrupt the orderly stacking of phospholipids in the plasma membrane. In a lipid layer composed entirely of saturated fatty acids ((A) in Figure 5:1(c)) the fatty acid chains contain only single bonds between carbon atoms and thus rest together to form rigid structures. In a lipid layer containing unsaturated fatty acids with one double bond ((B) in Figure 5:1 (c)) the double bonds introduce a deformation that interferes with orderly stacking and makes the fatty acid region somewhat fluid. When fatty acids with two double bonds are present ((C) in Figure 5:1 (c)), the deformation and consequent fluidity are greater still (Fox, 1972).

Since increasing unsaturation lowers the transition temperature of plasma membrane phospholipids (thereby increasing fluidity) it has been suggested that this phenomenon is an important factor in the adaptation to cold environments. Further, on the basis of their respective cardinal growth temperatures, the order of decreasing unsaturation in lipids of fungi having different growth temperature optima would be expected to be psychrophile > mesophile > thermophile. In addition, if these organisms are grown at temperatures at either extreme of their optima, the degree of unsaturation might also be expected to adjust accordingly (Weete, 1980).

The interaction between growth temperature and fatty acid composition has been extensively studied in bacteria (Chapman, 1967; Farrell and Rose, 1967; Esser and Souza, 1976; McElhaney, 1976; Amelunxen and Murdock, 1978). In comparison there are few reports concerned with filamentous fungi. Mumma et al., (1970) showed that the degree of lipid unsaturation was indeed higher in mesophilic fungi compared with thermophilic species. In addition, Sumner and Morgan (1969) showed that several mesophilic Mucor species had similar levels of unsaturation. This area needs further investigation since most investigations report on total lipids only. Further, there are very few comparisons between psychrophilic, mesophilic and thermophilic fungi from the same genus.

Previous work on bacteria (Esser and Souza, 1976) and mycoplasma (McElhaney, 1976) has indicated that the minimum growth temperature of an organism may be determined by its fatty acid composition. At low temperatures, the presence of phospholipid hydrocarbon chains in the gel state results in a solid membrane, a situation in which growth is impossible.

In contrast, the possible effects of the physical state of the phospholipids on the upper growth temperature limit are less precise. The lipids of many microorganisms exist entirely in the liquid crystalline phase at all growth temperatures, (McElhaney, 1976). Therefore, there

is no theoretical basis for suggesting that the maximum growth temperature would be directly determined by the upper boundary of the membrane phospholipid phase transition. Nevertheless, the possibility exists that at high temperatures, the molecular motion of the fatty acid hydrocarbon chains may be so great that the lipid bilayer structure becomes unstable, resulting in transient breakdowns in membrane permeability. This might cause death by preventing nutrient uptake and also by causing the irreversible efflux of essential metabolites from the cell (McElhaney, 1976).

However, membrane fluidity is not determined by phospholipid fatty acid composition alone. The inlcusion of a sterol, such as cholesterol, in a phospholipid bilayer tends to have a condensing effect on liquid cystalline phases, thereby reducing membrane fluidity (Oldfield and Chapman, 1972; Demel and Dekruyff, 1976). Further, carotenoids have also been shown to decrease membrane fluidity in a similar manner to sterols (Huang and Haug, 1974).

In this part of the investigation, the role of fungal lipids in delineating the cardinal growth temperatures of psychrophilic, mesophilic and thermophilic fungi is considered. The first element of this investigation involves the separation and identification of total cellular lipids. Subsequently fat components unique to the plasma membrane are identified and the

effects of growth temperature on total lipids and plasma membrane phospholipids are compared. Finally, the sterol and carotenoid compositions of these organisms are compared.

INTRODUCTION

There have been relatively few reports concerning the effect of growth temperature on fungal lipid composition. These have been reviewed by Weete (1980).

Increases in the degree of lipid unsaturation have been found in *Aspergillus niger*, *Rhizopus nigricans* (Pearson and Raper, 1927) and *A. nidulans* (Singh and Walker, 1956) when grown at reduced temperatures. In addition, the lipids of *Saccharomyces cerevisiae* (Chang and Matson, 1972) and *Candida utilis* (Meyer and Bloch, 1963) became less unsaturated when grown at elevated temperatures.

Sumner and Morgan (1969) and Sumner *et al.* (1969) compared the lipid composition of psychrophilic, mesophilic and thermophilic species of *Mucor* and *Rhizopus*. Although all species contained a greater quantity of unsaturated lipid at lower growth temperatures, the lipids of the psychrophiles and mesophiles were similarly unsaturated. The thermophiles produced lipid that was significantly more saturated at both high and low growth temperatures. In a comparison of the lipid composition of sporangiospores and vegetative mycelium, spores contained less lipid than mycelium, but spore lipids were always more highly saturated (Sumner and Morgan, 1969).

Mumma *et al.* (1970; 1971(a)) compared the lipid compositions of nine species of thermophilic fungi with an

equal number of mesophilic species of the same genera. In general, the thermophilic species contained more lipid than their mesophilic counterparts. In the thermophiles, the polar lipids were significantly more saturated than were the neutral lipids of the thermophiles or the polar or neutral lipids of the mesophiles. In mesophilic species, the predominant fatty acids were palmitic, linoleic and linolenic, as compared with oleic and other highly saturated fatty acids in the thermophiles. Mesophiles preferentially incorporated palmitic and linoleic acids into their polar lipids while thermophiles incorporated saturated fatty acids. Phosphatidic acid was a major component of the phospholipid of Humicola grisea var thermoidea and occurred in a greater concentration than had previously been reported in other fungi (Mumma et al., 1971(b)). Sterols and their derivatives also comprised an unusually large fraction of the lipid of this thermophile, suggesting a possible role in thermostability. The ability to alter fatty acid composition with growth temperature has also been reported in other species (McMurrough and Rose, 1973; Watson et al., 1976; Manocha and Campbell, 1978; Morton et al., 1978; Kerekes and Nagy, 1980).

In contrast, similar responses to changing temperatures have not been shown for other fungi tested including Pythium ultimum (Bowman and Mumma, 1967), Cunninghamella blakesleeanus and Rhizopus arrhizus (Shaw, 1966).

In this investigation, the effect of growth temperature on lipid composition is compared in psychrophilic, mesophilic and thermophilic Mucor species. Both total cellular lipids and plasma membrane lipids are considered.

PLASMA MEMBRANE ISOLATION

The structure and composition of the fungal plasma membrane has received very little attention in comparison to other cell types, due to the presence of a rigid cell wall in fungi which must be removed before the plasma membrane can be isolated. Two basic methods have been used to prepare intact plasma membranes from filamentous fungi and yeasts. The first of these involves the conversion of mycelium or cells to protoplasts, lysing these protoplasts and isolating plasma membranes from the lysate by differential centrifugation. The second method entails separation of plasma membranes by centrifugation, from a preparation of disrupted mycelium or cells, obtained by subjecting the organisms to mechanical disruption (Rose, 1976).

Undoubtedly, the use of protoplasts offers a more gentle method of disrupting the cell wall compared with mechanical disruption. Nevertheless, the use of protoplasts is not without drawbacks. Plasma membrane preparations prepared using protoplasts involve either single-celled yeasts (Boulton and Eddy, 1962;

Boulton, 1965; Longley, et al., 1968; Schibeci, Rattray and Kidby, 1973; Marriott, 1975(a)) or septate filamentous fungi or yeasts (Garcia-Acha et al., 1966; Marriott, 1975(a)), yielding a single protoplast per septum or per cell. However, Mucoraceous fungi are characterised by aseptate mycelia and the traditional methods of protoplast isolation may, therefore, prove inappropriate. Further, many of the lytic enzyme preparations used in protoplast isolation also contain varying amounts of contaminating phospholipases (Rose, 1976) and proteases (Hamlyn et al., 1981). Clearly, the presence of these enzymes might irreversibly disrupt normal membrane structure.

Using a mechanical method, Nombela et al. (1974) isolated plasma membrane - rich fractions from homogenised extracts of Fusarium culmorum. More recently, Galpin and Jennings (1980) demonstrated the production of plasma membranes from mycelium of Dendryphiella salina disrupted with a glass homogeniser (providing suitably gentle shearing) followed by a relatively simple centrifugation regime. These authors also demonstrated the presence of only limited contamination of the plasma membrane by mitochondrial fragments using both marker enzyme assays and transmission electron microscopy. This method compares well with plasma membrane preparations from Neurospora crassa (Scarborough, 1975) and Candida (Schneider et al., 1978) and, as such, was adopted for this investigation.

MATERIALS AND METHODS

(1) SPECIES

As Chapter 2.

(2) TOTAL LIPID EXTRACTION

Total lipids were extracted using a method based on that recommended for plant tissues by Nichols (1963).

Log phase cultures of each test species, grown in 100 Erlenmeyer flasks containing 25 ml sterile GAE medium (Appendix 2:1) on a Gallenkamp orbital incubator at 100 r.p.m., were harvested by suction filtration and washed in excess of distilled water. Mycelia were extracted in 3 ml hot isopropanol (80C water bath) for 15 minutes in order to inactivate lipolytic enzymes. Mycelia were then homogenised with a glass homogeniser in 3 ml isopropanol, placed in a centrifuge tube and rinsed twice with 3 ml isopropanol. The extract was centrifuged at 1000 xg for 5 minutes for each rinse and the supernatant decanted into a 150 ml evaporating flask. Mycelial pellets were further extracted with 5 ml chloroform : isopropanol (1:1 (v/v)) twice and finally with 2 ml chloroform. After each extraction the supernatant was decanted into the evaporating flask. The combined lipid extracts were reduced to a small volume (approximately 1 ml) on a rotary evaporator and taken up in 2 ml chloroform. This chloroform extract was then

washed three times against 1 ml 0.88 M KCl (Folch wash), the upper aqueous layer being discarded after each wash. The lipid extracts in chloroform were subsequently reduced in volume in 5 ml pear-shaped flasks on a rotary evaporator, transferred to small screw-top vials and reduced to dryness under a stream of nitrogen. Lipid extracts were stored in the dark at -20C.

For each strain, the above extraction was carried out on log phase mycelia grown over the temperature range for growth of that strain. Three replicate samples per strain per temperature were methylated for gas chromatographic analysis and three replicate samples were retained for thin layer chromatographic analysis of the lipid classes extracted.

(3) THIN LAYER CHROMATOGRAPHY (TLC) OF TOTAL LIPID EXTRACTS

Lipids were separated on pre-coated TLC plates of silica gel 60 incorporating a fluorescent indicator, F-254 (BDH). The solvent system was composed of hexane : diethyl ether : acetic acid : ethanol (90:20:2:3).

Plates were activated by heating at 80C for 1 hour. Samples, dissolved in chloroform, were applied as small spots using a microsyringe. Plates were then placed in a suitable chamber containing solvent and run until the solvent front reached the top of the plate (approximately 2 hours).

The plates were then air dried and sprayed lightly with a 0.7% (w/v) solution of 2-7 dichlorofluorescin in 95% methanol. When stained in this way, lipids appear as fluorescent yellow spots when viewed under ultraviolet light.

Lipids were identified by comparing R_f values of unknown sample lipids with those of known standards. At the optimal growth temperature of each strain, all of the identified lipid classes were scraped off the TLC plates. These individual lipid classes were subsequently methylated in order to determine the fatty acid composition. Phospholipid spots were treated in this way over the temperature range for growth of each strain. Using oleic acid (18:1) as a standard, up to 91% recovery was achieved using this method.

(4) METHYLATION OF FATTY ACIDS FOR GAS CHROMATOGRAPHY (GC)

The method used was that of Morrison and Smith (1964).

1 ml methylation solution composed of 25% boron trifluoride in methanol, 20% benzene and 55% methanol was added to the dried lipid extract. The sample was then heated in a boiling water bath for 45 minutes. Fatty acid methyl esters were extracted by adding 1 ml distilled water followed by 2 ml pentane. The top layer

was pipetted off into a fresh screw-top vial, reduced to dryness under a stream of nitrogen and stored in the dark at -20C prior to GC analysis.

(5) GAS CHROMATOGRAPHY (GC) OF FATTY ACID METHYL ESTERS

Fatty acid methyl esters were separated on 6 ft (1.8 m) columns of 10% DEGS (diethyleneglycol succinate) on Chromosorb 101 using a Pye series 304 Gas Chromatograph equipped with dual-flame ionization detector and computing integrator. Nitrogen (40 ml per minute) was employed as carrier gas with a column temperature of 190C. Samples taken up in pentane, were identified by comparison of retention times with those of known standards (Appendix 5:1).

The degree of lipid unsaturation, which is usually reflected in the number of double bonds in the acyl moieties of complex lipids, was calculated from the fatty acid composition. It is expressed as unsaturation per mole (Δ/mole):

$$\Delta/\text{mole} = 1 \times (\% \text{ Monoenes})/100 + 2 \times (\% \text{ Dienes})/100 + 3 \times (\% \text{ Trienes})/100$$

Results are recorded as percentage methyl fatty acid composition. However, in each case, a greater

proportion of percentages were below 20%, therefore the data do not conform to the normal distribution. To overcome this, data were transformed using the arcsin transformation. In this way data conform to the normal distribution, allowing statistical analysis (Bishop, 1966).

(6) PLASMA MEMBRANE ISOLATION AND LIPID EXTRACTION

Plasma membrane fractions were isolated using a similar method to that described by Galpin and Jennings (1980) for Dendryphiella salina.

Log phase mycelia of each strain were prepared by inoculating 250 ml flasks containing 100 ml sterile GAE medium (Appendix 2:1). Cultures were incubated on a Gallenkamp orbital incubator at 100 r.p.m. at the optimum growth temperature of the strain under investigation. Mycelia prepared in this way were harvested by suction filtration and rinsed with 2 x 20 ml portions of extraction medium containing 20 mM TES (Tris-(hydroxymethyl)-methyl-2-aminoethanesulphuric acid) buffer, 400 mM sucrose and 0.1% (v/v) 2-mercaptoethanol, pH 7.2 at 4C. 1 g batches of mycelia (up to 20 g total wet weight) in 2 ml extraction medium were disrupted with a glass homogeniser. The resulting homogenate was then centrifuged at 2000 x g for 10 minutes at 4C to remove nuclei and larger cell debris. All further

centrifugations were carried out at 4C. The 2000 x g supernatant was successively centrifuged at 12,000 x g for 20 minutes (to remove mitochondria) and 100,000 x g for 60 minutes. The pellet from the final spin was resuspended in 6 ml of extraction medium and gently layered onto 6 ml of sucrose solution, specific gravity 1.1713, and spun for a further 60 minutes at 100,000 x g. Membranes prepared in this way were assayed for mitochondrial contamination using the marker enzymes of succinate dehydrogenase and fumarase (see below).

In order to extract the lipid fraction of the plasma membrane, the final 100,000 x g pellet was resuspended in 3 ml hot isopropanol and heated at 80C for 15 minutes to inactivate lipolytic enzymes. Lipids were then extracted using a similar method to that described earlier for mycelium. The isopropanol/membrane solution was reduced to dryness under a stream of nitrogen. Lipids were subsequently extracted in 2 x 3 ml washes of chloroform : isopropanol (1:1 (v/v)) and a final wash in 3 ml chloroform, the extract being reduced to dryness under a stream of nitrogen after each wash. The lipid extracts were then taken up in 2 ml chloroform and washed three times against 1 ml 0.88M KCl (Folch wash), the upper aqueous layer being discarded after each wash. The lipid extracts in chloroform were subsequently reduced in volume in 5 ml pear-shaped flasks on a rotary evaporator, transferred to small

screw-top vials and reduced to dryness under a stream of nitrogen. Lipids extracted in this way were stored in the dark at -20C.

The above extraction was carried out in duplicate for each strain. Lipid extracts were fractionated using TLC and the fractions subsequently analysed by GC as previously described.

ENZYME ASSAYS

(I) SUCCINATE DEHYDROGENASE

Succinate dehydrogenase (EC.1.3.99.1) was assayed by the method of Pennington (1961). A total assay volume of 3 ml contained 100 mM potassium phosphate buffer, pH 7.4, 100 mM potassium succinate, 0.2 mg ml^{-1} iodonitrotetrazolium violet and 100 µg protein. Samples were incubated for 30 minutes at 30C after which the reaction was stopped with 0.3 ml of 50% (w/v) trichloroacetic acid. Extraction with 10 ml of ethyl acetate produced formazen which was read at 490 nm against controls incubated without succinate on an LKB "Ultrospec" spectrophotometer.

(II) FUMARASE

Fumarase (EC.4.2.1.2) was assayed by the method of Hill and Bradshaw (1969). A sample containing 100 - 200 µg protein was added to a 1 cm light path

cuvette containing 50 mM L-malate in 50 mM phosphate buffer, pH 7.3. The increase in absorbance at 250 nm was followed over 1 minute on an LKB "Ultrospec" spectrophotometer. The temperature was carefully maintained at 25C and linearity was ensured by setting up the reaction mixture to give a change of 0 - 0.5 O.D. units. Enzyme units are defined as 1 x 10^3 the initial rate of change in extinction per 10 seconds.

PROTEIN CONCENTRATION

Protein concentration was assayed by the method of Lowry, et al. (1951). 200 µl of sample was mixed with 1 ml of solution E (freshly prepared by mixing solutions A (2% Na_2 CO_3 in 0.1 N NaOH), B (1% $CuSO_4$) and C (2% Na tartrate) in the ratio 100:1:1) and allowed to stand for 10 minutes at room temperature. 100 µl of solution D (Folin and Ciocalteaus phenol reagent mixed 1:2 (v/v) with distilled water) was then added, mixed rapidly and allowed to stand for 30 minutes at room temperature. Finally, 5 ml of distilled water was added, mixed, and the absorbance read at 740 nm against a suitable blank. The protein concentration of the sample was determined by comparison with known values from a previously constructed standard curve (Appendix 5:2).

RESULTS

METHYL FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACTS

The methyl fatty acid compositions of the total lipid extracts of each species grown at optimum temperature are given in Figure 5:2 (data are given in Appendix 5:3). In general, each species produced a series of saturated and unsaturated (monoenoic, dienoic and trienoic), even-numbered carbon chain fatty acids ranging from 14 to 20 carbons in chain length. In each species, fatty acids with 18 carbons were most abundant, those with 14 carbons being least abundant. In each species oleic (18:1) and linoleic (18:2) were the most abundant unsaturated fatty acids. However, there were differences between species in terms of saturated fatty acid content. Each species incorporated palmitic acid (16:0) and arachidic acid (20:0) as their most abundant saturated fatty acids, but differed in the relative amounts incorporated. Thus, in the thermophilic strains of M. pusillus, palmitic acid (16:0) was the most abundant saturated fatty acid, with a ratio of approximately 2:1 to arachidic acid (20:0) in terms of percentage content. However, in both mesophilic strains of M. hiemalis and also in the psychrophile, M. psychrophilus, palmitic acid (16:0) and arachidic acid were incorporated in approximately equal amounts in terms of percentage content. In addition, the monoene gladoleic acid (20:1) was incorporated amongst the unsaturated

FIGURE 5.2

PERCENTAGE METHYL FATTY ACID COMPOSITION OF

TOTAL LIPID EXTRACTS AT OPTIMUM GROWTH TEMPERATURE

FIGURE 5·2

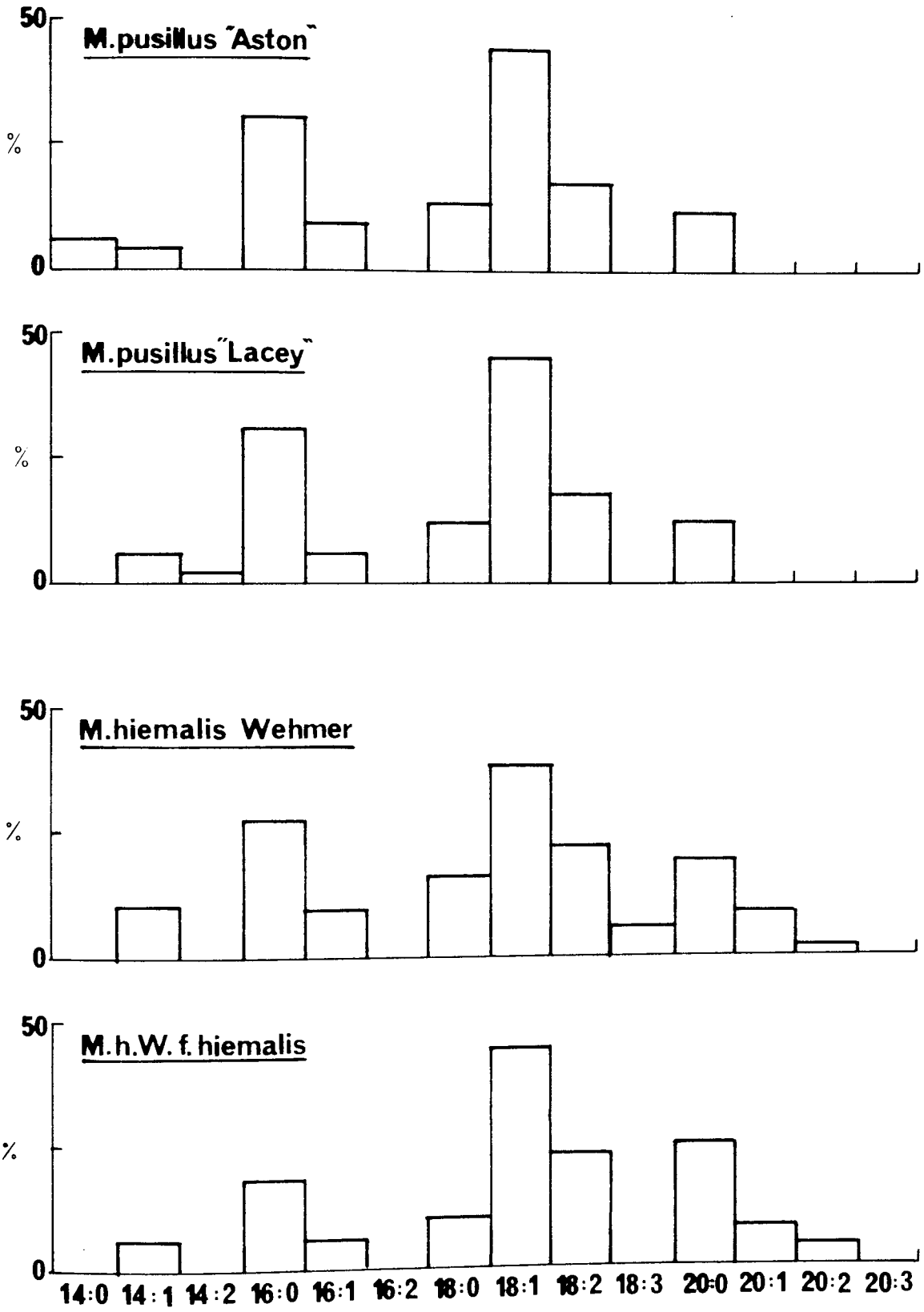
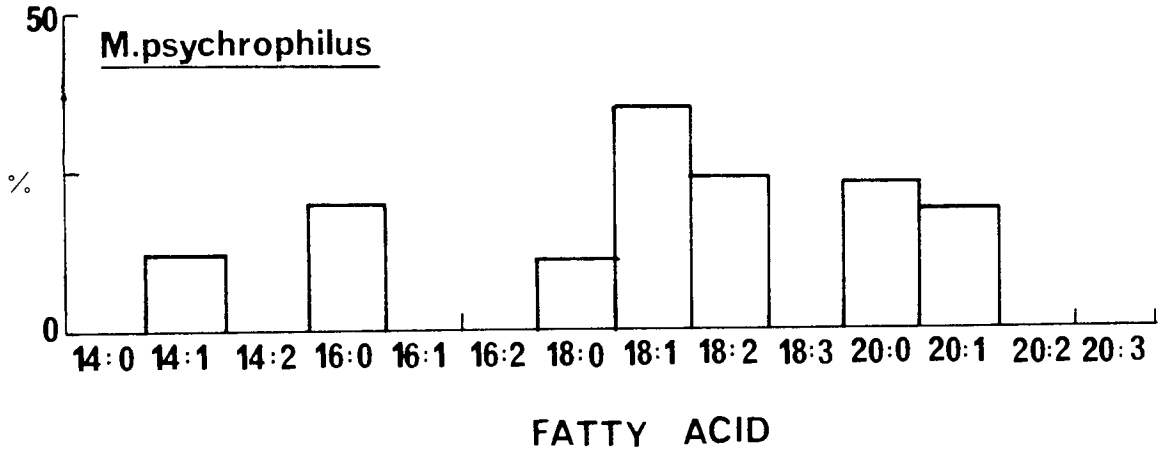

M.pusillus "Aston"
M.pusillus "Lacey"
M.hiemalis Wehmer
M.h.W. f. hiemalis
50
%
0
14:0 14:1 14:2 16:0 16:1 16:2 18:0 18:1 18:2 18:3 20:0 20:1 20:2 20:3
FATTY ACID

FIGURE 5·2


50
M.psychrophilus
%
0
14:0 14:1 14:2 16:0 16:1 16:2 18:0 18:1 18:2 18:3 20:0 20:1 20:2 20:3
FATTY ACID

fatty acids of the mesophilic and psychrophilic species, but was only a minor component (less than 1%) in the unsaturated fatty acid profile of the thermophilic species.

THE EFFECT OF GROWTH TEMPERATURE ON THE METHYL FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACTS

The effect of growth temperature on the methyl fatty acid composition of total lipid extracts in terms of lipid unsaturation is shown in Figure 5:3 (data are given in Appendix 5:4). In addition, Table 5:1 shows the effect of growth temperature on the chain length of the methyl fatty acids of total lipid extracts.

In general, the lipids of the psychrophilic and mesophilic species were similarly unsaturated, but were more unsaturated than the thermophilic lipids. Further, only the temperate mesophilic strain of M. hiemalis and the psychrophile, M. psychrophilus, showed significant changes in lipid unsaturation with temperature. In addition, there were no significant changes in fatty acid chain length with temperature.

Figure 5:3 shows the effect of growth temperature on the lipid unsaturation of the thermophilic strains of M. pusillus. Lipids of both strains were similarly unsaturated at all temperatures, but were significantly less unsaturated than the lipids of the mesophilic and psychrophilic species.

FIGURE 5.3

THE EFFECT OF GROWTH TEMPERATURE ON THE METHYL FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACTS IN TERMS OF LIPID UNSATURATION

KEY:

A	M. PUSILLUS "ASTON"
B	M. PUSILLUS "LACEY"
C	M. HIEMALIS WEHMER
D	M. HIEMALIS WEHMER F. HIEMALIS
E	M. PSYCHROPHILUS

FIGURE 5:3

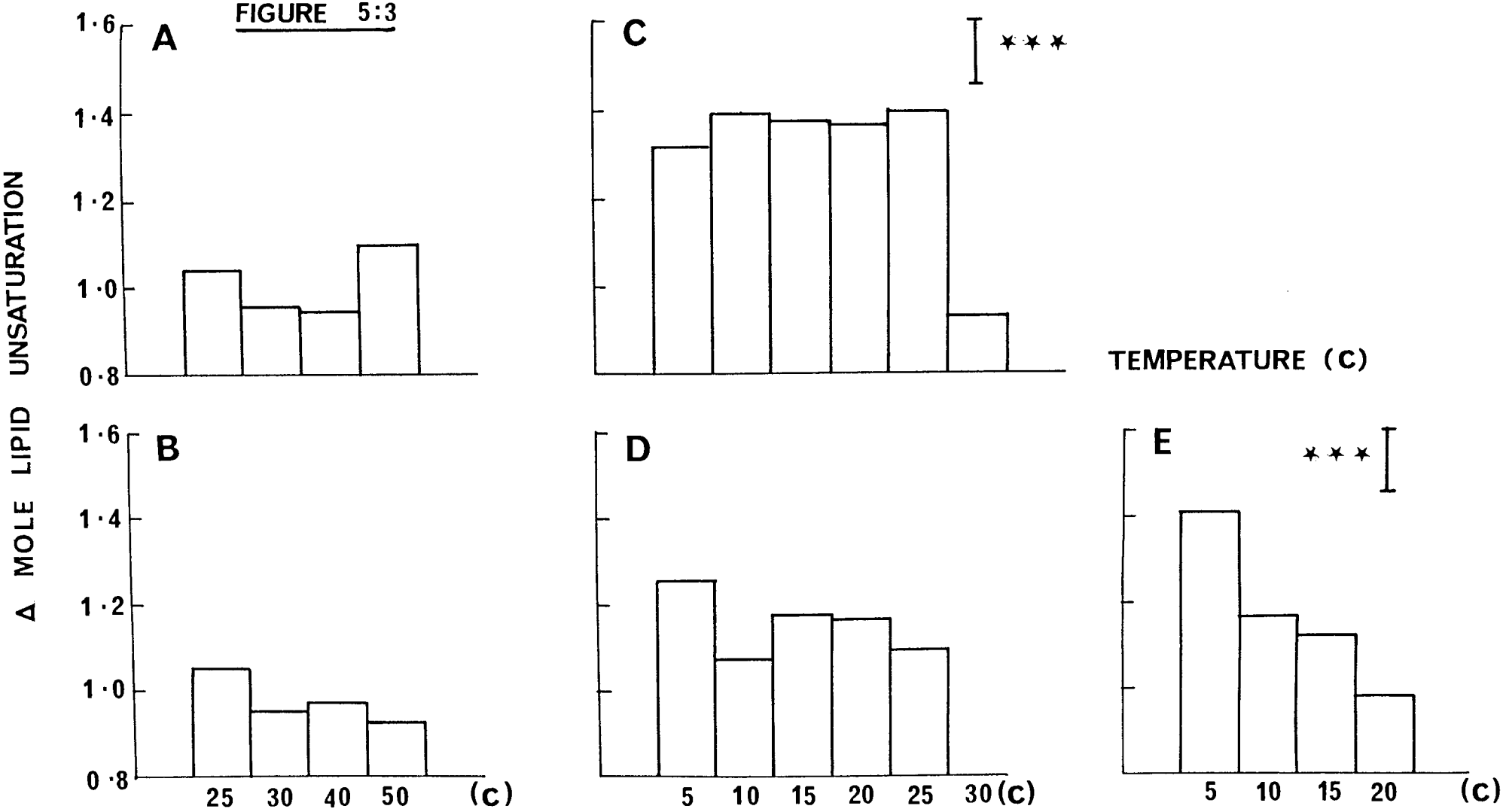

A
B
C
D
E
1·6
1·4
1·2
1·0
0·8
Δ MOLE LIPID UNSATURATION
25 30 40 50 (C)
5 10 15 20 25 30 (C)
5 10 15 20 (C)
TEMPERATURE (C)

Table 5:1 THE EFFECT OF GROWTH TEMPERATURE ON TOTAL FATTY ACID CHAIN LENGTH

GROWTH TEMPERATURE	CHAIN LENGTH Σ14	Σ16	Σ18	Σ20	LEAST SIGNIFICANT DIFFERENCE
MUCOR PUSILLUS "ASTON"					
25C	17.1	122.7	248.8	61.7	2.1
30C	17.1	103.3	222.5	73.9	0.8
40C	35.2	116.9	223.0	41.6	16.8
50C	40.1	109.9	224.1	39.9	8.1
MUCOR PUSILLUS "LACEY"					
25C	17.1	115.7	223.1	65.0	2.0
30C	17.1	103.3	223.1	73.9	1.2
40C	22.8	110.0	224.2	42.8	8.6
50C	28.5	120.3	231.8	30.0	7.9
MUCOR HIEMALIS WEHMER					
5C	35.7	111.5	193.3	87.9	5.2
10C	33.0	107.8	208.1	109.1	3.4
15C	24.3	96.8	211.4	114.3	1.5
20C	23.8	106.2	236.8	107.0	8.7
25C	30.0	105.9	245.8	92.4	5.5
30C	34.5	93.9	212.5	89.1	0.8
M.HIEMALIS W.F.HIEMALIS					
5C	24.3	70.0	229.0	112.7	4.4
10C	23.8	60.4	231.0	117.3	8.3
15C	17.1	70.5	232.3	85.2	4.1
20C	28.1	84.3	262.4	89.4	2.1
25C	28.1	81.5	232.1	87.4	4.1
MUCOR PSYCHROPHILUS					
5C	35.9	90.8	227.3	107.8	7.8
10C	35.9	59.8	225.2	125.3	8.4
15C	34.5	65.1	232.8	95.9	1.2
20C	25.7	81.9	204.3	88.9	9.0

FIGURES REFER TO THE PERCENTAGE CONTRIBUTION TO THE FATTY ACID PROFILE AND ARE THE SUM OF THREE REPLICATES

In addition, there were no significant changes in lipid unsaturation with temperature in either of the two thermophilic strains. Table 5:1 shows the effect of growth temperature on the chain length of the methyl fatty acids. Both thermophilic strains showed similar chain length profiles. The order of decreasing chain length in percentage contribution to the fatty acid profile was C18 > C16 > C20 > C14 in both strains. There were no significant changes in chain length with temperature.

From 5-25C, the lipids of the temperate and Antarctic strains of M. hiemalis were similarly unsaturated and there were no significant changes in lipid unsaturation with temperature in either strain. Levels of lipid unsaturation were similar to those of the psychrophilic species, but were more unsaturated than those of the thermophilic species. However, from 25-30C, the temperate strain of M. hiemalis showed a reduction in lipid unsaturation to a level typical of that found in the thermophilic species at 30C. Table 5:1 shows the effect of growth temperature on methyl fatty acid chain length. Both strains were similar, with the order of decreasing chain length in percentage contribution to the fatty acid profile being C18 > C16 ≥ C20 > C14. This shows that more C20 fatty acids were incorporated in the mesophilic strains compared with the thermophiles. There were no significant changes in chain length with temperature.

The lipids of the psychrophile, M. psychrophilus, were similarly unsaturated to those of the mesophilic species, but were more unsaturated than the thermophilic lipids. There were significant changes in lipid unsaturation with temperature. Specifically, there were significant reductions in lipid unsaturation from 5-10C and from 15-20C. The order of decreasing chain length in percentage contribution to the fatty acid profile was C18 > C16 ≥ C20 > C14, a similar order to that found in the mesophilic strains. There were no significant changes in fatty acid chain length with temperature.

Typical gas chromatograph traces of methyl fatty acid compositions of total lipid extracts are shown in Figure 5:4.

THIN LAYER CHROMATOGRAPHY OF TOTAL LIPID EXTRACTS

Fractionation of total lipid extracts showed that the same lipid classes were common to each of the species investigated. Neutral lipids consisted of acylglycerols and free fatty acids, with phospholipids comprising the polar lipid fraction. A typical separation is shown in Figure 5:5. Each class is considered in terms of fatty acid composition at optimum growth temperature.

FIGURE 5.4

TYPICAL GAS CHROMATOGRAPH TRACES SHOWING METHYL FATTY ACID COMPOSITION OF TOTAL LIPID EXTRACTS AT OPTIMUM GROWTH TEMPERATURE

FIGURE 5:4 M.pusillus "Aston"

1 14:0

2 14:1

3 16:0

4 16:1

5 18:0

6 18:1

7 18:2

8 20:0

SV SOLVENT FRONT

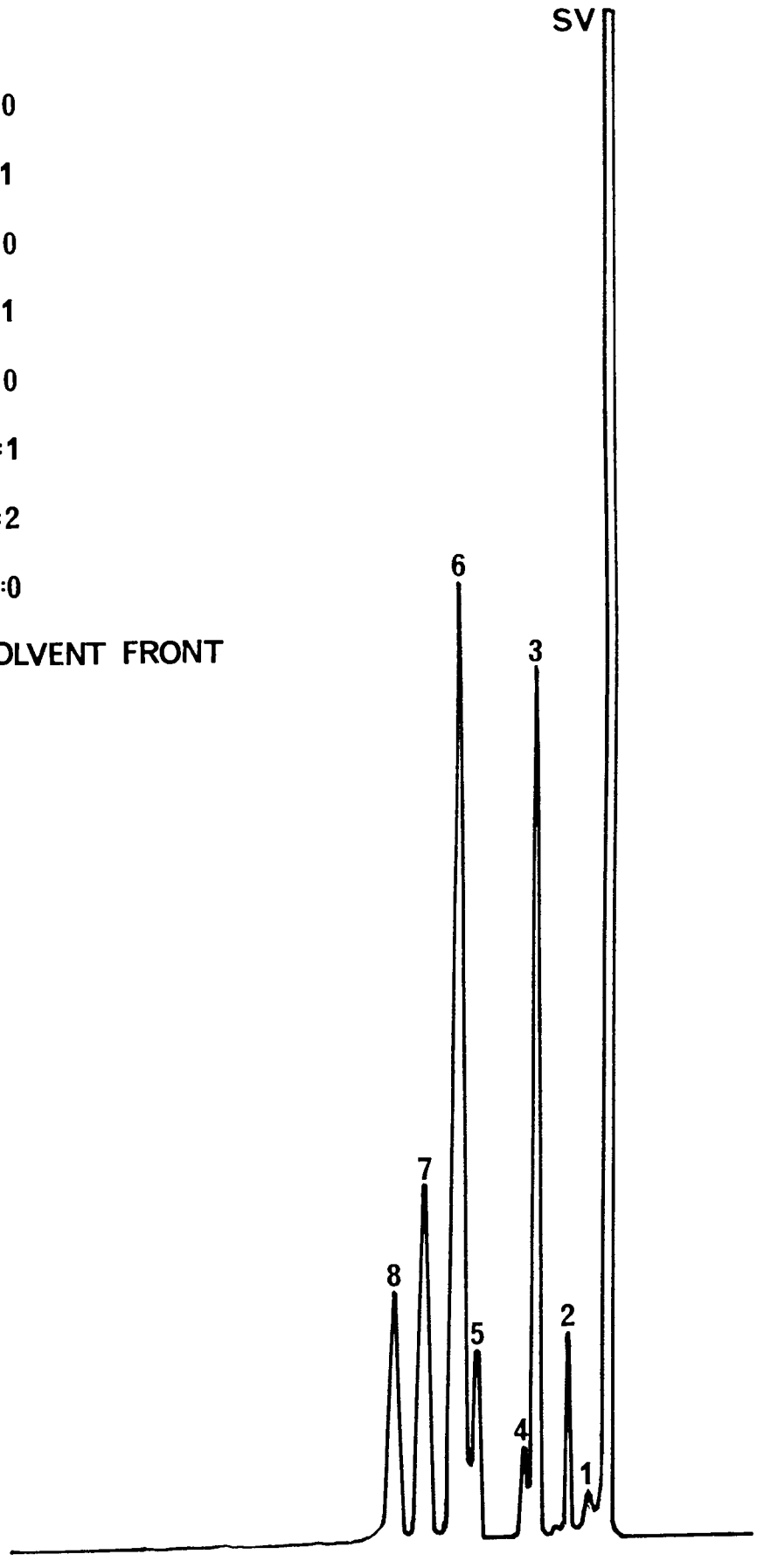


FIGURE 5:4 M. pusillus "Lacey"

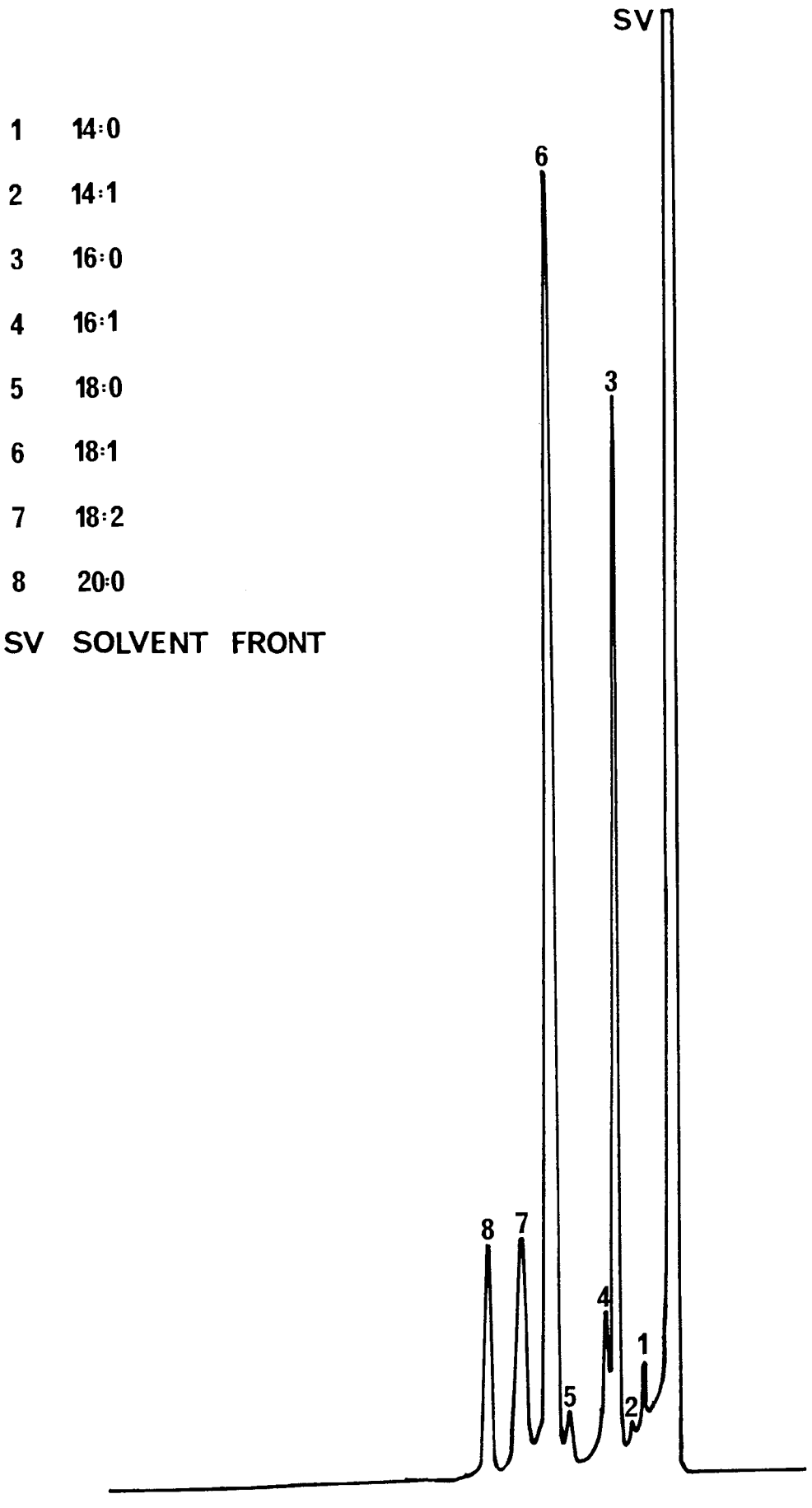


FIGURE 5:4 M.hiemalis Wehmer

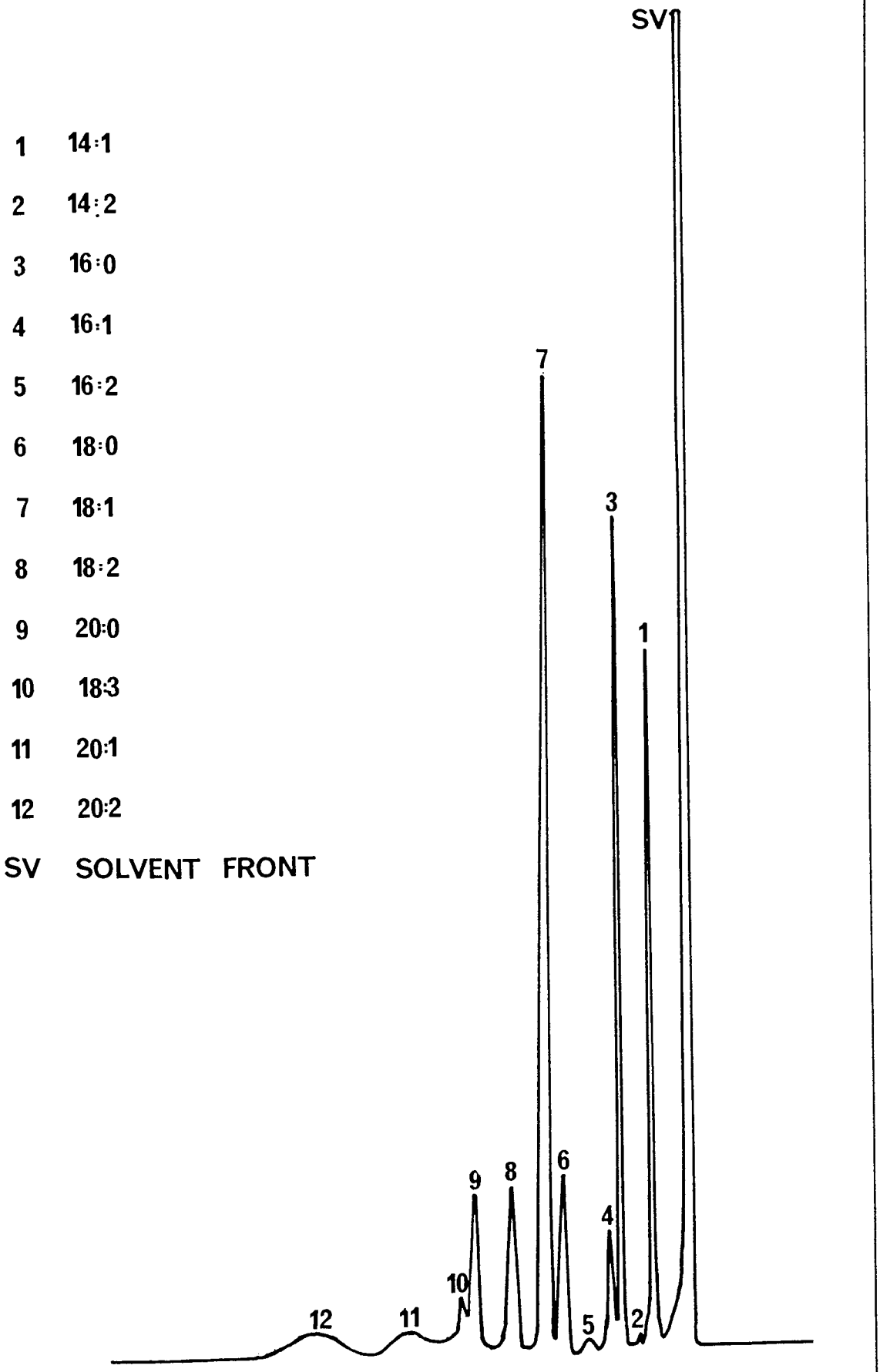


FIGURE 5:4 M.h.W.f. hiemalis

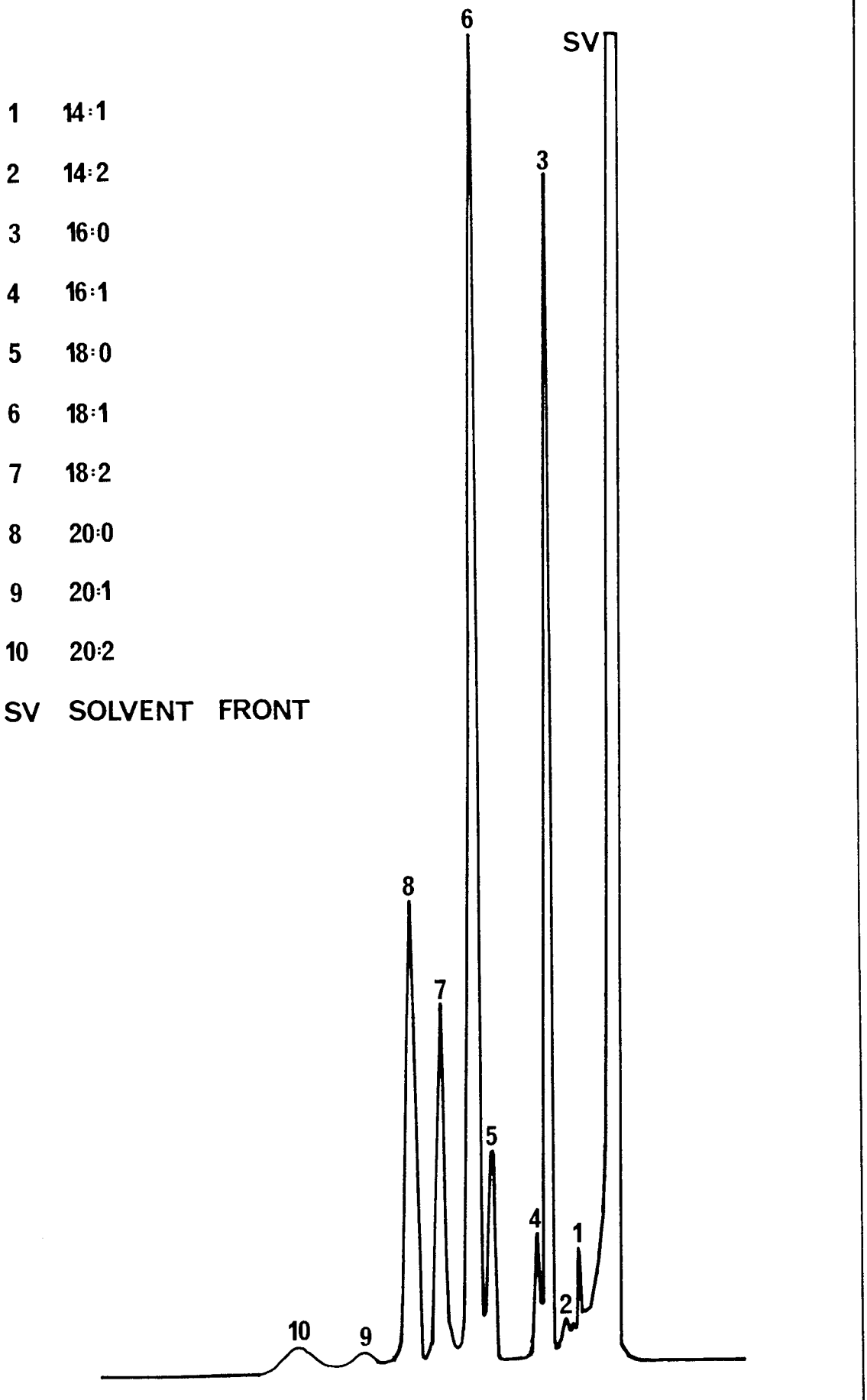


FIGURE 5:4 M.psychrophilus

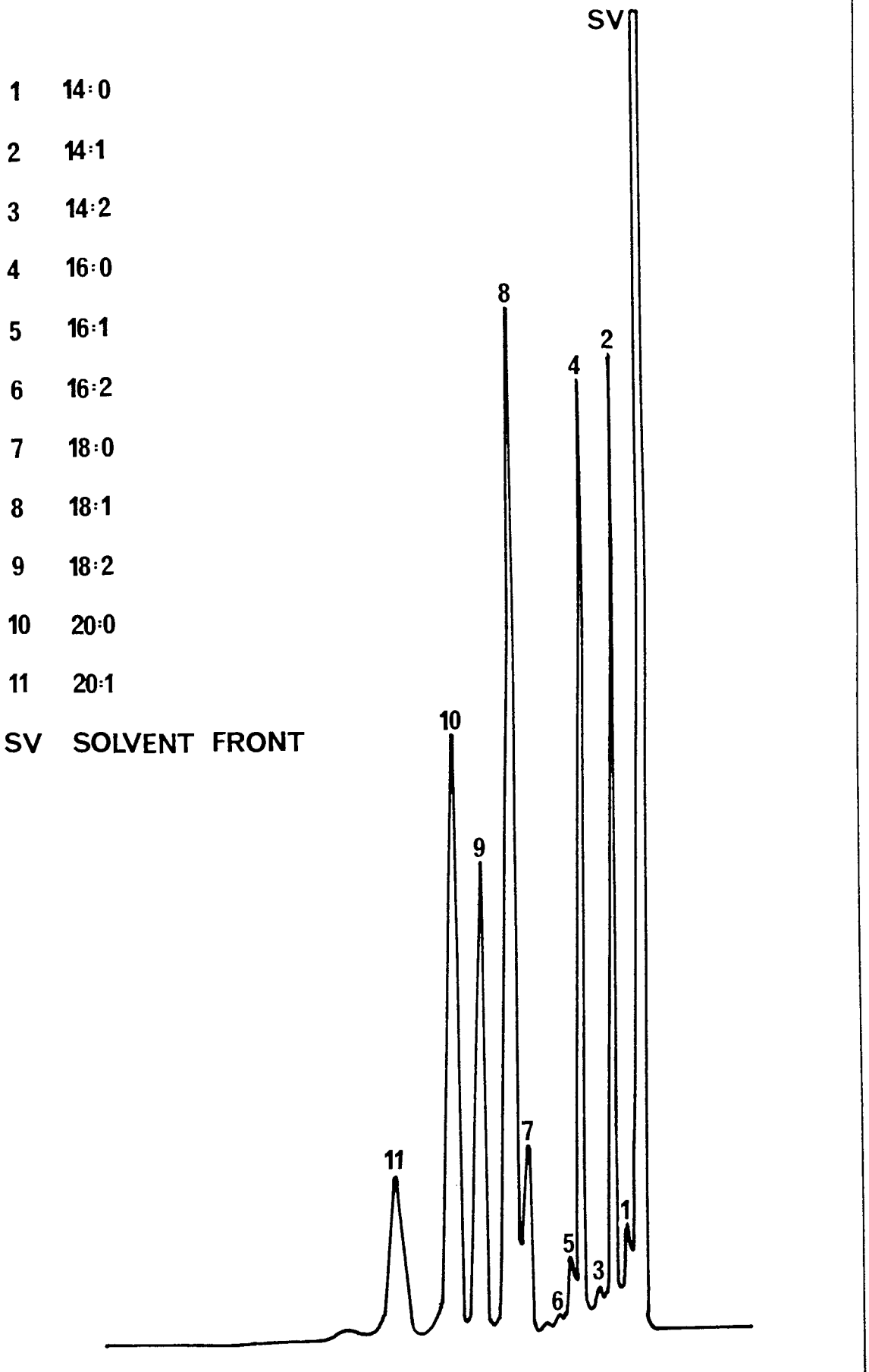


FIGURE 5.5

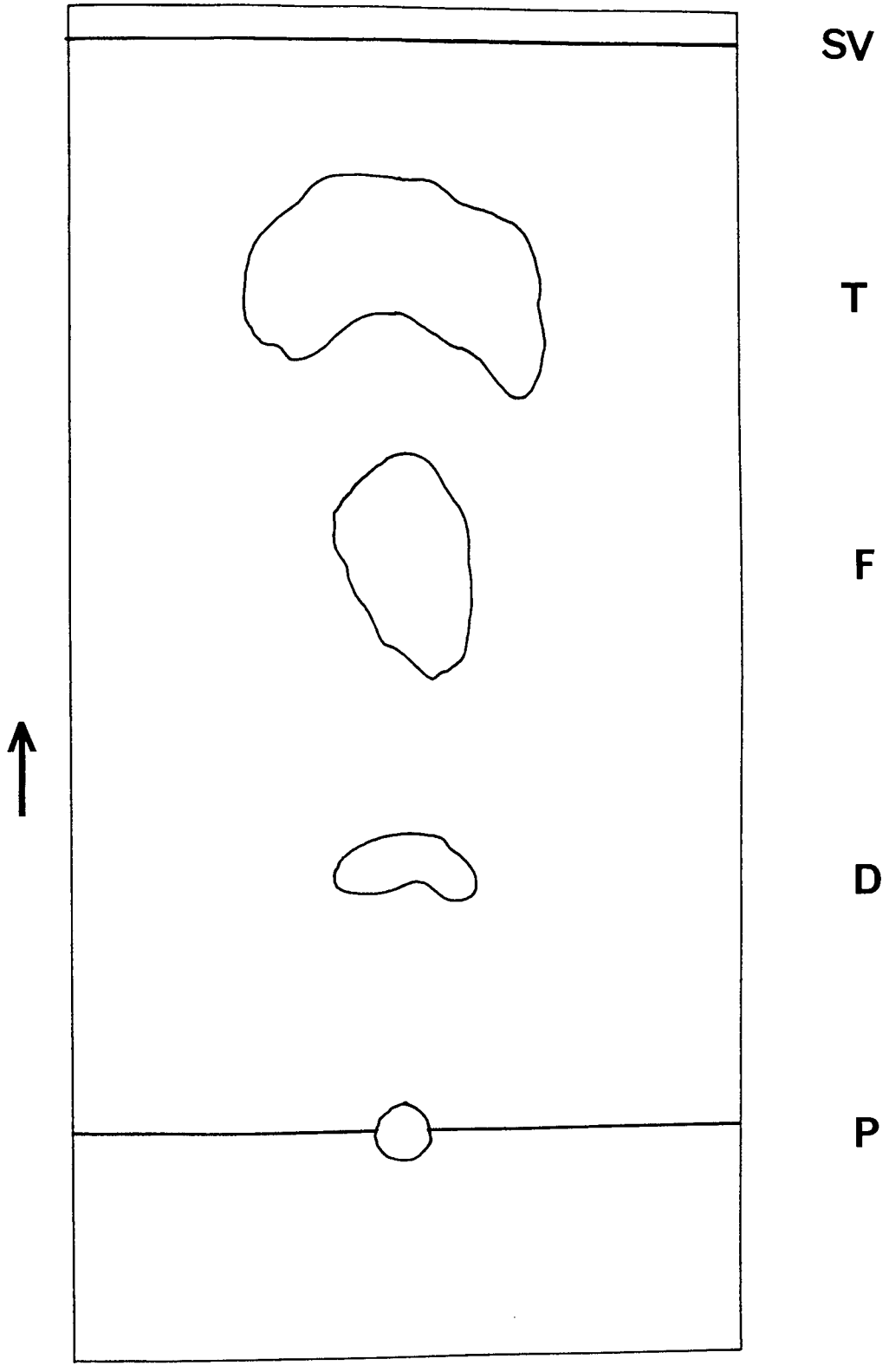
THIN LAYER CHROMATOGRAPHY OF TOTAL LIPID EXTRACTS:

A TYPICAL SEPARATION (M. PUSILLUS "ASTON" AT 40C)

KEY:

SV	SOLVENT FRONT
T	TRIACYLGLYCEROLS
F	FREE FATTY ACIDS
D	DIACYLGLYCEROLS
P	PHOSPHOLIPIDS

FIGURE 5:5



NEUTRAL LIPIDS : (I) ACYLGLYCEROLS

Mycelia of each species investigated contained triacylglycerols (triglycerides) and diacylglycerols (diglycerides). The methyl fatty acid compositions of the triglyceride component of each species grown at optimum temperature are given in Table 5:2. Each species produced a series of saturated and unsaturated, even-numbered carbon chain fatty acids ranging from 14 to 20 carbon atoms in chain length. In general, fatty acids with 16 and 18 carbon atoms were most abundant, with palmitic acid (16:0) being the major saturated fatty acid and oleic (18:1) and linoleic (18:2) being the most abundant unsaturated fatty acids. There were a few differences in composition between species. Both the Antarctic strain of M. hiemalis and the psychrophilic M. psychrophilus incorporated the monoene gladoleic acid (20:1) into their triglycerides in addition to oleic acid (18:1). In addition, neither the thermophilic nor mesophilic species incorporated trienoic fatty acids. However, M. psychrophilus incorporated the triene linolenic acid (18:3) into its triglyceride component.

Table 5:3 compares the lipid unsaturation of the triglyceride components with those of the total lipid extracts at optimum growth temperatures. In each strain the values are very similar. The importance of this will be discussed later.

Table 5:2 TRIGLYCERIDE METHYL FATTY ACID COMPOSITION AT OPTIMUM GROWTH TEMPERATURE

FATTY ACID	M.PUSILLUS "ASTON"	M.PUSILLUS "LACEY"	M.HIEMALIS WEHMER	M.H.W.F. HIEMALIS	M. PSYCHROPHILUS
14:0	5.7 ± 0.0	+	12.0 ± 0.8	12.4 ± 0.8	11.0 ± 0.9
14:1	±	+	±	±	±
14:2	5.7 ± 0.0	5.7 ± 0.0	13.8 ± 1.5	+	+
16:0	30.6 ± 0.9	31.1 ± 0.8	32.3 ± 1.0	22.2 ± 1.1	18.4 ± 2.0
16:1	±	6.7 ± 5.8	±	±	9.2 ± 2.0
16:2	+	±	13.8 ± 4.0	+	±
18:0	10.0 ± 0.0	10.0 ± 0.0	13.3 ± 0.8	10.0 ± 0.0	13.7 ± 2.0
18:1	39.8 ± 1.4	45.0 ± 0.0	38.6 ± 3.1	43.8 ± 3.1	41.0 ± 1.4
18:2	18.3 ± 1.0	20.6 ± 0.5	15.7 ± 1.7	24.6 ± 0.8	25.4 ± 1.1
20:0	11.5 ± 0.0	12.4 ± 0.8	10.5 ± 0.9	18.3 ± 0.4	19.4 ± 1.6
18:3	±	±	±	±	8.1 ± 0.0
20:1	+	+	+	12.1 ± 0.8	11.5 ± 0.0
20:2	+	+	+	±	±
20:3	+	+	+	+	+

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6 n-1) + MINOR COMPONENT (LESS THAN 1%)

Table 5:3 A COMPARISON BETWEEN THE LIPID UNSATURATION OF THE TRIGLYCERIDE COMPONENT WITH THE TOTAL LIPID EXTRACT AT OPTIMUM GROWTH TEMPERATURE

	LIPID UNSATURATION - Δ MOLE	
	TRIGLYCERIDE	TOTAL LIPID
MUCOR PUSILLUS "ASTON"	0.922 ± 0.059	0.952 ± 0.040
MUCOR PUSILLUS "LACEY"	0.955 ± 0.030	0.9975 ± 0.088
MUCOR HIEMALIS WEHMER	1.350 ± 0.024	1.392 ± 0.110
M. HIEMALIS W.F. HIEMALIS	1.211 ± 0.066	1.178 ± 0.076
MUCOR PSYCHROPHILUS	1.250 ± 0.019	1.166 ± 0.030
	MEAN OF 2 REPLICATES + STANDARD DEVIATION (6 n-1)	MEAN OF 3 REPLICATES + STANDARD DEVIATION (6 n-1)

Table 5:4 DIGLYCERIDE METHYL FATTY ACID COMPOSITION AT OPTIMUM GROWTH TEMPERATURE

FATTY ACID	M.PUSILLUS "ASTON"	M.PUSILLUS "LACEY"	M.HIEMALIS WEHMER	M.H.W.F. HIEMALIS	M. PSYCHROPHILUS
14:0	8.7 ± 1.1	8.1 ± 0.0	9.4 ± 1.1	8.7 ± 1.1	11.5 ± 0.0
14:1	±	±	±	±	±
14:2	10.0 ± 0.0	10.5 ± 0.9	8.1 ± 0.0	8.7 ± 1.1	10.8 ± 3.1
16:0	24.4 ± 1.0	25.6 ± 0.4	26.1 ± 0.5	19.4 ± 0.0	22.2 ± 1.3
16:1	±	±	±	±	±
16:2	10.8 ± 0.8	11.0 ± 0.9	5.7 ± 0.0	8.1 ± 0.0	12.3 ± 2.7
18:0	8.1 ± 0.0	8.1 ± 0.0	11.0 ± 0.9	5.7 ± 0.0	3.8 ± 3.3
18:1	41.0 ± 1.4	42.7 ± 0.0	40.2 ± 0.3	33.4 ± 0.3	32.0 ± 1.3
18:2	23.3 ± 0.5	23.3 ± 0.5	24.6 ± 0.4	23.3 ± 0.5	25.7 ± 0.2
20:0	19.4 ± 1.6	17.1 ± 1.2	18.4 ± 1.0	18.1 ± 0.4	24.3 ± 2.3
18:3	±	±	±	±	±
20:1	+	+	+	27.5 ± 0.4	16.7 ± 1.6
20:2	+	+	5.7 ± 0.0	±	±
20:3	+	+	5.7 ± 0.0	+	+

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6 n-1) + MINOR COMPONENT (LESS THAN 1%)

The methyl fatty acid compositions of the diglyceride component of each species grown at optimum temperature are given in Table 5:4. Palmitic acid (16:0) and arachidic acid (20:0) were the predominant saturated fatty acids, with oleic (18:1) and linoleic (18:2) being the most abundant unsaturated fatty acids. In general, saturated, monoenoic and dienoic fatty acids with even-numbered carbon chains ranging from 14 to 20 carbons were incorporated into the diglycerides of each of the test species. There were only minor differences between species. Both M. psychrophilus and the Antarctic strain of M. hiemalis incorporated gladoleic acid (20:1) as part of their monoenoic diglycerides in addition to oleic acid (18:1). Only the temperate mesophile, M. hiemalis Wehmer, incorporated a trienoic fatty acid, a small amount of 20:3.

NEUTRAL LIPIDS : (II) FREE FATTY ACIDS

The free methyl fatty acid compositions of each species grown at optimum temperature are given in Table 5:5. Again, saturated, monoenoic, dienoic and trienoic fatty acids with even-numbered carbon chains ranging from 14 to 20 carbons in chain length were incorporated. Further, fatty acids with 16 and 18 carbons were most abundant with palmitic acid (16:0) being the most abundant saturated acid and oleic (18:1) and linoleic (18:2) acids being the major unsaturated acids. There

Table 5:5 FREE METHYL FATTY ACID COMPOSITION AT OPTIMUM GROWTH TEMPERATURE

FATTY ACID	M.PUSILLUS "ASTON"	M.PUSILLUS "LACEY"	M.HIEMALIS WEHMER	M.H.W.F. HIEMALIS	M. PSYCHROPHILUS
14:0	5.7 ± 0.0	1.9 ± 1.0	15.3 ± 0.0	11.0 ± 0.9	9.2 ± 2.0
14:1	±	±	±	±	±
14:2	5.7 ± 0.0	3.8 ± 3.3	24.4 ± 0.0	15.3 ± 0.0	6.5 ± 1.4
16:0	30.6 ± 0.9	32.0 ± 0.0	30.2 ± 0.4	28.2 ± 1.0	26.2 ± 1.0
16:1	±	±	±	±	±
16:2	+	2.7 ± 0.5	26.8 ± 0.4	16.4 ± 0.0	5.7 ± 0.0
18:0	10.0 ± 0.0	9.4 ± 1.1	±	14.2 ± 0.0	28.0 ± 0.7
18:1	41.0 ± 1.4	45.0 ± 1.0	24.6 ± 1.1	29.8 ± 1.4	39.8 ± 1.5
18:2	20.6 ± 0.5	20.0 ± 0.5	±	8.1 ± 0.0	18.1 ± 0.5
20:0	11.0 ± 0.9	12.4 ± 0.8	16.8 ± 1.3	13.3 ± 0.8	7.3 ± 1.4
18:3	±	±	±	5.7 ± 0.0	±
20:1	+	+	11.0 ± 0.9	±	+
20:2	+	+	±	21.3 ± 2.7	+
20:3	+	5.7 ± 0.0	+	14.2 ± 0.0	+

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6 n-1) + MINOR COMPONENT (LESS THAN 1%)

were few differences between species, although the Antarctic strain of M. hiemalis was exceptional in producing more fatty acids with 20 carbons than the remainder of the strains investigated.

POLAR LIPIDS - PHOSPHOLIPIDS

The methyl fatty acid compositions of the phospholipid components of each species grown at optimum temperature are shown in Figure 5:6 (data are given in Appendix 5:5). Figure 5:6 also shows the methyl fatty acid compositions of the plasma membrane phospholipids at optimum growth temperature. These will be considered later.

Each species produced a series of saturated and unsaturated, even-numbered carbon chain fatty acids, ranging from 14 to 20 carbons in chain length. Palmitic acid (16:0) and arachidic acid (20:0) were the most abundant saturated fatty acids with oleic (18:1) and linoleic (18:2) acids being the major unsaturated acids. Both mesophilic strains and the psychrophilic species incorporated gladoleic acid (20:1) in addition to oleic acid (18:1), whereas both thermophilic strains did not.

FIGURE 5.6

A COMPARISON BETWEEN THE PERCENTAGE METHYL FATTY ACID COMPOSITION OF PLASMA MEMBRANE PHOSPHOLIPIDS WITH TOTAL PHOSPHOLIPIDS AT OPTIMUM GROWTH TEMPERATURE

FIGURE 5·6

P PLASMA MEMBRANE

T TOTAL

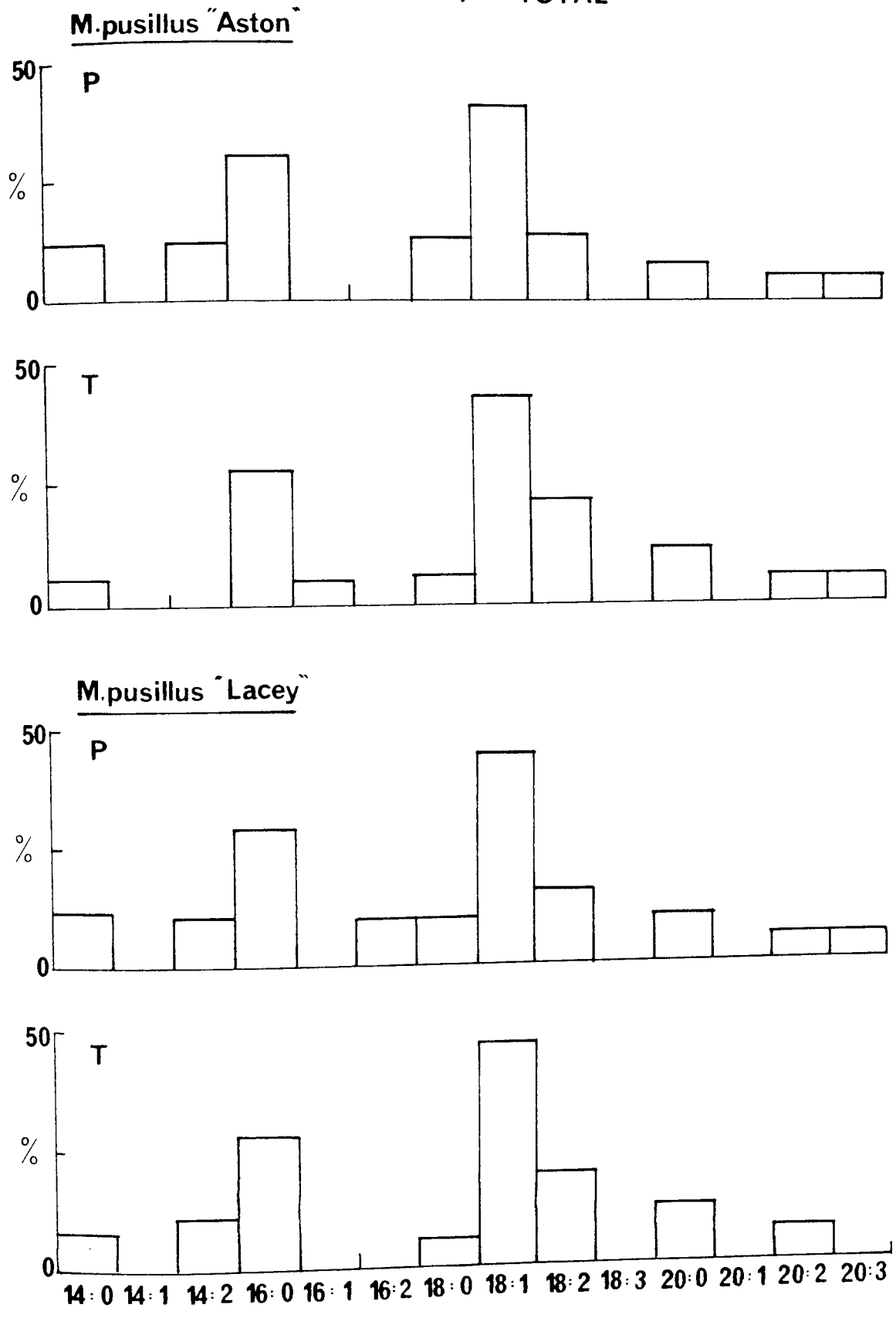


FIGURE 5·6

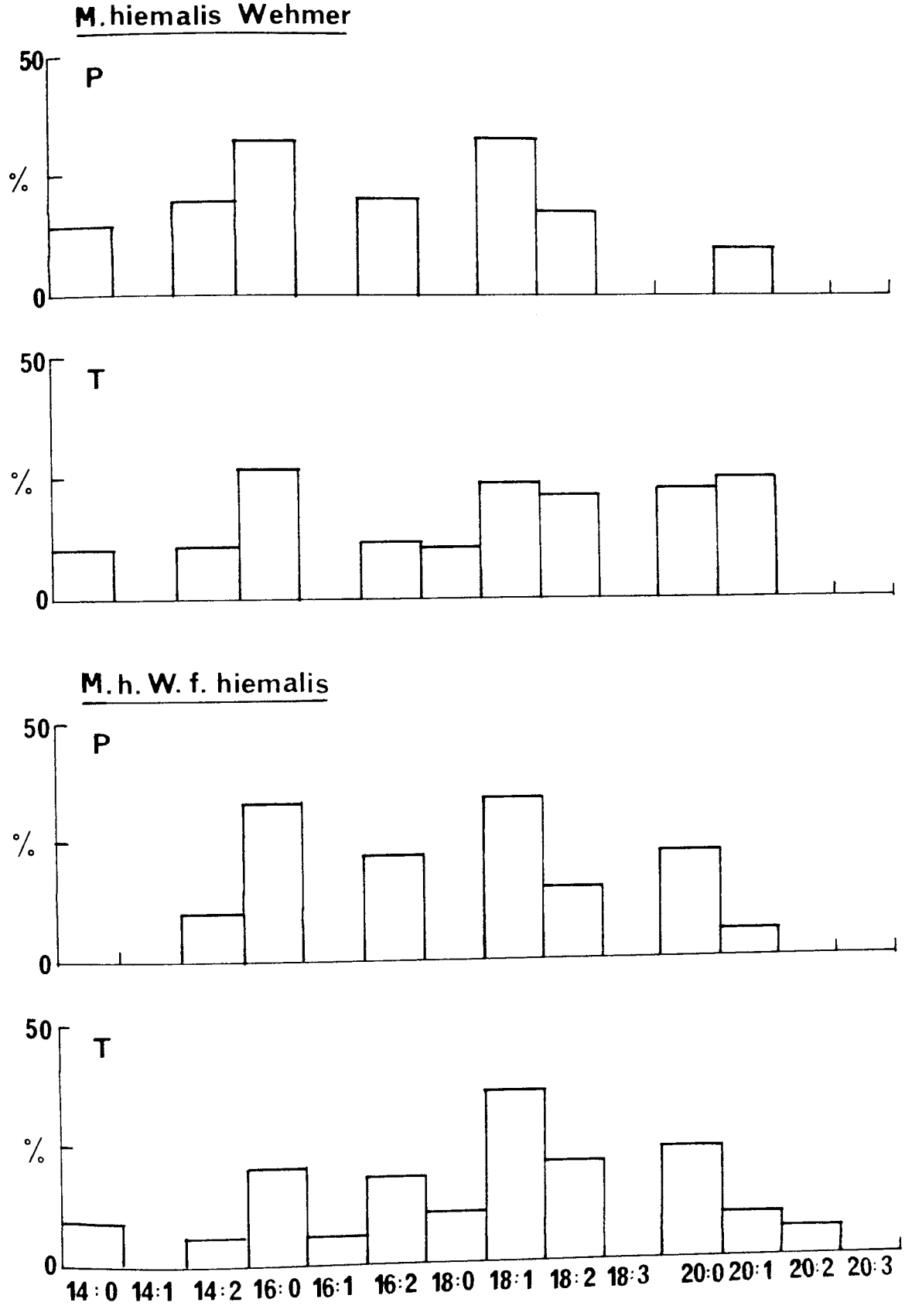
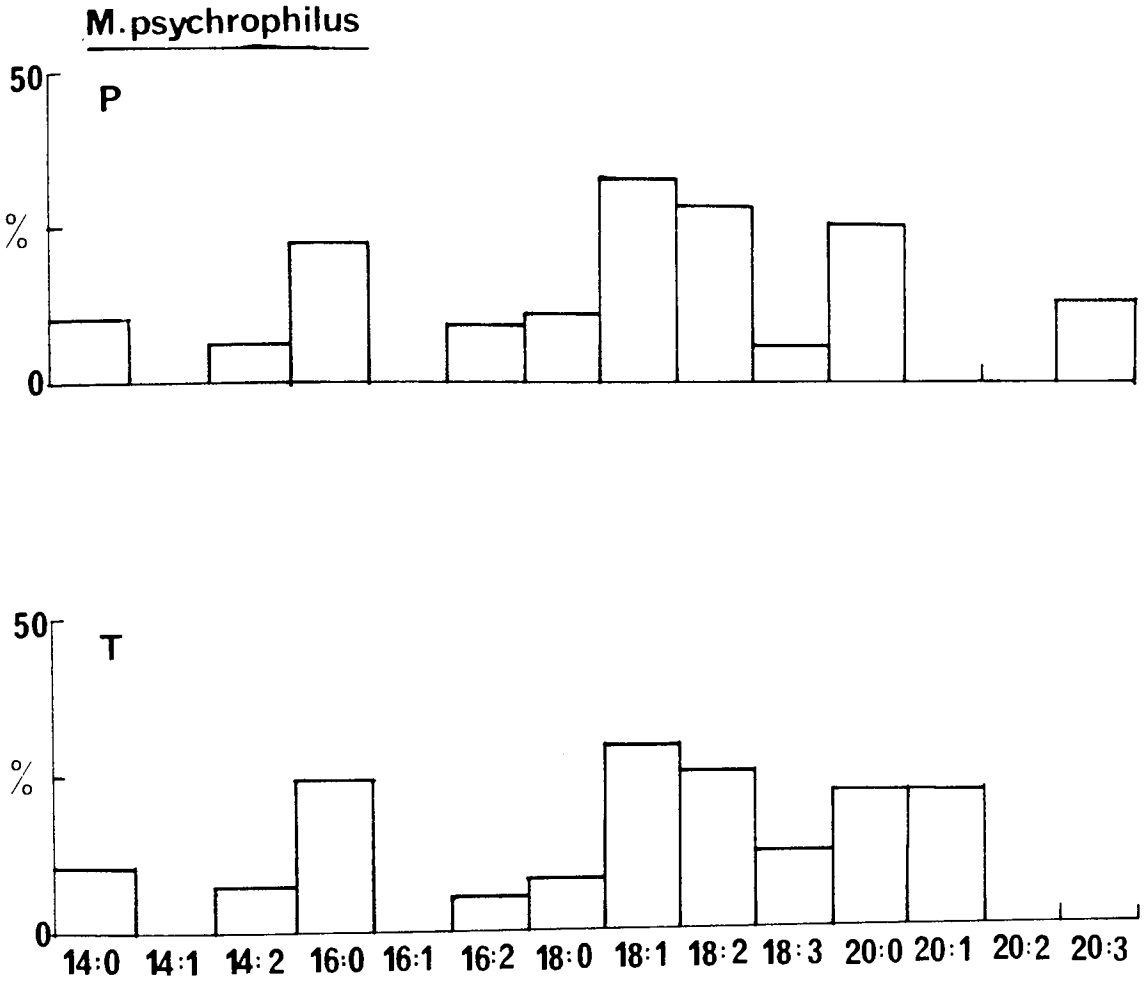

M.hiemalis Wehmer
50
P
%
0
50
T
%
0
M.h.W.f. hiemalis
50
P
%
0
50
T
%
0
14:0 14:1 14:2 16:0 16:1 16:2 18:0 18:1 18:2 18:3 20:0 20:1 20:2 20:3
FATTY ACID

FIGURE 5·6


M.psychrophilus
50
P
%
0
50
T
%
0
14:0 14:1 14:2 16:0 16:1 16:2 18:0 18:1 18:2 18:3 20:0 20:1 20:2 20:3
FATTY ACID

FRACTIONATION OF PLASMA MEMBRANE LIPIDS

(I) PLASMA MEMBRANE ISOLATION

In order to justify lipid analysis, it was necessary to assay the plasma membrane fraction for contamination by other organelles, particularly mitochondria. Table 5:6 compares the total homogenate, mitochondrial and plasma membrane fractions with respect to the relative activities of fumarase and succinate dehydrogenase. Fumarase activity was detected in only one of the plasma membrane fractions, that of *M. pusillus* "Lacey", but was only 1% of that of the total homogenate activity. However, fumarase is only active in the intact mitochondrion and cannot be used to detect mitochondrial membrane fragments that may sediment along with the plasma membrane. In contrast, succinate dehydrogenase is active in both intact and disrupted mitochondria and may, therefore, be used as a marker for contaminating mitochondrial membrane fragments. Table 5:6 shows that there was very little contamination of plasma membrane fractions with mitochondrial membrane fragments as determined by relative succinate dehydrogenase activity. In *M. psychrophilus*, succinate dehydrogenase activity was too low to be detected in any of the fractions.

These results are similar to those reported for *D. salina* plasma membranes by Galpin and Jennings (1980) who, in addition to measuring marker enzyme activities,

Table 5:6 A COMPARISON BETWEEN TOTAL HOMOGENATE, MITOCHONDRIAL AND PLASMA MEMBRANE FRACTIONS WITH RESPECT TO FUMARASE AND SUCCINATE DEHYDROGENASE ACTIVITIES

RELATIVE FUMARASE ACTIVITY(%)	M.PUSILLUS "ASTON"	M.PUSILLUS "LACEY"	M.HIEMALIS WEHMER	M.H.W.F. HIEMALIS	M. PSYCHROPHILUS
Total Homogenate	100	100	100	100	100
Mitochondria	53	48	59	45	56
Plasma Membrane	0	1	0	0	0
RELATIVE SUCCINATE DEHYDROGENASE ACTIVITY(%)					
Total Homogenate	16	18	18	18	-
Mitochondria	100	100	100	100	-
Plasma Membrane	4	3	1	2	-

FIGURES ARE THE MEAN OF 2 REPLICATES
100% RELATIVE ACTIVITY REFERS TO THE MAXIMUM ENZYME ACTIVITY OF ALL FRACTIONS
- NO ACTIVITY RECORDED

also demonstrated limited mitochondrial contamination by ultrastructural examination of D. salina plasma membranes prepared by the same method as the plasma membrane fractions of the Mucoraceous fungi in this investigation. Therefore, there was very limited contamination of the plasma membrane fractions by mitochondria or mitochondrial membrane fragments in the psychrophilic, mesophilic and thermophilic Mucoraceous species investigated.

PLASMA MEMBRANE LIPID COMPOSITION

Fractionation of plasma membrane lipid extracts by TLC separated two components, triglycerides and phospholipids, in each of the species investigated. Each component will be considered in terms of methyl fatty acid composition.

(I) TRIGLYCERIDES

Table 5:7 shows the methyl fatty acid composition of the plasma membrane triglycerides of each species at optimum growth temperature. In general, the plasma membrane triglyceride compositions were very similar to those of the total cellular triglycerides (Table 5:2). Each species produced a series of saturated and unsaturated even-numbered carbon chain fatty acids ranging from 14 to 20 carbon atoms in chain length. Fatty acids with 16 and 18 carbons were most abundant,

Table 5:7 METHYL FATTY ACID COMPOSITION OF PLASMA MEMBRANE TRIGLYCERIDE AT OPTIMUIM GROWTH TEMPERATURE

FATTY ACID	M.PUSILLUS "ASTON"	M.PUSILLUS "LACEY"	M.HIEMALIS WEHMER	M.H.W.F. HIEMALIS	M. PSYCHROPHILUS
14:0	10.0 ± 0.0	11.5 ± 0.0	12.9 ± 0.0	11.5 ± 0.0	12.9 ± 0.0
14:1	+	+	+	+	+
14:2	12.4 ± 1.0	12.2 ± 1.0	18.9 ± 0.7	12.9 ± 0.0	5.7 ± 0.0
16:0	30.2 ± 0.8	29.0 ± 0.4	30.4 ± 0.5	31.4 ± 0.9	2 2.4 ± 0.8
16:1	+	+	+	+	+
16:2	10.0 ± 0.0	12.9 ± 0.0	23.2 ± 0.6	12.9 ± 0.0	5.7 ± 0.0
18:0	10.0 ± 0.0	15.9 ± 0.8	8.1 ± 0.0	15.9 ± 1.0	18.0 ± 1.6
18:1	45.0 ± 1.0	40.1 ± 0.3	32.3 ± 0.4	37.8 ± 0.4	45.3 ± 1.2
18:2	15.9 ± 0.8	14.2 ± 0.0	16.4 ± 0.0	12.9 ± 0.0	22.4 ± 0.6
20:0	+	+	+	+	9.3 ± 5.1
18:3	+	+	+	+	5.7 ± 0.0
20:1	10.0 ± 0.0	11.5 ± 0.0	9.1 ± 1.3	10.0 ± 0.0	+
20:2	5.7 ± 0.0	5.7 ± 0.0	+	5.7 ± 0.0	+
20:3	5.7 ± 0.0	10.0 ± 0.0	6.9 ± 1.7	10.8 ± 1.1	5.7 ± 0.0

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6 n-1) + MINOR COMPONENT (LESS THAN 1%)

with palmitic acid (16:0) being the most abundant saturated acid and oleic (18:1) and linoleic (18:2), being the major unsaturated acids. However, the plasma membrane triglycerides of each species incorporated a higher percentage of fatty acids with 20 carbon atoms than the total triglyceride component.

(II) PHOSPHOLIPIDS

The methyl fatty acid composition of the plasma membrane phospholipids of each species at optimum growth temperature are shown in Figure 5:6 (data are given in Appendix 5:6). Each species produced a series of saturated and unsaturated, even-numbered carbon chain fatty acids, ranging from 14 to 20 carbons in chain length. Palmitic acid (16:0) and arachidic acid (20:0) were the most abundant saturated acids, with oleic (18:1) and linoleic (18:2) acids being the major unsaturated acids. Table 5:8 compares the plasma membrane phospholipid fatty acid chain lengths at optimum growth temperatures. In the thermophilic strains of M. pusillus, fatty acids of 16 and 18 carbons in chain length were most abundant, those with 14 and 20 carbons being least abundant. However, the thermophilic strains incorporated significantly higher amounts of 20 carbon chain length fatty acids than the mesophilic and psychrophilic species. In the mesophiles, the order of decreasing chain length in percentage contribution

Table 5:8 PLASMA MEMBRANE PHOSPHOLIPID CHAIN LENGTH AT OPTIMUM GROWTH TEMPERATURE

SPECIES	CHAIN LENGTH Σ14	Σ16	Σ18	Σ20	LEAST SIGNIFICANT DIFFERENCE
MUCOR PUSILLUS "ASTON"	51.1	96.8	108.8	56.0	2.2
MUCOR PUSILLUS "LACEY"	46.0	77.3	139.3	62.8	2.0
MUCOR HIEMALIS WEHMER	46.3	103.6	101.4	31.4	0.5
M.HIEMALIS W.F. HIEMALIS	39.7	110.2	97.6	35.8	2.2
MUCOR PSYCHROPHILUS	31.4	61.0	142.6	38.2	2.3

FIGURES REFER TO THE PERCENTAGE CONTRIBUTION TO THE FATTY ACID PROFILE AND ARE THE SUM OF 2 REPLICATES

to the fatty acid profile was C18 ≥ C16 > C14 ≥ C20 in both strains. In M. psychrophilus, the order of decreasing chain length in percentage contribution to the fatty acid profile was C18 > C16 > C14 ≥ C20. In both the mesophilic and psychrophilic strains, the amount of C20 incorporated was approximately half of that incorporated into the thermophilic plasma membrane phospholipids.

A COMPARISON BETWEEN TOTAL AND PLASMA MEMBRANE PHOSPHOLIPID COMPOSITIONS

It is assumed that phospholipids extracted from mycelia are true membrane components (Rose, 1976). Figure 5:6 compares the methyl fatty acid composition of the plasma membrane phospholipids with the total phospholipid fractions at optimum growth temperature. This shows that in each strain there are only minor differences in methyl fatty acid composition between these phospholipid fractions. In addition, Figure 5:7 (data are given in Appendix 5:7) shows that in each strain, the degree of unsaturation of the total phospholipid component is very similar to that of the plasma membrane phospholipid component at optimum growth temperature. Therefore, it may be assumed that the total phospholipid compositions of the test species provide reliable, indirect determinations of the plasma membrane phospholipid compositions. It follows that

FIGURE 5.7

A COMPARISON BETWEEN THE DEGREE OF UNSATURATION OF PLASMA MEMBRANE PHOSPHOLIPIDS WITH TOTAL PHOSPHOLIPIDS AT OPTIMUM GROWTH TEMPERATURE

KEY:

A M. PUSILLUS "ASTON"

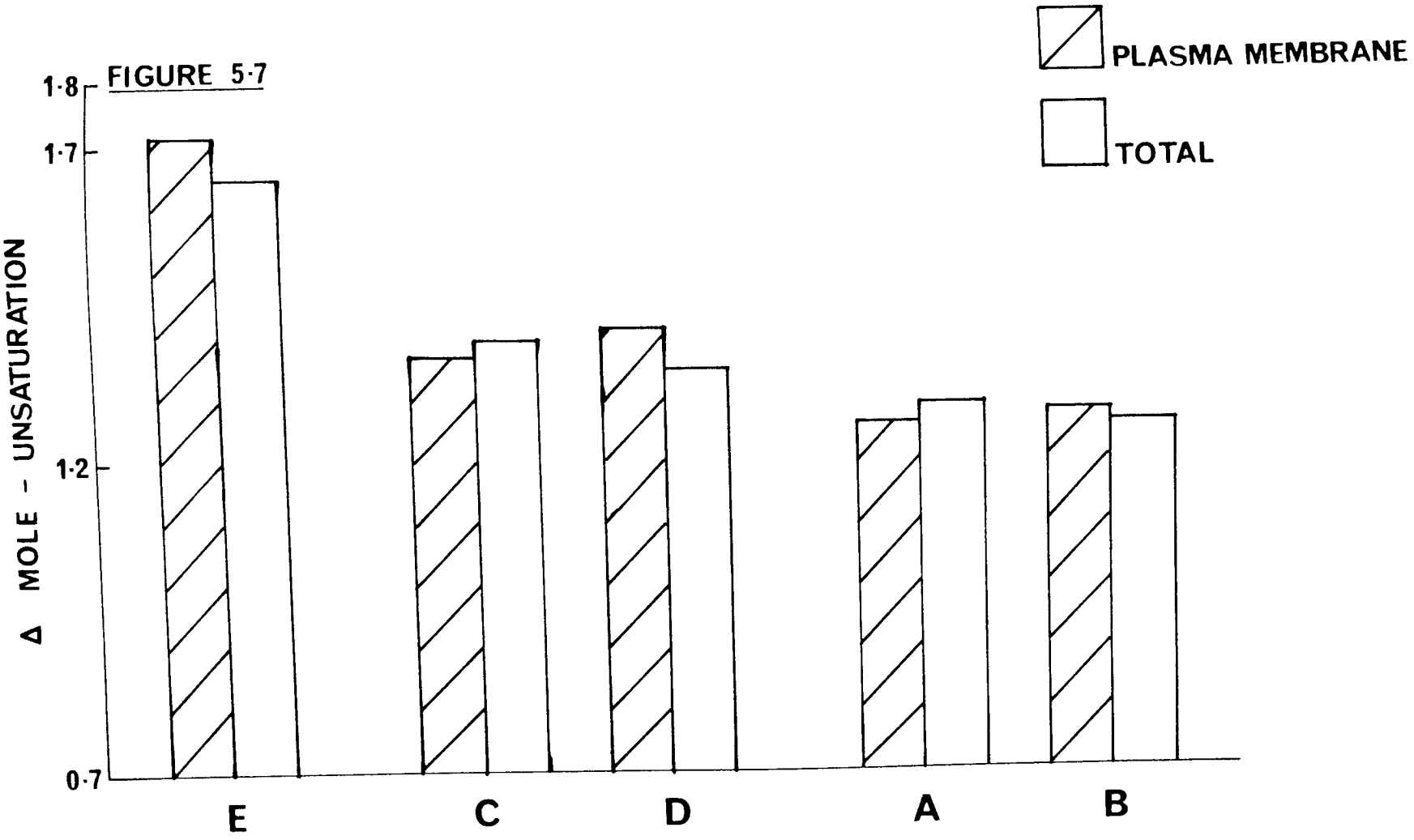
B M. PUSILLUS "LACEY"

C M. HIEMALIS WEHMER

D M. HIEMALIS WEHMER F. HIEMALIS

E M. PSYCHROPHILUS

FIGURE 5·7



any change in total phospholipid composition with temperature will reflect a change in plasma membrane phospholipid composition. In particular, a change in total phospholipid unsaturation with temperature will reflect a change in plasma membrane phospholipid unsaturation and, therefore, a change in plasma membrane fluidity.

THE EFFECT OF GROWTH TEMPERATURE ON PHOSPHOLIPID UNSATURATION

The effect of growth temperature on phospholipid unsaturation is demonstrated by Figure 5:8 (data are given in Appendix 5:8). In general, the phospholipids of each of the test species were more unsaturated than the total lipids. However, the phospholipids of the psychrophilic species were more unsaturated than those of the mesophiles which, in turn, were more unsaturated than those of the thermophilic species. Only the temperate strain of the mesophile, M. hiemalis, and the psychrophile, M. psychrophilus, showed significant changes in phospholipid unsaturation with temperature.

Figure 5:8 shows that the phospholipids of the thermophilic strains of M. pusillus were similarly unsaturated at all growth temperatures, but were significantly less unsaturated than the mesophilic and pscyhrophilic phospholipids. In addition there were no significant changes in phospholipid unsaturation with temperature in either strain.

FIGURE 5.8

THE EFFECT OF GROWTH TEMPERATURE ON THE METHYL FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS IN TERMS OF PHOSPHOLIPID UNSATURATION

KEY:

A M. PUSILLUS "ASTON"

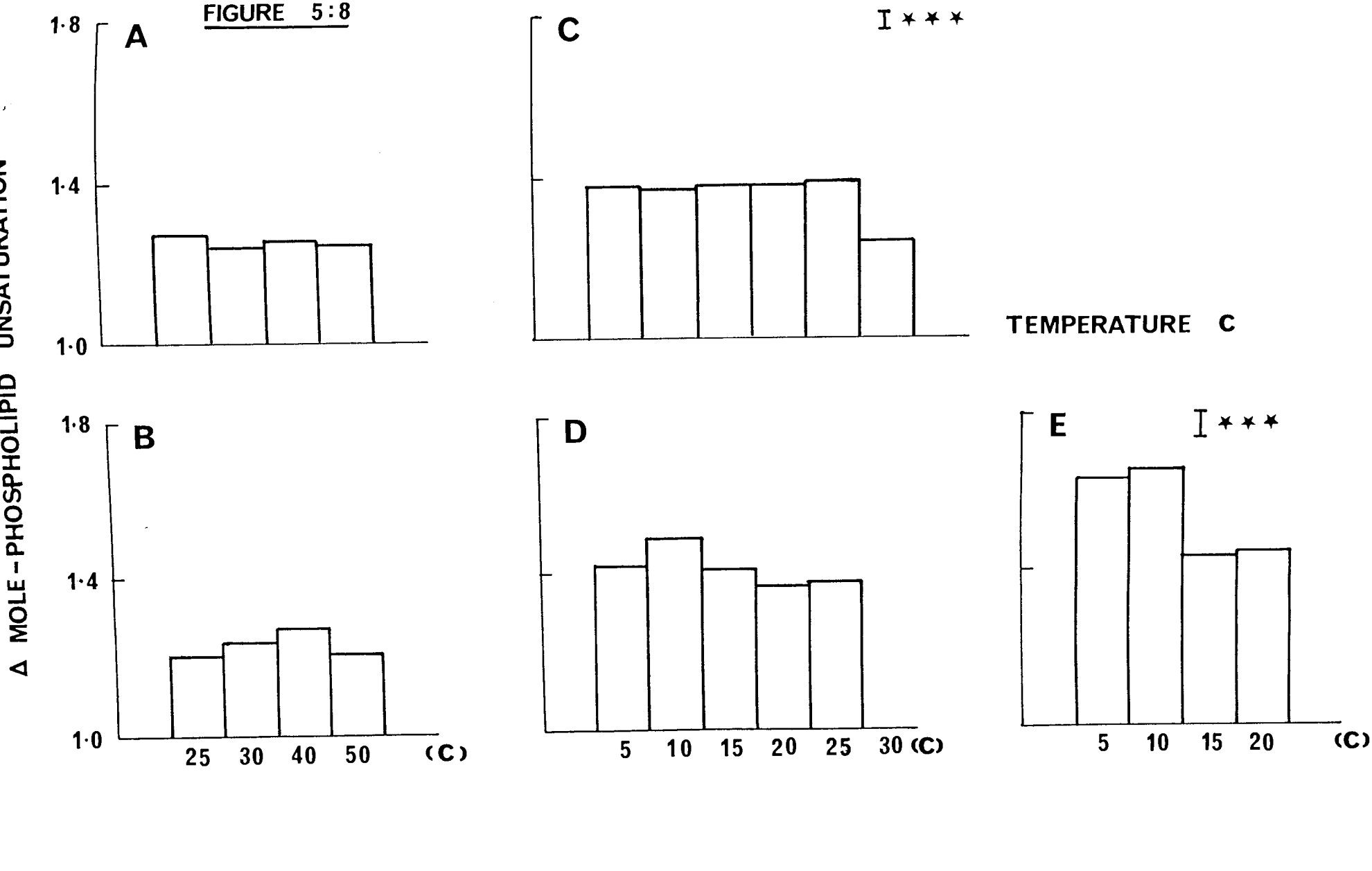
B M. PUSILLUS "LACEY"

C M. HIEMALIS WEHMER

D M. HIEMALIS WEHMER F. HIEMALIS

E M. PSYCHROPHILUS

FIGURE 5:8


A
1·8
1·4
1·0
C
I * * *
TEMPERATURE C
Δ MOLE-PHOSPHOLIPID UNSATURATION
B
1·8
1·4
1·0
25 30 40 50 (C)
D
5 10 15 20 25 30 (C)
E
I * * *
5 10 15 20 (C)

The phospholipids of the mesophilic strains of M. hiemalis were significantly more unsaturated than the thermophilic phospholipids, but were significantly less unsaturated than the psychrophilic phospholipids. From 5-25C, both the temperate and Antarctic strains were similarly unsaturated and there were no significant changes in phospholipid unsaturation with temperature in either strain. However, from 25-30C, the temperate strain showed a significant reduction in phospholipid unsaturation to a level typical of that found in the thermophilic species at 30C.

The phospholipids of the psychrophile, M. psychrophilus, were the most highly unsaturated of all the species investigated. In addition, there was a significant decrease in phospholipid unsaturation with increasing temperature. From 5-10C, the phospholipids were similarly unsaturated. However, from 10-15C there was a significant reduction in phospholipid unsaturation to a level typical of that found in the mesophiles at 15C. This reduced level of phospholipid unsaturation was maintained at the maximum growth temperature of 20C.

DISCUSSION

The fatty acid compositions of fungi have been previously reviewed by Shaw (1966), Wassef (1977) and Weete (1974, 1976, 1980). The total methyl fatty acid compositions of the psychrophilic, mesophilic and thermophilic Mucoraceous fungi investigated are similar to those reported for similar fungi by Sumner *et al.* (1969) and are typical of Phycomycete fungi in general (Weete, 1980). Also, in terms of lipid unsaturation, the results are similar to the findings of Sumner and Morgan (1969) and Sumner *et al.* (1969) who compared the lipid composition of psychrophilic, mesophilic, thermotolerant and thermophilic species of *Mucor* and *Rhizopus*. They reported that the lipids of the psychrophiles and mesophiles were similarly unsaturated but were significantly more unsaturated than the thermophilic lipids. Similar differences in total lipid unsaturation were apparent between species from the same thermal groups in this investigation. The effect of changes in lipid unsaturation on the physical properties of lipids, particularly melting point, were outlined earlier. As a direct consequence of such effects it follows that the order of decreasing unsaturation in lipids of fungi having different growth temperature optima should be psychrophile > mesophile > thermophile. It should also follow that if such organisms are grown at temperatures at either extreme of their optima, then the degree of

lipid unsaturation would adjust accordingly (Weete, 1980). Here, although the thermophilic lipids were less unsaturated (more saturated) than those of the mesophiles and psychrophile, the lipids of the latter two groups were similarly unsaturated. Therefore, it is difficult to explain the differences in growth temperature optima between these thermal groups simply on the basis of differences in the degree of total lipid unsaturation. Indeed, although the thermophilic strains of M. pusillus show a decrease in unsaturation and concomitant increase in optimum growth temperature compared with the mesophilic and psychrophilic species, the latter two groups have similar degrees of lipid unsaturation but nevertheless show significant differences in growth temperature optima.

An increase in fatty acid chain length has the same effect on lipid melting point as a decrease in lipid unsaturation, so the order of decreasing fatty acid chain lengths in these organisms should be thermophile > mesophile > psychrophile. Therefore, it might have been possible to explain the different growth temperature optima of the psychrophilic and mesophilic species if the mesophiles had incorporated longer chain length fatty acids than the psychrophilic species. In fact, the mesophilic and psychrophilic species incorporated fatty acids of similar chain lengths. Moreoever, the fatty acid chain lengths of these organisms were greater than those of the thermophiles.

In general, changing growth temperature did not result in the expected adjustments in the degree of lipid unsaturation in the mesophiles and thermophiles. At temperatures below and above the optimum growth temperature, the thermophilic strains of M. pusillus did not show any significant changes in either total lipid unsaturation or fatty acid chain length. This was also true of the Antarctic strain of the mesophile, M. hiemalis. On the other hand, the temperate strain of this species showed a significant decrease in total lipid unsaturation at supraoptimal growth temperatures. In contrast, the psychrophile, M. psychrophilus showed a significant increase in lipid unsaturation at suboptimal growth temperatures.

Conflicting reports of the effect of growth temperature on lipid unsaturation have been reported in other fungi. Thus, increases in the degree of lipid unsaturation have been found in species of Aspergillus and Rhizopus (Pearson and Raper, 1927; Singh and Walker, 1956) when grown at reduced temperatures, while the lipids of Saccharomyces cerevisiae (Chang and Matson, 1972) and Candida utilis (Meyer and Bloch, 1963) became less unsaturated when grown at elevated temperatures. In contrast, there were no changes in the degree of lipid unsaturation with temperature in Pythium ultimum (Bowman and Mumma, 1967), Cunninghamella blakesleeanus and Rhizopus arrhizus (Shaw, 1966).

All of the above work is based on total lipid extracts only. However, fungal lipids are distributed between two distinct regions of the cell. The major concentration of lipid is found in the cytoplasm, occurring as acylglycerols. The remaining lipids comprise the membrane systems of the cell, occurring as phospholipids. In a recent series of papers Ellis (1981(a); 1981(b); 1981(c); 1981(d); 1981(e); 1982) has shown that there are no structural or developmental patterns in thermophilic fungi that have not been observed in mesophilic species, although there may sometimes be a different mode of cytoplasmic lipid storage in thermophiles grown at higher temperatures compared with that observed in the same fungi grown at lower temperatures (Ellis, 1982). Since cytoplasmic lipids serve mainly as energy storage materials, such compositional changes with temperature are probably of minimal importance with respect to the thermostability of the cell (Crisan, 1973).

In this study, fractionation of total lipid extracts by TLC yielded neutral lipids composed of acylglycerols (triglycerides and diglycerides) and free fatty acids and polar lipids composed of phospholipids, in each of the test species. Triglycerides are often the most abundant fungal lipid, comprising over 90% of the total lipid in some species (Weete, 1980). Although the amounts of triglyceride incorporated into the lipids of the test species were not determined quantitatively,

Figure 5:5 shows that the triglyceride fractions were the predominant lipids in all of the test species. Since triglycerides are often the most abundant lipid, the fatty acids of the total lipid extract often reflect the fatty acid composition of the triglyceride component (Weete, 1980). Indeed, a comparison between the total (Figure 5:2, Appendix 5:3) and triglyceride (Table 5:2) fatty acid compositions shows that the two are very similar. Further, Table 5:3 compares the lipid unsaturation of the triglyceride component with the total lipid extract and demonstrates that these were very similar in each of the test species. Therefore, it is reasonable to assume that the total fatty acid compositions of the test species reflect those of the triglyceride components. It follows that, since triglycerides are cytoplasmic and do not contribute to the thermostability of the cell, it is misleading to attempt to explain changes in total lipid composition with temperature as physiological adaptations to growth at extreme temperatures. In contrast, the phospholipids comprising the membrane systems of the cell are more likely to contribute significantly to thermostability. In this case, a positive correlation between changing phospholipid composition with changing growth temperature in psychrophilic, mesophilic and thermophilic fungi may well reflect a physiological mechanism that enables these organisms to grow optimally at high and low temperatures.

Earlier work established that a breakdown in plasma membrane integrity occurs at supramaximal growth temperatures in the psychrophilic, mesophilic and thermophilic Mucoraceous fungi investigated. This implies that maximum growth temperature may be determined, in part at least, by plasma membrane structure, of which the phospholipids form an integral part.

The lipid composition of the fungal plasma membrane has been reviewed by Rose (1976) and Weete (1980). At present, more data are available on the composition of the yeast plasma membrane than on the plasma membranes of filamentous fungi. In addition, these data are difficult to compare, firstly because of the different methods used to isolate the plasma membranes, secondly because a wide variety of different analytical methods have been used and finally because many workers do not report detailed analyses of the lipid fractions (Rose, 1976). In general, plasma membrane lipids are composed of phospholipids and sterols, although several workers have reported the presence of neutral lipids including triglycerides and sterol esters (Marriott, 1975(a); Nurminen and Suomalainen, 1973). Similarly, the plasma membrane fractions of the Mucoraceous species investigated all contained a triglyceride component in addition to a phospholipid component. The presence of neutral lipids is not easy to explain since they are not amphipathic molecules and cannot therefore be

incorporated into membranes. However, there is evidence that triglycerides may play a role in membrane biogenesis (Rose, 1976; Weete, 1981).

At optimum growth temperatures, the order of decreasing phospholipid unsaturation was psychrophile > mesophile > thermophile in both the total and plasma membrane phospholipids of the test species. Therefore, in contrast to the total lipid unsaturation, there is a positive correlation between the degree of plasma membrane phospholipid unsaturation and optimum growth temperature. In addition, the plasma membrane phospholipids of the thermophilic species incorporated fatty acids of significantly longer chain length than the mesophilic and psychrophilic species. This, in turn, will also tend to increase the melting temperature of the membrane lipids in common with the decrease in phospholipid unsaturation.

As outlined earlier, any change in plasma membrane phospholipid unsaturation with temperature is manifest in a similar change in total phospholipid unsaturation. Since there were few changes in plasma membrane phospholipid unsaturation with temperature in the test species, these observations suggest that the physical state of the plasma membrane phospholipids in psychrophilic, mesophilic and thermophilic fungi may be a factor in determining the different cardinal growth temperatures characteristic of such organisms.

In particular, the differences in phospholipid unsaturation between the thermal groups will be manifest in the thermophilic plasma membrane phospholipids having higher melting points than those of the mesophiles which, in turn, will have higher melting points than those of the psychrophiles. Membrane phospholipids must be at or near to the point of transition between a thermotropic gel phase and a liquid-crystalline phase in order to function correctly (Byrne and Chapman, 1964; Chapman, 1969). It follows that, as the melting temperature of the membrane phospholipids increases, so too does the point at which the gel to liquid-crystalline phase transition occurs.

On this basis, the optimum growth temperatures of thermophiles would be expected to be higher than mesophiles which, in turn, should be higher than psychrophiles and indeed this is the case. Moreoever, the ability of an organism to change membrane phospholipid unsaturation with temperature (thereby maintaining optimum membrane fluidity) may well delineate both the upper and lower temperature limits for growth.

It is quite possible that the minimum growth temperature could be directly determined by the lower boundary of the gel to liquid-crystalline phase transition (McElhaney, 1976). In other words, the fungal plasma membrane may cease to function when all, or a large part of the membrane lipid becomes solid. Indeed, there

is evidence to suggest that the rigid, tightly packed gel state will not support normal membrane function (Luzzati and Husson, 1962; Chapman 1967; Steim et al. 1969; Overath et al. 1970; McElhaney, 1984). The thermophile, M. pusillus, did not show any significant changes in phospholipid unsaturation with temperature. Therefore, as the growth temperature falls, the plasma membrane lipids will become progressively less fluid and will eventually exist entirely in the gel state. This will result in a solid membrane, a situation in which growth is impossible. In addition, earlier work established that at temperatures below the minimum growth temperature, M. pusillus showed little or no metabolite efflux, whereas over its temperature range for growth, this thermophilic species was considerably more "leaky" than the mesophilic and psychrophilic species. This is consistent with the plasma membrane solidifying, thereby preventing growth in this species at even moderate temperatures.

The temperate strain of the mesophile, M. hiemalis is capable of growth at 30C whereas the maximum growth temperature of its Antarctic counterpart is 25C. Therefore, it is interesting to note that from 25-30C the temperate strain showed a significant reduction in phospholipid unsaturation to a level typical of that found in the thermophilic species at 30C. This could be an adaptation allowing growth at

30C in that the resultant higher melting temperature of the phospholipids might allow the formation of a plasma membrane that would have otherwise been too unstable at this temperature. In contrast, since there were no other significant changes in phospholipid unsaturation with temperature, the minimum growth temperature of both strains might also be expected to be determined by the point at which the plasma membrane becomes too rigid to support growth. Moreover, having phospholipids of lower melting points than the thermophiles, this would be expected to occur at a lower temperature and, indeed, this is the case.

The psychrophile, M. psychrophilus, having the highest degree of phospholipid unsaturation of all of the test species, consequently has the lowest optimum growth temperature. This species was also capable of adjusting phospholipid unsaturation (and therefore membrane fluidity) at temperatures both above and below optimum growth temperature. Again, however, the minimum growth temperature is likely to reflect the lower boundary of the liquid crystalline phase transition.

In contrast, the possible effects of the physical state of the plasma membrane lipids on the upper temperature limit for growth are less precise. Indeed, there is no theoretical basis for supposing that the maximum growth temperature would be directly determined

by the upper boundary of the membrane phase transition (McElhaney, 1976). In fact the lipids of many microorganisms exist entirely in the liquid-crystalline state, even at their optimum growth temperatures (Singer and Nicolson, 1972; Bretscher, 1973; Capaldi, 1974). However, the physical state of the membrane lipids may influence the maximum growth temperature in other ways. For example, at temperatures above the phase transition, the thermal motion of the fatty acid chains increases markedly with temperature (Fox and Keith, 1972). McElhaney et al. (1973) have shown that the hydrophobic core of the lipid bilayer appears to be the major cellular permeability barrier and indeed this would explain why the rates at which many ions and non electrolytes passively diffuse across cell membranes have been observed to increase very rapidly with increasing temperature (Stein, 1967; De Gier et al. 1971; McElhaney et al., 1973). It follows that at high temperatures, the plasma membrane may become sufficiently "leaky" so that an adequate intracellular concentration of low molecular weight metabolites can no longer be maintained (McElhaney, 1976).

There is experimental evidence from two sources in this investigation to support the above hypothesis. Firstly, earlier work showed that there was a significant efflux of low molecular weight metabolites at supramaximal temperatures in all of the test species. Secondly, analysis of membrane lipids has shown that the order of

decreasing melting temperature of the membrane phospholipids was thermophile > mesophile > psychrophile. Therefore, it follows that the upper boundary of the membrane phase transition and subsequent breakdown in permeability due to increased thermal motion of membrane fatty acids with temperature (manifest in increased metabolite efflux) would be expected to occur at a higher temperature in thermophiles than in mesophiles, and at a higher temperature in mesophiles than in psychrophiles. Indeed, this was true of the psychrophilic, mesophilic and thermophilic Mucoraceous species investigated.

In addition the physical state of the membrane lipids might also determine the maximum growth temperature in an indirect manner by influencing the conformation of membrane proteins (McElhaney, 1976). Indeed, there is evidence which suggests that many integral membrane proteins require lipids which exist in a particular physical state for correct enzymatic or transport functions (Singer and Nicolson, 1972; Bretscher, 1973; Capaldi, 1974) or for protection from thermal denaturation (Wisdom and Welker, 1973). It follows that membrane lipids which exist in an excessively fluid state may no longer be capable of stabilising certain membrane-bound proteins in a fully functional state. The effects of temperature on plasma membrane-bound proteins in general and plasma membrane ATPase activity in particular are considered later in the investigation.

In summary, there is evidence to suggest that the temperature range for growth of psychrophilic, mesophilic and thermophilic fungi may be determined by the physical state of the plasma membrane lipids. The minimum growth temperature would appear to be delineated by the lower boundary of the gel to liquid-crystalline phase transition of the plasma membrane lipids, below which the membrane exists in a rigid gel state incompatible with growth. In contrast, the maximum growth temperature does not appear to reflect the upper boundary of the gel to liquid-crystalline phase transition. Nevertheless, at temperatures above this phase transition, the thermal motion of the membrane fatty acids may be so great as to cause breakdowns in the membrane permeability barrier, resulting in the type of efflux of low molecular weight metabolites observed earlier at supramaximal growth temperatures in psychrophilic, mesophilic and thermophilic Mucoraceous fungi.

However, the inclusion of sterols (Oldfield and Chapman, 1972; Demel and Dekruyff, 1976) or carotenoids (Huang and Haug, 1974) may also influence membrane fluidity. Therefore, having determined the effect of temperature on fatty acid composition in psychrophilic, mesophilic and thermophilic fungi, an investigation of the sterol and carotenoid compositions of these organisms is necessary.

CHAPTER SIX

THE EFFECT OF GROWTH TEMPERATURE ON THE STEROL AND CAROTENOID COMPOSITION OF VEGETATIVE MYCELIUM

INTRODUCTION

In addition to effects on phospholipid composition, the interaction between growth temperature and fungal plasma membrane function may be further complicated by the presence in the membrane of terpenoid compounds such as sterols or carotenoids. Investigations on both artificial lipid bilayers (Ladbrooke *et al.*, 1968; Oldfield and Chapman, 1972; Demel and Dekruyff, 1976; Elliott, 1976) and the plasma membrane of *Acholeplasma laidlawii* (Demel *et al.*, 1972; Dekruyff *et al.*, 1972, 1973; McElhaney *et al.*, 1973) have shown that the inclusion of a sterol, such as cholesterol, tends to decrease membrane fluidity. At the point of insertion, lateral movement of the fatty acid hydrocarbon chains is restricted, thereby decreasing the fluidity of the lipid bilayer when in the liquid crystalline state. Further, sterols may be ranked in order of effectiveness in reducing membrane permeability. For maximum effectiveness, a sterol must include a planar sterol nucleus, a 3 ß-hydroxygroup and an inert side chain. On this basis, cholesterol is more effective than stigmasterol, with ergosterol being less effective and coprastanol ineffective (Butler, *et al.*, 1970).

Fungal sterol composition has been reviewed by Weete (1980). Ergosterol has been considered to be the principal fungal sterol since first being identified by Tarret in 1889 (quoted in Weete (1980)) in extracts of ergot and later in other fungi. Earlier identifications of ergosterol

and other sterols have been confirmed using the modern techniques of gas chromatography, mass spectroscopy and nuclear magnetic resonance spectroscopy. McCorkindale et al., (1969) demonstrated the presence of cholesterol, ergosterol and 22-dihydroergosterol, in M. hiemalis and M. dispersus. In addition, Gordon et al., (1971) and Safe (1973) found episterol, 22-dihydroergosterol, ergosta-8, 14, 24 (28) trienol, 5-dehydroepisterol, ergosterol and ergosta-5, 7, 9 (11), 22-trienol in M. rouxii, whilst cholesterol, ergosterol and maybe ß-sitosterol and stigmasterol were found in M. genevensis.

Carotenoid pigments have also been shown to decrease membrane fluidity in a similar manner to sterols (Huang and Haug, 1974). Fungal carotenoid compsosition has been reviewed by Goodwin (1976) and Weete (1980). In general, Phycomycete fungi produce only carotenes. Indeed, ß carotene is the only carotenoid present in those Mucoraceous species that have been investigated (Valadon, 1976).

Although, Friend and Goodwin (1954) demonstrated that carotenoid synthesis in Phycomyces blakesleeanus is qualitatively the same in cultures grown from 5-25C, the effect of growth temperature on fungal carotenoid composition has been neglected. Further, a similar situation is apparent with respect to the interaction between growth temperature and sterol composition, indicating that further investigation is necessary.

The aim of this chapter, therefore, is to determine the effect of growth temperature on the sterol and carotenoid composition of psychrophilic, mesophilic and thermophilic fungi. The interaction between growth temperature and sterol and carotenoid composition is considered in relation to possible effects on plasma membrane function in these organisms and, therefore, complements previous work on the interaction between growth temperature and plasma membrane phospholipid composition.

MATERIALS AND METHODS

(1) SPECIES

As Chapter 2.

(2) THE EFFECT OF GROWTH TEMPERATURE ON THE STEROL COMPOSITION OF VEGETATIVE MYCELIUM

Log phase mycelia of each species were produced in 100 ml Erlenmeyer flasks containing 25 ml sterile GAE medium (Appendix 2:1) at growth temperatures over the range 5-50C as described in Chapter 4.

Mycelia were subsequently harvested by suction filtration and washed with 100 ml distilled water. 1 g batches (up to 20 g wet weight) of mycelia were disrupted in 2 ml methanol with a glass homogeniser. The homogenate was then shaken with 2 volumes of chloroform and filtered onto preweighed Whatman No. 1 filter paper. Flasks, filter paper and mycelia were then washed three times in 5 ml chloroform : methanol (2:1 (v/v)) to ensure quantitative transfer of lipid extracts. 0.9% NaCl (w/v) in distilled water was then added to the filtrate (the volume being equivalent to one fifth of the filtrate volume). The extract was shaken vigorously and allowed to stand overnight in the dark at 20C (mycelial extracts were dried overnight at 80C in order to obtain the extracted dry weight as previously described). This produced two phases, the upper of which (water-methanol-salt) was removed and discarded. The remaining chloroform phase

was then washed with 10 ml chloroform-methanol-saline (3:47:48 (v/v/v)) in order to remove traces of the water phase.

The extract was then transferred to a 150 ml evaporating flask and reduced to approximately 1 ml on a rotary evaporator and transferred to a 5 ml pear-shaped flask. The 150 ml flask was then washed in 1 ml ethyl acetate and the washings also added to the 5 ml pear-shaped flask. The lipid extracts were further reduced in volume on a rotary evaporator, transferred to small screw-top vials and reduced to dryness under a stream of nitrogen. Lipid extracts were stored in the dark at -20C.

For each strain, the above extraction was carried out on log phase mycelia grown over the temperature range for growth of that strain. Three replicate samples were silylated in order to identify sterol components and three replicate samples were retained for carotenoid analysis.

(3) SILYLATION OF STEROLS FOR GAS CHROMATOGRAPHIC ANALYSIS

Sterols were silylated using a similar method to that described for carbohydrates by Holligan and Drew (1971).

Thus 0.35 ml anhydrous pyridine, 0.10 ml BSFTA (bis(transmethylsilyl)-trifluoracetamide) and 0.05 ml TMCS (trimethylchlorosilane) were added to each extract in turn and as quickly as possible. The stoppered vials were then shaken briefly and allowed to stand overnight.

(4) GAS CHROMATOGRAPHY OF STEROLS

Sterols were separated on 6 ft. (1.8 m) columns of OV17 (methyl phenyl silicone) on Diatomite CLQ (100-120 mesh) using a Pye series 304 Gas Chromatograph equipped with dual flame ionization detector and computing integrator. Nitrogen (40ml per minute) was employed as carrier gas with a column temperature of 270C. Sterols were tentatively identified by comparison of retention times with those of standard sterols and by consulting tables of relative retention times of standard sterols (Heftmann, 1976).

(5) THE EFFECT OF GROWTH TEMPERATURE ON THE CAROTENOID COMPOSITION OF VEGETATIVE MYCELIUM

Lipid extracts were prepared as described above. For each strain, at each growth temperature, three of the six replicate samples were analysed for carotenoid composition. Carotenoids were determined using ß carotene as an indicator of total carotenoid composition. Thus, 2 ml chloroform was added to each vial and the absorbance read at 461 nm against a chloroform blank on an LKB "Ultrospec" Spectrophotometer. ß carotene content was determined by comparison with known values from a previously constructed standard curve (Appendix 6:1).

RESULTS

(1) THE EFFECT OF GROWTH TEMPERATURE ON THE STEROL COMPOSITION OF VEGETATIVE MYCELIUM

The effect of growth temperature on the sterol composition of the test species is summarised in Table 6:1. In addition, the effect of growth temperature on the ergosterol and ß-sitosterol composition of the test species is demonstrated by Figures 6:1 and 6:2 respectively (data are given in Table 6:1).

Table 6:1 shows that, in general, the sterols cholesterol, ergosterol, ß-sitosterol and stigmasterol were incorporated into each of the test species. In terms of percentage contribution to the total sterol content, ergosterol and ß-sitosterol were the major sterols present in each species, together comprising over 92% of total sterol composition at all growth temperatures. In contrast, cholesterol and stigmasterol may be considered as minor components in terms of percentage contribution to the total sterol content. Indeed, levels of these sterols could only be quantified in the thermophilic species of M. pusillus, with only trace amounts being recorded in the mesophilic and psychrophilic species. In general, there were few significant changes in individual sterol compositions with temperature. Each species is considered in turn below.

TABLE 6:1 THE EFFECT OF GROWTH TEMPERATURE ON THE STEROL CONTENT OF VEGETATIVE MYCELIUM

TEMPERATURE (C)	PERCENTAGE STEROL COMPOSITION CHOLESTEROL	ERGOSTEROL	ß-SITOSTEROL	STIGMASTEROL
M. PUSILLUS "ASTON"				
25	0.830	83.380	13.783	2.030
30	1.203	88.165	10.149	0.607
40	1.639	83.977	6.762	5.603
50	0.868	78.421	16.839	2.535
F	0.425	3.826	4.068*	1.827
LSD	-	-	9.323	-
M. PUSILLUS "LACEY"				
25	2.244	87.008	10.748	+
30	1.792	85.109	13.099	+
40	2.037	83.360	14.613	+
50	2.296	86.812	10.892	+
F	0.171	2.446	3.379	-
LSD	-	-	-	-
M. HIEMALIS WEHMER				
5	+	53.920	46.080	+
10	+	49.103	50.897	+
15	+	35.612	64.388	+
20	+	44.251	55.748	+
25	+	32.218	67.782	+
30	+	39.170	60.830	+
F	-	19.838***	19.834***	-
LSD	-	6.623	6.623	-
M. HIEMALIS W.F. HIEMALIS				
5	0.600	40.064	58.373	0.841
10	+	35.843	64.157	+
15	0.240	30.848	69.062	+
20	+	33.510	66.490	+
25	+	44.808	55.192	+
F	-	18.891***	17.081***	-
LSD	-	4.902	5.369	-
M. PSYCHROPHILUS				
5	+	42.828	57.172	+
10	+	44.493	55.506	+
15	+	43.275	56.725	+
20	+	43.954	56.046	+
F	-	0.008	0.009	-
LSD	-	-	-	-

+ Trace Amount

FIGURE 6.1

THE EFFECT OF GROWTH TEMPERATURE ON ß-SITOSTEROL COMPOSITION

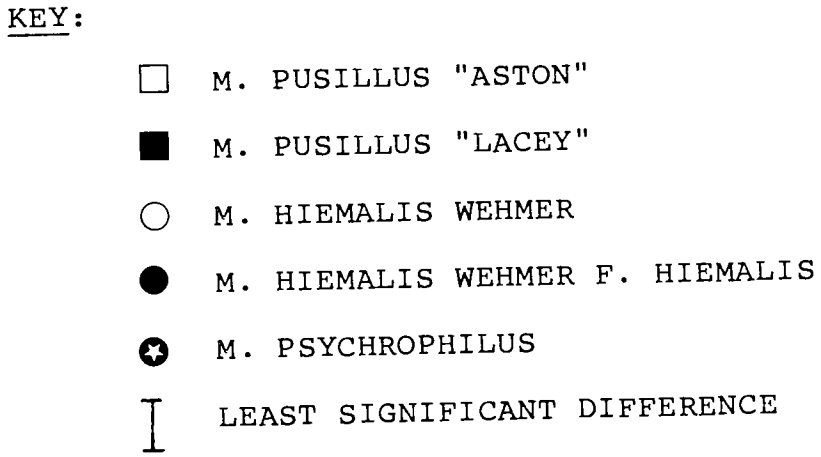


FIGURE 6:1

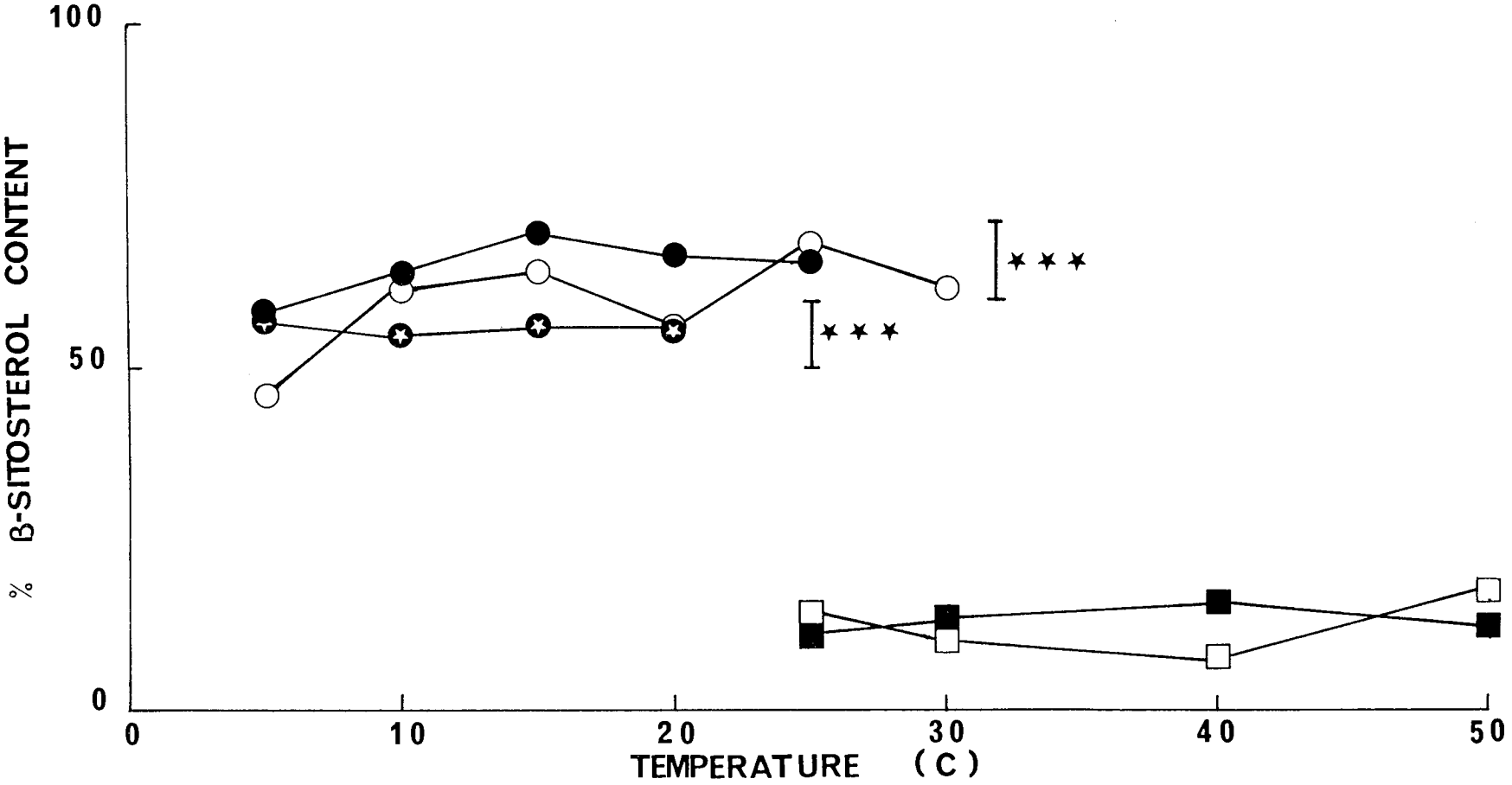


FIGURE 6.2

THE EFFECT OF GROWTH TEMPERATURE ON ERGOSTEROL COMPOSITION

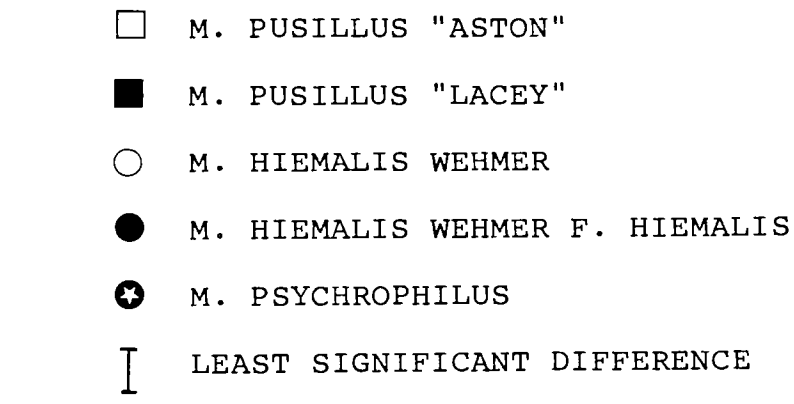
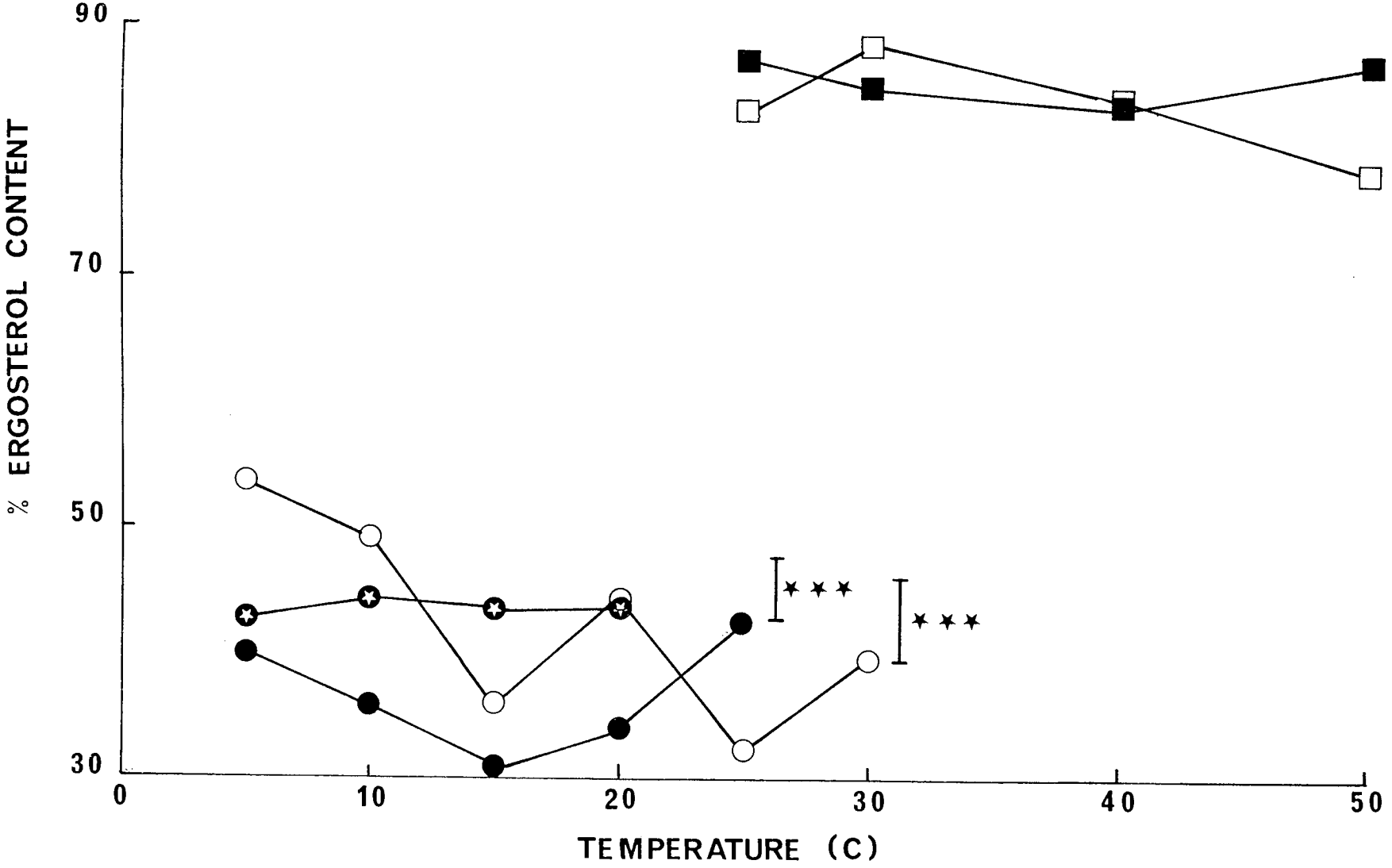


FIGURE 6:2



(I) M. PUSILLUS

Table 6:1 shows that the sterols of both thermophilic strains of M. pusillus comprised ergosterol, ß-sitosterol, cholesterol and stigamasterol. Further, Figure 6:1 shows that both strains incorporated higher levels of ergosterol compared with the mesophilic and psychrophilic species. However, there were no significant changes in ergosterol content with temperature in the thermophiles. In contrast, Figure 6:2 shows that the thermophiles incorporated lower levels of ß-sitosterol compared with the mesophiles and psychrophiles. In contrast to M. pusillus "Lacey" which did not show any signficant changes in ß-sitosterol content with temperature, M. pusillus "Aston" showed a significant decrease in ß-sitosterol content from 40-50C. In addition, there were no significant changes in cholesterol or stigmasterol content with temperature in either thermophilic strain.

(II) M. HIEMALIS

Table 6:1 shows that, in common with the thermophilic and psychrophilic species, both the temperate and Antarctic mesophilic strains of M. hiemalis incorporated ergosterol, ß-sitosterol, cholesterol and stigmasterol at all growth temperatures. Figure 6:1 shows that both mesophilic strains contained similar levels of ergosterol compared with the psychrophilic species, but lower levels of ergosterol compared with the thermophilic species. However, in contrast with the thermophiles, both mesophilic strains showed highly significant changes in ergosterol content with temperature. In particular, the temperate strain of M. hiemalis showed highly significant decreases in ergosterol content from 10-15C and from 20-25C, in contrast to the Antarctic strain, which showed a highly significant increase in ergosterol content from 20-25C. Figure 6:2 shows that both mesophilic strains also incorporated similar levels of ß-sitosterol compared with the psychrophilic species, but higher levels of ß-sitosterol compared with the thermophilic species. Again, there were highly significant, yet contrasting, changes in ß-sitosterol content with temperature in the mesophilic strains. Thus, the temperate strain showed highly significant increases in ß-sitosterol content from 10-15C and from 20-25C in contrast to the Antarctic strain, which showed a significant decrease

in ß-sitosterol content from 20-25C. However, in common with the thermophilic species, there were no significant changes in either cholesterol or stigmasterol content with temperature in both mesophilic strains.

(III) M. PSYCHROPHILUS

In common with the thermophilic and mesophilic species, the sterols of the psychrophile, M. psychrophilus comprised ergosterol, ß-sitosterol, cholesterol and stigmasterol (Table 6:1). Figure 6:1 shows that M. psychrophilus incorporated similar levels of ergosterol compared with the mesophilic species, but lower levels compared with the thermophilic species. In contrast, Figure 6:2 shows that the psychrophilic species contained similar levels of ß-sitosterol compared with thermophiles, but higher levels compared with the thermophiles. However, there were no significant changes in the content of any individual sterol with temperature in M. psychrophilus.

(2) THE EFFECT OF GROWTH TEMPERATURE ON THE CAROTENOID COMPOSITION OF VEGETATIVE MYCELIUM

The effect of growth temperature on the carotenoid composition of the test species in terms of ß carotene content is summarised in Table 6:2.

In general, the mesophilic species incorporated much higher levels of ß carotene than both the thermophilic and psychrophilic species, at all growth temperatures.

TABLE 6:2 THE EFFECT OF GROWTH TEMPERATURE ON THE ß CAROTENE CONTENT OF VEGETATIVE MYCELIUM

SPECIES	TEMPERATURE (C) 5	10	15	20	25	30	40	50	F	L.S.D.
M. PUSILLUS "ASTON"	-	-	-	-	7.97	3.41	3.60	3.65	*** 36.16175828	1.58
M. PUSILLUS "LACEY"	-	-	-	-	7.41	7.16	6.61	3.41	*** 21.91997666	1.26
M. HIEMALIS WEHMER	17.93	8.38	42.88	63.88	33.90	37.85	-	-	*** 68.81971262	9.54
M. HIEMALIS WEHMER F. HIEMALIS	45.04	45.84	39.74	34.86	60.58	-	-	-	** 8.691330394	13.50
M. PSYCHROPHILUS	4.15	6.72	4.11	4.30	-	-	-	-	* 7.567262648	1.99

FIGURES REFER TO THE ß CAROTENE CONTENT IN µg mg-1 DRY WEIGHT

\- NO GROWTH

In addition, there were significant changes in ß carotene content with temperature in all of the test species. However, there were no consistent trends in these changing ß carotene levels either between similar strains of a specific thermal group or between differing thermal groups. Thus, despite having similarly high levels of ß carotene at all growth temperatures, the temperate and Antarctic mesophilic strains of M. hiemalis did not show similar changes in ß carotene content with temperature. This is demonstrated by Table 6:2, which shows that, although the temperate strain showed highly significant increases in ß carotene content from 10-20C, there was a significant decrease in ß carotene content from 20-25C. In contrast the Antarctic strain showed a highly significant increase in ß carotene content from 20-25C. The thermophilic strains of M. pusillus also demonstrated contrasting responses to temperature in terms of ß carotene content. Thus, M. pusillus "Aston" showed a highly significant decrease in ß carotene content from 25-30C, in contrast to M. pusillus "Lacey", which showed a similar reduction, albeit from 40-50C (Table 6:2). In the psychrophile, M. psychrophilus, ß carotene content was maximal at the optimum growth temperature of 10C, falling significantly at both suboptimal and supraoptimal growth temperatures, (Table 6:2).

DISCUSSION

These data clearly demonstrate a significant interaction between sterol and carotenoid composition and growth temperature in psychrophilic, mesophilic and thermophilic fungi. However, since there were no consistent trends in this interaction, either between different thermal groups and even between closely related strains of a specific thermal group, it is difficult to correlate these differences with possible effects on plasma membrane function in these organisms.

One explanation for this might be that total sterol and carotenoid contents do not reflect those of the plasma membrane. Indeed, previous work has established this to be true of total fatty acids and, considering the roles of terpenoid lipids in fungal metabolism, this may also be true of total sterols and carotenoids.

Fungal sterols serve mainly as precursors to hormones (Barksdale, 1969) and are predominantly cytoplasmic in origin. Indeed, bound sterols of *Mucor rouxii* represent only 0.2% of the total sterols (Safe, 1973). Therefore, it is unlikely that the total sterols of the Mucoraceous species investigated will accurately represent plasma membrane sterols. Sterols comprise a small proportion (if any) of the plasma membrane, which itself is a small subset of the fungal cell, consequently very large initial amounts of mycelium

are needed to generate quantifiable levels of sterols in isolated plasma membrane fractions. Unfortunately, the nature of the isolation method used in this investigation is incompatible with high yields of plasma membranes, therefore total sterols only were investigated.

However, Kramer et al. (1978) have shown that the plasma membrane sterols of yeasts such as Saccharomyces cerevisiae are composed entirely of ergosterol. It follows therefore, that any significant changes in the plasma membrane ergosterol content of the test species with temperature might be manifest in similar changes in total ergosterol content.

Sterols tend to reduce plasma membrane fluidity by reducing the lateral motion of phospholipid fatty acid hydrocarbon chains (Ladbrooke et al., 1968; Demel et al., 1972; Oldfield and Chapman, 1972; Dekruyff et al., 1972, 1973; McElhaney, et al., 1973; Demel and Dekruyff, 1976; Elliott, 1976). Therefore in order to maintain optimal membrane fluidity with increasing growth temperature, an organism might be expected to incorporate increasing amounts of ergosterol into the plasma membrane. On this basis, the order of decreasing plasma membrane ergosterol content in fungi demonstrating different growth temperature optima should be thermophile > mesophile > psychrophile. However, in a specific thermal group, the level of plasma membrane ergosterol might also be expected to increase from

minimal to maximal growth temperatures. In this investigation, although the thermophiles incorporated the highest levels of ergosterol at all growth temperatures, the mesophiles and psychrophiles incorporated similar levels. Further, within each thermal group, ergosterol content did not change significantly with temperature in a consistent manner. Thus, both the thermophilic and pscyhrophilic species did not show any significant changes in ergosterol content with temperature. In contrast, although the mesophilic strains of M. hiemalis showed significant changes in ergosterol content with temperature, the temperate strain showed a significant decrease from 20-25C while the Antarctic strain showed a signficant increase from 20-25C. Clearly, further research in this area is necessary. In particular, that fraction of the total sterol composition representative of the plasma membrane fraction must be delineated. Only then will it be possible to accurately determine the interaction between growth temperature and plasma membrane sterol composition in psychrophilic, mesophilic and thermophilic fungi.

Fungal carotenoids are thought to be mainly involved in photochemical reactions. (Goodwin, 1976; Weete, 1980). In addition, although carotenoids have been shown to decrease membrane fluidity in a similar manner to sterols (Huang and Haug, 1974), they are unlikely to be incorporated into the plasma membrane should sterols be available. This is because exogenous

sterols will replace carotenoids in membranes (Smith, 1963). Therefore, although there was a significant interaction between carotenoid content (in terms of ß carotene content) and growth temperature in the psychrophilic, mesophilic and thermophilic fungi investigated, these changes will probably reflect changes in cytoplasmic carotenoid content, which are unlikely to contribute to the thermostability of such organisms.

In summary, the contribution of terpenoids to the thermostability of the plasma membrane in psychrophilic, mesophilic and thermophilic fungi remains unclear. Although there was a signficant interaction between both sterol and carotenoid content and growth temperature, only the sterols are likely to be true plasma membrane components and, therefore, contribute to the thermal stability of the cell. Of the sterols investigated, ergosterol is the most likely to be a true membrane component. However, there were no consistent trends in the changes observed in ergosterol content with temperature. However, the data refer to total ergosterol content only and there is evidence to suggest that this may not be representative of plasma membrane ergosterol content. Clearly, further investigations into the interaction between plasma membrane sterol content and growth temperature in psychrophilic, mesophilic and thermophilic fungi are needed in the future.

CHAPTER SEVEN

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON PLASMA MEMBRANE PROTEINS

INTRODUCTION

Earlier work has established the interaction between temperature and plasma membrane lipid composition in psychrophilic, mesophilic and thermophilic fungi. An investigation of the plasma membrane protein fraction of these organisms is therefore also warranted and is potentially of some consequence. As outlined earlier (Chapter 5), the study of the fungal plasmalemma is a relatively new area with many contemporary investigations concentrating on the membrane lipid composition rather than the protein component. Nevertheless, attempts to delineate the protein composition of fungal plasma membranes have been initiated and both protein and glycoprotein components have been reported (Santos et al., 1978; Bussey et al., 1979; Schneider et al., 1979). However, numbers of proteins reported are dependent on the resolution of the particular electrophoretic technique employed. Thus, whereas one-dimensional gels yield 17 to 25 protein bands, two dimensional gels yield up to 200 peptide spots.

The enzymatic profile of the plasma membrane is consistent with its role as the mediator of cell surface phenomena. Enzymes present include Mg^{++} dependent ATPases, mannan and chitin synthetases, invertase and other hydrolytic enzymes (Marriott, 1975(b), Nurminen et al., 1970; Santos, et al., 1978).

There have been many comparative investigations of the structure and thermostability of cytoplasmic proteins and extracellular enzymes between thermophilic and mesophilic microorganisms (Zuber, 1976; Amelunxen and Murdock, 1978). However, these relate mainly to prokaryotes and the thermostability of enzymes and other proteins has not been extensively studied in filamentous fungi. On the basis of electrophoretic analysis of cellular extracts of Penicillium dupontii and Humicola lanuginosa, Crisan (1969) indicated that the production of thermostable enzymes in thermophilic fungi may be an exception rather than the rule. Loginova and Tashpulatov (1967) compared the cellulotyic enzymes produced by mesophilic and thermotolerant strains of Aspergillus fumigatus. Although the thermotolerant strain showed optimum activity at 60C compared with 55C in the mesophilic strain, the optimum activity of the mesophilic enzyme occurred at temperatures 25C in excess of the growth temperature optimum. Similarly, in a comparison of the ribonucleases of eight mesophilic and eight thermophilic fungi, Craveri and Colla (1966) demonstrated that the average temperature for optimum enzyme activity was 55C in the mesophiles and 60.5C in the thermophiles. Thus in both these examples, enzymes derived from mesophilic organisms do not demonstrate marked thermal instability compared to those from thermophiles, therefore, such

enzymes are unlikely to account for the characteristic differences in temperature optima or regimes manifest between these two groups.

Although the thermostability of cytoplasmic enzymes and proteins has been more extensively documented in prokaryotic microorganisms than in eukaryotic filamentous fungi, there remains a paucity of data relating to the effect of temperature on membrane proteins in both groups. In the mycoplasma, Acholeplasma laidlawii, a reduction in growth temperature from 37 to 25C has been reported to result in the disappearance of two membrane proteins as determined by electrophoretic patterns (Amar, et al, 1979). In addition, from preliminary experiments on protoplasts of Bacillus stearothermophilus, Welker (1976) has suggested that growth temperature may modify the activity and or biosynthesis of enzymes concerned with maintaining the structure and function of the plasma membrane, thereby enhancing the thermal stability of membrane transport processes.

Therefore, in this part of the investigation the thermostability of plasma membrane proteins from psychrophilic, mesophilic and thermophilic fungi was compared. In the first stage of this investigation, the thermostability of detergent-solubilised plasma membrane proteins from these organisms was compared using

isoelectric focusing profiles. Next, the thermostability of a specific plasma membrane-bound enzyme was investigated, that of adenosine triphosphatase (ATPase). This enzyme was considered to be a particularly apt choice. ATP is the primary cellular energy carrier. According to the chemiosmotic theory of Mitchell (1963, 1967), energy released by oxidation of suitable organic substrates can be transduced into an electrochemical potential difference of protons across the plasma membrane which, in turn, can be used to drive secondary active transport processes moving solutes into the cell via the so-called H^+ symport (Harold, 1977; Eddy, 1982). In filamentous fungi, Jennings (1976) has shown that both transport extrusion and the coupled influx of potassium is brought about by the free energy of hydrolysis of ATP, which implicates the involvement of ATPase as the pump molecule. Indeed, such ion-translocating plasma membrane-bound ATPases have been isolated from both filamentous fungi (Goffeau and Slayman, 1981) and yeasts (Aldermann and Höfer, 1984). It follows that significant differences in the degree of denaturation of plasma membrane ATPase with increasing growth temperature, with the concomitant reduction in the capacity to uptake solutes via active transport processes, may be a determinant of the differing cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi.

MATERIALS AND METHODS

(1) SPECIES

As Chapter 2.

(2) THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE THERMOSTABILITY OF PLASMA MEMBRANE PROTEINS

(i) PLASMA MEMBRANE ISOLATION AND PROTEIN SOLUBILISATION

Plasma membranes were isolated from log phase mycelia of each species grown at optimum temperature by the method of Galpin and Jennings (1980), as described in Chapter 5.

In order to solubilise plasma membrane proteins, the final 100,000 xg pellet was resuspended in 6 ml extraction medium containing 1% Triton X-100 (v/v). Plasma membrane/detergent solutions were then shaken for 3 hours at 5C. The solubilised proteins were separated from insoluble debris by centrifugation at 100,000 xg for 60 minutes. Subsequently, 2 ml of supernatant was heated at 60C for one hour, a further 2 ml was heated at 70C for one hour, the remaining 2 ml was retained as a control. Membrane proteins were then separated by isoelectric focusing as described below. The above procedure was carried out in duplicate.

(ii) ISOELECTRIC FOCUSING OF PLASMA MEMBRANE PROTEINS

Following heat treatment, membrane protein samples were concentrated using Minicon-B (Amicon) clinical sample concentrators and separated by isoelectric focusing in 0.5 mm polyacrylamide (T = 5%, C = 3%) ampholine plates, pH range 3.5 - 9.5 (LKB, PAG 1818-101). Electrode solutions and running conditions are given in Appendix 7:1. The pH gradient across the gel was measured using isoelectric point markers (BDH pI calibration kit, pH range 5.65 - 8.30). Protein bands were visualised by silver staining (Merril et al., 1981; Sammons et al., 1981). The silver staining protocol is given in Appendix 7:1).

(iii) THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITY

Plasma membranes were prepared from log phase mycelia of each species grown at optimum temperature by the method of Galpin and Jennings (1980), as described in Chapter 5.

The final 100,000 xg pellet was resuspended in 6 ml resuspension medium (extraction medium made 60% (v/v) with glycerol) and stored at -20C prior to the assessment of ATPase activity (the addition of glycerol prevents freezing and minimises loss of ATPase activity) as described below.

ATPase activity of the plasma membrane samples was determined by measuring the release of phosphate from adenosine triphosphate (ATP) in the presence of membrane samples. Reaction medium composed of 1.25 ml 80 mM tris/acetate buffer, pH 9.0, 0.50 ml 2 mM ATP, 0.05 ml 2mM $MgCl_2$ and 0.25 ml 50 mM KCl was aliquoted into 10 ml conical flasks. 0.2 ml of plasma membrane sample was added to the reaction medium and the final volume made up to 3 ml with distilled water. Flasks were incubated for 60 minutes over a range of temperatures between 10-70C. The reaction was halted by the addition of ice-cold 25% (w/v) trichloroacetic acid. The mixture was subsequently centrifuged at 2000 xg for 10 minutes to remove any protein precipitate and the phosphate concentration of the supernatant was determined as described in Chapter 4.

The above procedure was carried out in duplicate and the results analysed using two way analysis of variance.

RESULTS

(1) THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE THERMOSTABILITY OF PLASMA MEMBRANE PROTEINS

Isoelectric focusing of Triton-solubilised plasma membrane proteins resulted in the separation of some 49-52 protein bands with pI values ranging from 3.5 - 9.5 when visualised by silver staining (Figure 7:1). Major bands were apparent at pH 5.0 and also at pH 8.0 and pH 8.5. There were only minor variations in banding patterns both between closely related strains of the same species and also between different species from different thermal groups, at optimum growth temperatures. Further, there were no apparent changes in banding patterns following heat treatment at 60C and 70C. Protein banding patterns, typical of thermophilic, mesophilic and psychrophilic species at optimum growth temperature are shown diagrammatically in Figure 7:1.

(2) THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITY

The effect of environmental temperature on the activity of plasma membrane adenosine triphosphatase (ATPase) activity as determined by the concentration of inorganic phosphate liberated from ATP is shown in the form of an Arrhenius plot in Figure 7:2. This shows that all of the species investigated demonstrated ATPase activity,

FIGURE 7.1

ISOELECTRIC FOCUSING OF PLASMA MEMBRANE PROTEINS :

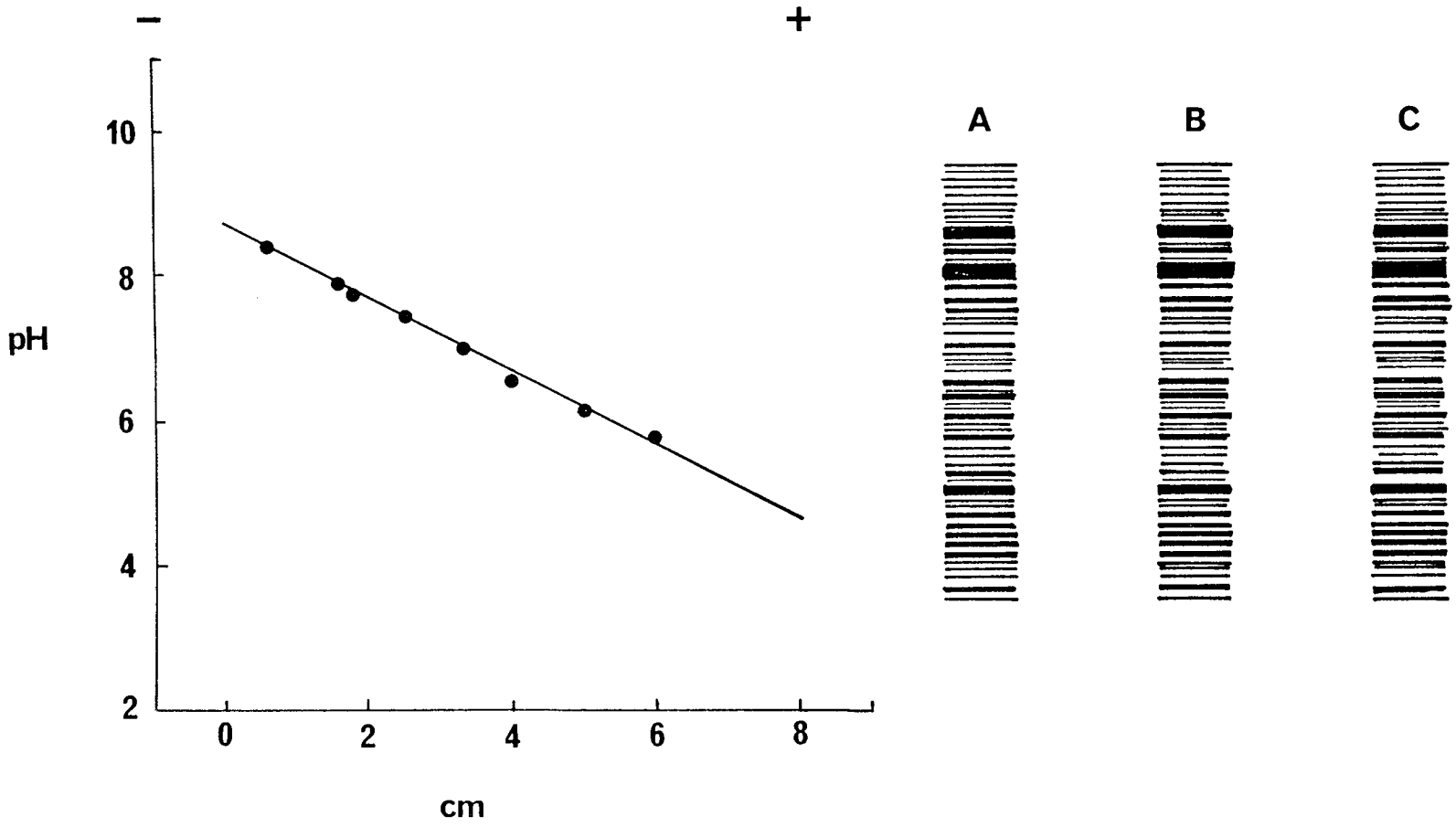
A DIAGRAMMATIC REPRESENTATION OF A TYPICAL SEPARATION

AT OPTIMUM GROWTH TEMPERATURE

KEY:

A	M. PUSILLUS "ASTON"
B	M. HIEMALIS WEHMER
C	M. PSYCHROPHILUS

FIGURE 7:1



even at temperatures in excess of maximum growth temperatures. In general, the mesophilic species showed significantly higher ATPase activities compared with the thermophilic species which, in turn, showed signifcantly higher ATPase activities compared with the psychrophilic species, at most of the temperatures investigated. Further, only the thermophilic species showed optimum ATPase activity at optimum growth temperature. In contrast, the mesophilic and psychrophilic species showed optimum ATPase activities at temperatures in excess of their respective maximum growth temperatures. Each species is considered in turn below.

Figure 7:2 shows that the ATPase activity of the thermophilic strains of M. pusillus increased from a minimum at 20C to a maximum at 40C, the optimum growth temperature. From 40-70C there was a linear decrease in ATPase activity in both strains. ATPase activity in the thermophilic species was significantly less than that observed in the mesophilic species, but was significantly higher than in the psychrophilic species at most of the temperatures investigated.

The mesophilic strains of M. hiemalis showed the highest ATPase activities of all the thermal groups, at most of the temperatures investigated (Figure 7:2). Both the temperate and Antarctic strains showed optimum ATPase activity at 50C, 20C and 25C above their respective maximum growth temperatures. From 50-70C there was a

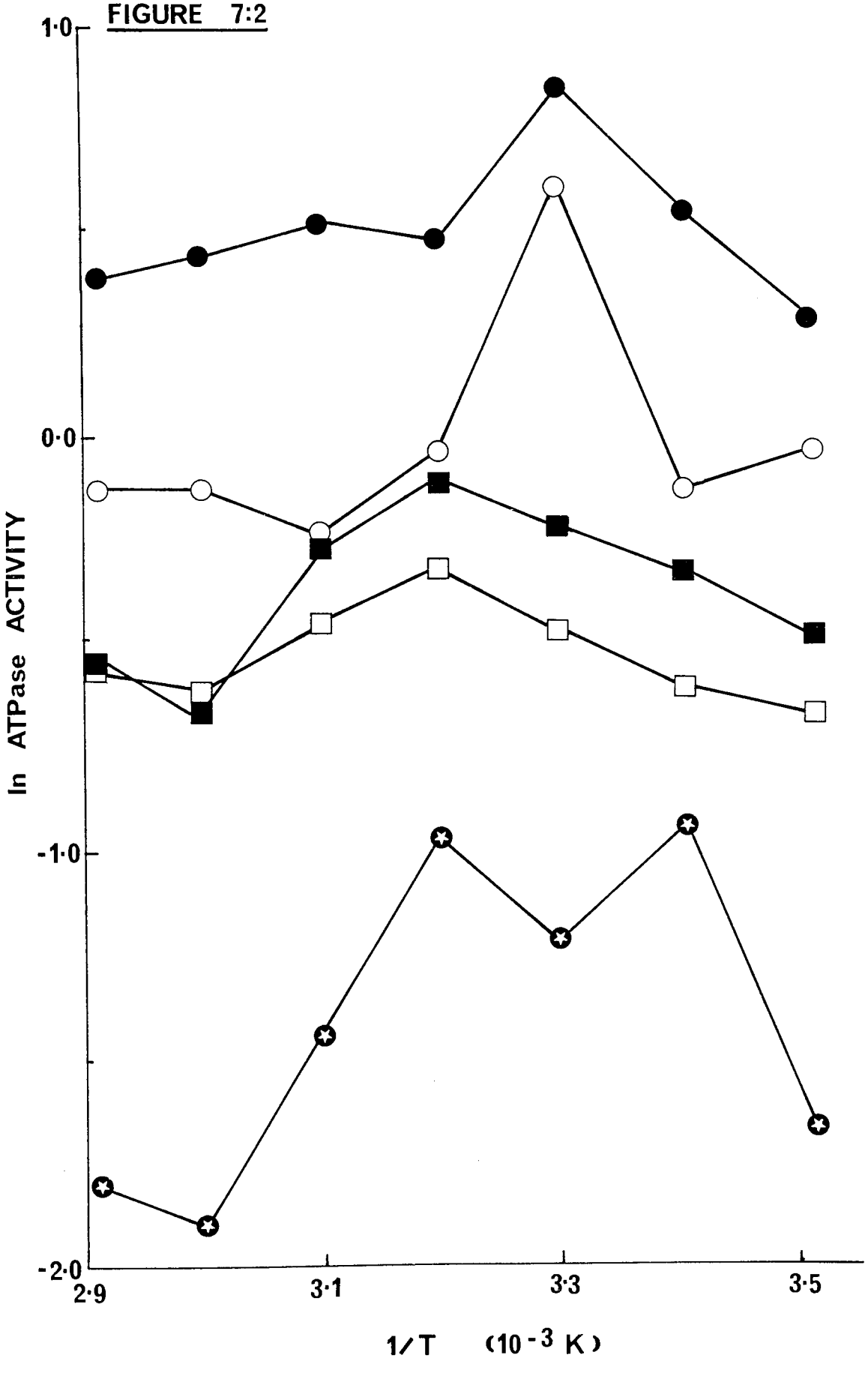
FIGURE 7.2

ARRHENIUS PLOT FOR PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITY

KEY:

- □ M. PUSILLUS "ASTON"
- ■ M. PUSILLUS "LACEY"
- ○ M. HIEMALIS WEHMER
- ● M. HIEMALIS WEHMER F. HIEMALIS
- ✪ M. PSYCHROPHILUS

FIGURE 7:2



loss in ATPase activity in both strains. Figure 7:2 also shows that over the temperature ranges for growth of the mesophilic strains, ATPase activity was significantly less than at 50C.

The psychrophile, M. psychrophilus, showed the lowest ATPase activity of all the thermal groups, at all the temperatures investigated. Figure 7:2 shows that ATPase activity was lowest from 10-20C, the temperature range for growth. From 20-40C, however, there was a significant increase in ATPase activity, with optimum ATPase activity from 40-60C. From 60-70C, there was a loss in ATPase activity.

DISCUSSION

These data indicate that thermal denaturation of the protein component of the plasma membrane is not a limiting factor of fungal growth at high temperatures.

In all of the species investigated, solubilisation of plasma membrane proteins with Triton X-100 followed by isoelectric focusing yielded 50 plus protein bands at optimum growth temperatures. None of the bands observed at optimum growth temperatures disappeared following heat treatment at 60C and 70C, demonstrating the thermostable nature of membrane proteins at these temperatures. Since these membrane proteins were stable at temperatures in excess of the respective growth temperature maxima, it is unlikely that thermolabile lesions in the protein component of the plasma membrane delineate the cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi. It may be argued that the behaviour of detergent-solubilised material is not truly representative of behaviour *in vivo*, particularly *in situ* within the plasma membrane. Indeed, complex membrane proteins may dissociate into their component subunits during solubilisation and electrophoresis. Thus, the ATPase of *Acholeplasma laidlawii* has been shown to disocciate into at least 5 subunits in the presence of detergents (Lewis and McElhaney, 1983). Even if it is assumed that a single band is representative of a single protein, the presence

of that band in a gel following heat-treatment gives no guarantee that the three dimensional conformation of a protein molecule is preserved at that particular temperature. Ljungdahl and Sherod (1976) demonstrated that there is little or no difference in amino acid composition between thermostable thermophilic cytoplasmic proteins and their potentially more thermolabile counterparts derived from mesophilic organisms. The common nature of the amino acid residues may, in turn, limit any differences in protein conformation between the two groups, thereby accounting for the marginal differences in thermostability that have been observed. Of greater consequence is the close association which occurs between certain enzymes and membrane phospholipids whose concentration or nature can alter enzyme activity. Indeed, this has been shown to be the case for plasma membrane ATPase (Metcalfe et al., 1976), therefore the isoelectric focusing data is not in itself sufficient proof of the thermostable nature of fungal membrane proteins.

The plasma membrane-bound ATPase fraction from all of the species investigated demonstrated a thermostable nature. Thus, in each species, ATPase activity was measured at temperatures in excess of the respective growth temperature maxima. This implies that denaturation of the enzyme portion of the plasma membrane is not a limiting factor of fungal growth at high temperatures.

Despite the fact that there was only slight correlation between optimum growth temperature and optimum ATPase activity, it is interesting to note certain similarities between the Arrhenius plot for growth (Figure 2:2) and the Arrhenius plot for ATPase activity (Figure 7:2). In particular, the trend in specific growth rates was mesophile > thermophile > psychrophile in order of decreasing specific growth rates. Likewise, the trend in ATPase activities was also mesophile > thermophile > psychrophile in order of decreasing ATPase activities. In general, there was also a linear increase in both specific growth rates and ATPase activities to maxima, followed by deviations from linearity manifest in reductions in specific growth rates and ATPase activities respectively, at high temperatures.

The efficiency of plasma membrane processes and in particular, the active uptake across the membrane of solutes essential for growth processes at a given temperature, will inevitably affect the specific growth rate at that temperature. Since the active uptake of solutes is driven by the H^+ symport (Harold, 1977; Eddy, 1982), itself driven by energy derived from the splitting of ATP by ATPase (Jennings, 1976), it follows that ATPase activity may be a factor in determining the specific growth rate at a given temperature.

The role of plasma membrane phospholipids in determining both the cardinal growth temperatures and the specific growth rates over the temperature ranges for growth characteristic of psychrophilic, mesophilic and thermophilic fungi has been considered in detail (Chapter 5). In addition, plasma membrane phospholipids can also alter the conformation and, therefore the activity of membrane-bound ATPase (Metcalfe et al., 1976). In consequence, at a given growth temperature, the interaction between the physical state of the plasma membrane phospholipid, as governed by fatty acid composition, and ATPase activity, might be a significant factor in determining the specific growth rate at that temperature. The relationships between plasma membrane phospholipid composition, ATPase activity and the specific growth rates and cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi are considered in the General Discussion.

In summary, in contrast to plasma membrane phospholipid composition, thermal denaturation of the membrane protein component is unlikely to be a limiting factor of fungal growth at high temperatures. However, the activity of plasma membrane-bound ATPase may be a significant factor in determining the specific growth rates characteristic of psychrophilic, mesophilic and thermophilic fungi at a given temperature.

CHAPTER EIGHT

GENERAL DISCUSSION

The aim of the present investigation was to characterise the interaction between temperature and growth in psychrophilic, mesophilic and thermophilic fungi in order to gain further insight into the physiological mechanisms underlying fungal growth at extreme temperatures.

The experimental evidence outlined in earlier chapters strongly suggests that differences in plasma membrane structure and function delineate the upper and lower growth temperature limits characteristic of these thermal groups. In those Mucoraceous species investigated, these differences in plasma membrane structure and function appear to be the consequence of a disparity in the lipid composition of the membrane rather than in the protein component, which appears both uniform in character and stability. However, the nature of the interaction between the more uniform membrane protein component and the relatively unique lipid component may be significant in establishing the dissimilar specific growth rates apparent between the thermal groups.

The unique nature of the plasma membrane lipids of each thermal group is the result of modifications in the fatty acid composition of the membrane phospholipid molecules. The importance of phospholipids to plasma membrane structure is emphasised by the fluid mosaic

model of Singer and Nicolson (1972) which views the plasma membrane as a dynamic structure in which the membrane proteins "float" in a fluid phospholipid bilayer. Phospholipid molecules exhibit multiphasic properties and the composition of the hydrophobic chains in particular, determines the level of membrane fluidity. In order to function correctly, membrane phospholipids must be at or near to the point of transition between a thermotropic gel phase and a liquid-crystalline phase (Byrne and Chapman, 1964; Chapman, 1969). Phospholipid phase transition is a function of the melting temperature of the lipid moiety, which is determined by the molecular characteristics of the hydrocarbon chains of its component fatty acids. The hydrocarbon fatty acid tails may either be saturated, with a hydrogen atom linked to every carbon bond, or unsaturated with free carbons. The degree of unsaturation is an important determinant of the melting point of the phospholipid and also, therefore, the temperature at which the gel to liquid-crystalline phase transition occurs. In general, as growth temperatures are increased, phospholipids tend to incorporate fatty acids that are more saturated and of greater chain lengths. Consequently, as growth temperature increases, so too does the point at which the gel to liquid-crystalline phase transition occurs. It follows that the temperature range for fungal growth may reflect the temperature range over which a species can maintain a membrane that is fluid and flexible enough to promote membrane-associated growth processes.

In the present investigation, there was a positive correlation between decreasing plasma membrane phospholipid unsaturation and increasing optimum growth temperature in the psychrophilic, mesophilic and thermophilic Mucoraceous species investigated. Thus, at optimum growth temperature, the order of decreasing plasma membrane phospholipid unsaturation was psychrophile > mesophile > thermophile. In addition, the plasma membrane phospholipids of the thermophilic species incorporated fatty acids of significantly longer chain lengths than the mesophilic and psychrophilic species. This, in turn, will also tend to increase the melting temperature of thermophilic plasma membrane phospholipids.

Clearly, if an organism was able to demonstrate the ability to adjust the degree of phospholipid unsaturation accordingly, in response to temperature, then this would tend to offset deleterious changes in membrane fluidity, thereby extending the temperature range for growth. However, the psychrophilic, mesophilic and thermophilic Mucoraceous species investigated demonstrated very few such changes in phospholipid unsaturation with changing temperature. Together, these observations support the view that the cardinal growth temperatures characteristic of psychrophilic, mesophilic and thermophilic fungi do indeed reflect the temperature ranges over which these thermal groups

exhibit levels of plasma membrane fluidity and flexibility sufficient to maintain vital membrane-associated growth processes. Therefore, it is pertinent to consider how compositional changes in plasma membrane phospholipids (and, therefore, membrane fluidity) may set the differing minimum and maximum growth temperatures characteristic of these thermal groups.

The minimum growth temperature may be determined by the lower boundary of the gel to liquid-crystalline phase transition of the plasma membrane phospholipids, below which the membrane exists in a rigid gel form, incompatible with growth. This may explain the inability of thermophiles to grow at the moderate temperatures at which mesophiles thrive. In the present investigation, the plasma membrane lipids of the thermophile, M. pusillus, were the most saturated of all the thermal groups. Therefore, at moderate temperatures, the presence of fatty acids with higher melting points will result in a solid membrane, characteristic of the rigid, tightly-packed gel state, thereby preventing growth. The metabolite efflux data for M. pusillus are consistent with this hypothesis in that at temperatures suitable for growth, this species was considerably more "leaky" than the mesophilic and psychrophilic species, whereas at temperatures below the minimum growth temperature of 20C, there was little or no efflux. Clearly, below the

minimum growth temperature the plasma membrane of *M. pusillus* demonstrates a considerable reduction in permeability consistent with a rigid membrane in which the phospholipids exist predominantly in the gel state.

As growth temperature is raised above this minimum, membrane fluidity and flexibility are restored, together with membrane-associated growth processes, thereby resuming metabolic activities. In each thermal group, this is characterised by increasing specific growth rates with increasing growth temperatures, culiminating in maximum specific growth rates at the optimum temperatures for growth. In the context of the hypothesis of ultrastructural thermostability, the optimum growth temperature may be defined as that temperature which is optimal in achieving an integration of all essential metabolic activities (Crisan, 1973). Although, some individual processes may be capable of more efficient operation at higher or lower growth temperatures, the overall physiology of the fungal cell is controlled by the most rate-limiting reaction. Plasma membrane thermostability is clearly a limiting factor of fungal growth at extreme temperatures.

As the maximum growth temperature is approached, excessive membrane fluidity and flexibility affect the physical integrity of the membrane, thereby disrupting permeability barriers and membrane-bound enzymatic

activity. This is characterised by increasing metabolic efflux and concomitant reductions in specific growth rate. Although the maximum growth temperature is unlikely to correspond with the upper boundary of the membrane phase transition, irreversible changes in the physical state of the membrane lipids may influence the maximum growth temperature in other ways. For example, the thermal motion of the fatty acid chains increases markedly with temperature, and since the hydrophobic core of the lipid bilayer appears to be the major cellular permeability barrier (McElhaney et al., 1973), the rates at which many low molecular weight metabolites passively diffuse across the cell membrane would be expected to increase rapidly with increasing temperature. Thus, when maximum growth temperatures are exceeded, disorganisation of the plasma membrane may reach an irreparable level and the membrane may become so "leaky" that an adequate intracellular concentration of low molecular weight metabolites can no longer be sustained. Indeed, in the present investigation, all of the thermal groups demonstrated excessive efflux of such metabolites at temperatures exceeding their respective growth temperature maxima. This increased permeability may also allow extracellular toxins access to sensitive metabolic sites within the fungal cell, and so eventually death is inevitable.

The interaction between growth temperature and fungal plasma membrane function may be further complicated by the presence in the membrane of terpenoid compounds such as sterols or carotenoids, which may offset temperature-induced changes in membrane fluidity. In the Mucoraceous species investigated only the sterols, and ergosterol in particular, are likely to be true plasma membrane components and, therefore, contribute to the thermostability of the cell. However, there were no consistent trends in the changes observed in ergosterol content with temperature. This may be because the data refer to total ergosterol levels only and there is evidence to suggest that these may not be representative of plasma membrane levels. In summary, the contribution of terpenoids to the thermostability of the fungal cell remains unclear. Further investigations are clearly needed in the future.

The physical state of the membrane lipids may also influence upper and lower growth temperature limits by influencing membrane proteins. In particular, there is evidence to suggest that many integral membrane proteins require lipids which exist in a particular physical state for correct enzymatic or transport functions (Singer and Nicolson, 1972; Bretscher, 1973; Capaldi, 1974) or for protection from thermal denaturation (Wisdom and Welker, 1973). Here, in all of the species

investigated, plasma membrane ATPase activity increased from minimum to optimum growth tempera
optimum to maximum growth temperature
the trend in specific growth rates ov
temperature range. The active uptake
the fungal cell is driven by the so-c
(Harold, 1977; Eddy, 1982), itself dr
derived from the splitting of ATP by
ATPase (Jennings, 1976). In the mycc
laidlawii, plasma membrane ATPase act
provided that the membrane lipids ren
crystalline phase (McElhaney, 1984).
possible that these observed increases in specific growth rates with temperature may be due to increased ATPase activity as the membrane becomes increasingly fluid. However, all of the species investigated demonstrated ATPase activity at temperatures in excess of their respective growth temperature maxima. Therefore, differences in the thermostability of membrane-bound enzymes are unlikely to limit fungal growth at high temperatures.

In the psychrophilic, mesophilic and thermophilic species investigated, the cardinal temperatures for growth and sporangiospore germination were identical for each species. This implies that the physical nature of

the sporangiospore plasma membrane phospholipids of these organisms resembles that of the equivalent mycelial structures. Unlike the thermophilic strains in which the temperature optima for growth and sporangiospore germination were identical, the mesophilic strains of M. hiemalis showed a reduction in both the optimum temperature for growth and sporangiospore germination in the Antarctic mesophile, M. hiemalis Wehmer f. hiemalis, compared with its temperate counterpart, M. hiemalis Wehmer. The Antarctic strain has been shown to occur as part of the phylloplane mycoflora on two plant species predominant on South Georgia, namely Festuca contracta T. Kirk (a meadow grass) and Poa flabellata (Lam.) Hook (a tussock grass). The extreme diurnal temperature ranges prevalent on South Georgia cause rapid freeze-thaw conditions. As a consequence, carbohydrates are leached from the leaves of these grasses and provide a nutrient source for the mesophilic phylloplane microfungi (Hurst, 1982). These plants are most abundant during the Antarctic summer where, although the mean air temperature is 5C, daily maxima are often as high as 15C. Clearly, a lowering of the optimum temperatures for both vegetative growth and sporangiospore germination from 25C in temperate regions to 15C in the Antarctic, coupled with the rapid growth rates characteristic of Mucoraceous fungi, confers a considerable advantage on the Antarctic strain in that

it will be able to compete for the limited available carbohydrates more rapidly than non-cold adapted mesophilic fungi that make up the remainder of the phylloplane microfungi.

In summary, ultrastructural thermostability, particularly that of the plasma membrane, would appear to be of greater significance than individual macromolecular thermostability in delineating fungal growth limits at extreme temperatures. Further, in the present investigation it is a disparity in plasma membrane lipid composition, rather than protein composition, that delineates the differing upper and lower growth temperature limits characteristic of those psychrophilic, mesophilic and thermophilic Mucoraceous fungi investigated.

APPENDICES

APPENDIX 2:1 MEDIA AND STATISTICAL NOTATION

(a) MEDIA

(1) MALT EXTRACT AGAR (MEA)

FORMULA (PER LITRE)

Malt Extract	30.0 g
Mycological Peptone	5.0 g
Agar No. 1 (Oxoid)	15.0 g

METHOD

Suspend 50g in 1 litre of distilled water. Stir to dissolve. Sterilise by autoclaving at 10,342 Nm^{-2} (15 p.s.i.), 121C, for 15 minutes.

(2) GLUCOSE-ASPARAGINE MEDIUM (GAE)

FORMULA (PER LITRE)

Glucose	10.00 g
Asparagine	1.00 g
Yeast Extract	0.50 g
K_2HPO_4	0.50 g
$MgSO_4 . 7H_2O$	0.50 g
$FeSO_4 . 7H_2O$	0.01 g

METHOD

Add ingredients to 1 litre of distilled water. Stir to dissolve. Sterilise by autoclaving at 10,342 Nm^{-2} (15 p.s.i.), 121C, for 15 minutes (Benitez et al., 1975). For Agar add 15.0g Agar No. 1 (Oxoid) per litre.

(3) DISTILLED WATER AGAR

FORMULA (PER LITRE)

Agar No.1	15.0 g

METHOD

Add to 1 litre of distilled water. Stir to dissolve. Sterilise by autoclaving at 10,342 Nm^{-2} (15 p.s.i.), 121C, for 15 minutes.

(b) STATISTICAL NOTATION

The following notation is used throughout:

PROBABILITY (p)		SYMBOL AND INTERPRETATION
$p > 0.05$		None i.e. Not Significant
$p < 0.05$	*	Significant
$p < 0.01$	**	Very Significant
$p < 0.001$	***	Highly Significant

APPENDIX 2:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - LINEAR EXTENSION

(I) M. PUSILLUS "ASTON"

TEMPERATURE (C)	MEDIUM	GROWTH RATE (mm DAY^{-1}) 1	2	3	$\bar{x}$	TREATMENT TOTAL
25	M_1	11.76	11.60	12.30	11.89	35.66
	M_2	4.85	4.92	4.92	4.89	14.69
30	M_1	23.30	21.60	21.60	22.16	66.50
	M_2	15.00	15.00	15.00	15.00	45.00
40	M_1	51.00	48.00	48.00	49.00	147.00
	M_2	31.20	31.20	31.20	31.20	93.60
50	M_1	16.00	15.60	16.20	15.93	47.80
	M_2	7.20	4.80	7.20	6.40	19.20

M_1 = MALT M_2 = GLUCOSE-ASPARAGINE

TWO WAY ANOVAR TABLE

TEMPERATURE (C)	M_1	M_2	TEMPERATURE TOTAL
25	35.66	14.69	50.35
30	66.50	45.00	111.50
40	147.00	93.60	240.60
50	47.80	19.20	67.00
MEDIA TOTAL	296.96	172.49	

APPENDIX 2:2 (Continued) ANOVAR SUMMARY TABLE (I) M. PUSILLUS "ASTON"

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	4482.295296	23	194.8824041	255.05
TREATMENTS (Tr)	4470.069629			
TEMPERATURE (Te)	3708.151146	3	1236.050382	*** 1617.65
MEDIA (Me)	645.532537	1	645.532537	*** 844.55
Te x Me (Tr-(Te+Me))	116.385946	3	38.79531533	*** 50.77
ERROR (T - Tr)	12.225667	16	0.7641041	

APPENDIX 2:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - LINEAR EXTENSION

(II) M. PUSILLUS "LACEY"

TEMPERATURE (C)	MEDIUM	GROWTH RATE (mm DAY^{-1}) 1	2	3	$\bar{x}$	TREATMENT TOTAL
25	M_1	12.26	11.76	11.11	11.71	35.13
	M_2	4.85	4.92	4.92	4.89	14.69
30	M_1	22.48	18.55	20.62	20.55	61.65
	M_2	13.80	13.80	13.80	13.80	41.40
40	M_1	36.54	41.43	41.78	39.92	119.75
	M_2	34.80	33.60	34.80	34.40	103.20
50	M_1	8.27	8.81	10.15	9.08	27.23
	M_2	9.60	9.60	9.60	9.60	28.80

M_1 = MALT M_2 = GLUCOSE-ASPARAGINE

TWO WAY ANOVAR TABLE

TEMPERATURE (C)	M_1	M_2	TEMPERATURE TOTAL
25	35.13	14.69	49.82
30	61.65	41.40	103.05
40	119.75	103.20	222.95
50	27.23	28.80	56.03
MEDIA TOTAL	243.76	188.09	

APPENDIX 2:2 (Continued) ANOVAR SUMMARY TABLE (II) M. PUSILLUS "LACEY"

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	3433.065363	23	149.2637114	84.10
TREATMENTS (Tr)	3404.669363			
TEMPERATURE (Te)	3220.632113	3	1073.544037	*** 604.90
MEDIA (Me)	129.1312046	1	129.1312046	*** 72.76
Te x Me (Tr-(Te+Me))	54.906046	3	18.30201533	*** 10.31
ERROR (T - Tr)	28.396	16	1.7747499	

APPENDIX 2:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - LINEAR EXTENSION

(III) M. PSYCHROPHILUS

TEMPERATURE (C)	MEDIUM	GROWTH RATE (mm DAY^{-1}) 1	2	3	$\bar{x}$	TREATMENT TOTAL
5	M_1	1.92	1.92	2.04	1.96	5.88
	M_2	2.04	2.16	2.04	2.08	6.24
10	M_1	3.06	3.24	3.12	3.14	9.42
	M_2	2.64	2.76	2.88	2.76	8.28
15	M_1	3.24	3.24	3.36	3.28	9.84
	M_2	2.64	2.64	2.64	2.64	7.92
20	M_1	0.96	1.20	1.08	1.08	3.24
	M_2	1.02	0.96	0.99	0.99	2.96

M_1 = MALT M_2 = GLUCOSE-ASPARAGINE

TWO WAY ANOVAR TABLE

TEMPERATURE (C)	M_1	M_2	TEMPERATURE TOTAL
5	5.88	6.24	12.12
10	9.42	8.28	17.70
15	9.84	7.92	17.76
20	3.24	2.96	6.20
MEDIA TOTAL	28.38	25.40	

APPENDIX 2:2 (Continued) ANOVAR SUMMARY TABLE (III) M. PSYCHROPHILUS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	16.13238334	23	0.70147971	106.825
TREATMENTS (Tr)	16.02731673			
TEMPERATURE (Te)	15.16165006	3	5.053883353	*** 769.635
MEDIA (Me)	0.37001673	1	0.37001673	*** 56.348
Te x Me (Tr-(Te+Me))	0.49564994	3	0.165216646	*** 25.1601
ERROR (T - Tr)	0.10506661	16	0.00656666	

APPENDIX 2:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - LINEAR EXTENSION

(IV) M. HIEMALIS WEHMER

TEMPERATURE (C)	MEDIUM	GROWTH RATE (mm DAY^{-1}) 1	2	3	$\bar{x}$	TREATMENT TOTAL
5	M_1	8.00	8.40	8.40	8.26	24.80
	M_2	6.60	6.60	6.60	6.60	19.80
10	M_1	14.40	15.00	13.20	14.20	42.60
	M_2	9.00	9.00	9.00	9.00	27.00
15	M_1	16.20	16.20	15.60	16.00	48.00
	M_2	13.20	13.20	13.30	13.23	39.70
20	M_1	24.00	23.40	24.00	23.80	71.40
	M_2	24.00	22.80	22.80	23.20	69.60
25	M_1	27.16	24.60	24.00	25.25	75.76
	M_2	19.80	19.20	19.80	19.60	58.80
30	M_1	5.40	5.40	5.40	5.40	16.20
	M_2	4.62	5.19	5.43	5.08	15.24

M_1 = MALT M_2 = GLUCOSE-ASPARAGINE

TWO WAY ANOVAR TABLE

TEMPERATURE (C)	M_1	M_2	TEMPERATURE TOTAL
5	24.80	19.80	44.60
10	42.60	27.00	69.60
15	48.00	39.70	87.70
20	71.40	69.60	141.00
25	75.76	58.80	134.56
30	16.20	15.24	31.44
MEDIA TOTAL	278.76	230.14	

APPENDIX 2:2 (Continued) ANOVAR SUMMARY TABLE (IV) M. HIEMALIS WEHMER

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	1837.174056	35	52.49068731	133.273
TREATMENTS (Tr)	1827.721456			
TEMPERATURE (Te)	1722.879256	5	344.5758512	*** 874.873
MEDIA (Me)	65.664011	1	65.664011	*** 166.720
Te x Me (Tr-(Te+Me))	39.178179	5	7.8356358	*** 19.895
ERROR (T - Tr)	9.4526	24	0.393858	

APPENDIX 2:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - LINEAR EXTENSION

(V) M. HIEMALIS WEHMER F. HIEMALIS

TEMPERATURE (C)	MEDIUM	GROWTH RATE (mm DAY^{-1}) 1	2	3	$\bar{x}$	TREATMENT TOTAL
5	M_1	6.33	5.72	6.21	6.09	18.26
	M_2	7.80	8.40	7.20	7.80	23.40
10	M_1	14.00	15.61	15.02	14.88	44.63
	M_2	10.20	10.20	10.30	10.23	30.70
15	M_1	18.67	19.32	18.14	18.71	56.13
	M_2	13.80	12.60	13.80	13.40	40.20
20	M_1	22.90	24.20	23.60	23.56	70.76
	M_2	21.60	22.80	21.60	22.00	66.00
25	M_1	25.33	26.32	26.01	25.89	77.66
	M_2	21.60	21.00	22.20	21.60	64.80
30	M_1	7.71	6.21	6.38	6.76	20.30
	M_2	5.32	6.01	4.89	5.41	16.22

M_1 = MALT M_2 = GLUCOSE-ASPARAGINE

TWO WAY ANOVAR TABLE

TEMPERATURE (C)	M_1	M_2	TEMPERATURE TOTAL
5	18.26	23.40	41.66
10	44.63	30.70	75.33
15	56.13	40.20	96.33
20	70.76	66.00	136.76
25	77.66	64.80	142.46
30	20.30	16.22	36.52
MEDIA TOTAL	287.74	241.32	

APPENDIX 2:2 (Continued) ANOVAR SUMMARY TABLE (V) M. HIEMALIS WEHMER F. HIEMALIS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	1849.492289	35	52.84263682	207.258
TREATMENTS (Tr)	1843.373222			
TEMPERATURE (Te)	1730.221055	5	346.044211	*** 1357.243
MEDIA (Me)	61.61944	1	61.61944	*** 241.682
Te x Me (Tr-(Te+Me))	51.532723	5	10.3065446	*** 40.424
ERROR (T - Tr)	6.119067	24	0.254961125	

APPENDIX 2:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - DRY WEIGHT

SPECIES	TEMPERATURE (C)	SPECIFIC GROWTH RATE, $\mu(h^{-1})$ 1	2	$\bar{x}$	TREATMENT TOTAL
M.PUSILLUS "ASTON"	5	-	-	-	-
	10	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	25	0.0244	0.0235	0.0230	0.0479
	30	0.0270	0.0215	0.0243	0.0485
	40	0.0325	0.0331	0.0328	0.0656
	50	0.0190	0.0210	0.0200	0.0310
	60	-	-	-	-
M.PUSILLUS "LACEY"	5	-	-	-	-
	10	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	25	0.0196	0.0362	0.0216	0.0558
	30	0.0216	0.0211	0.0279	0.0427
	40	0.0356	0.0438	0.0397	0.0794
	50	0.0180	0.0140	0.0160	0.0320
	60	-	-	-	-
M.PSYCHROPHILUS	5	0.0100	0.0147	0.0124	0.0247
	10	0.0258	0.0350	0.0304	0.0608
	15	0.0124	0.0110	0.0172	0.0234
	20	0.0092	0.0058	0.0075	0.0150
	25	-	-	-	-
	30	-	-	-	-
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-
M.HIEMALIS WEHMER	5	0.0239	0.0180	0.0210	0.0419
	10	0.0285	0.0325	0.0305	0.0610
	15	0.0573	0.0541	0.0557	0.1114
	20	0.0740	0.0630	0.0685	0.1370
	25	0.0941	0.0965	0.0953	0.1906
	30	0.0848	0.0905	0.0877	0.1753
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-
M.HIEMALIS WEHMER F. HIEMALIS	5	0.0248	0.0209	0.0229	0.0457
	10	0.0249	0.0260	0.0255	0.0509
	15	0.0552	0.0523	0.0538	0.1075
	20	0.0423	0.0432	0.0428	0.0855
	25	0.0395	0.0391	0.0393	0.0686
	30	-	-	-	-
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-

- No Growth.

APPENDIX 2:3 (Continued) TWO WAY ANOVAR TABLE

SPECIES	5C	10C	15C	20C	25C	30C	40C	50C	60C	SPECIES TOTAL
M. PUSILLUS "ASTON"	-	-	-	-	0.0479	0.0485	0.0656	0.0310	-	0.1930
M. PUSILLUS "LACEY"	-	-	-	-	0.0558	0.0427	0.0794	0.0320	-	0.2099
M. PSYCHROPHILUS	0.0247	0.0608	0.0234	0.0150	-	-	-	-	-	0.1239
M. HIEMALIS WEHMER	0.0419	0.0610	0.1114	0.1370	0.1906	0.1753	-	-	-	0.7172
M. HIEMALIS WEHMER F. HIEMALIS	0.0457	0.0509	0.1075	0.0885	0.0686	-	-	-	-	0.3582
TEMPERATURE TOTAL:	0.1123	0.1727	0.2423	0.2375	0.3629	0.2665	0.1450	0.0630	-	

APPENDIX 2:3 (Continued) ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	0.051520539	89	5.7888246×10^{-4}	34.553
TREATMENTS (Tr)	0.05076663			
SPECIES (Sp)	0.012230456	4	3.057614×10^{-3}	*** 182.508
TEMPERATURE (Te)	0.009682418	8	1.2103022×10^{-3}	*** 72.243
Sp x Te (Tr-(Te+Sp))	0.028853756	32	9.0167987×10^{-4}	*** 53.821
ERROR (T - Tr)	0.000753909	45	1.67533×10^{-5}	

APPENDIX 2:4 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON GROWTH RATE - MOLAR GROWTH YIELD

SPECIES	TEMPERATURE (C)	MOLAR GROWTH YIELD 1	2	$\bar{x}$	TREATMENT TOTAL
M.PUSILLUS "ASTON"	5	-	-	-	-
	10	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	25	78.66	61.81	70.24	140.47
	30	66.00	73.20	69.60	139.20
	40	86.23	73.47	79.85	159.70
	50	76.95	74.20	75.57	151.15
	60	-	-	-	-
M.PUSILLUS "LACEY"	5	-	-	-	-
	10	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	25	67.57	59.20	63.39	126.77
	30	69.49	66.16	67.83	135.65
	40	74.75	63.19	68.97	137.94
	50	75.07	83.98	79.52	159.05
	60	-	-	-	-
M.PSYCHROPHILUS	5	20.32	21.13	20.73	41.45
	10	39.53	38.15	38.84	77.68
	15	53.11	49.62	51.37	102.73
	20	45.07	49.02	47.05	94.09
	25	-	-	-	-
	30	-	-	-	-
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-
M.HIEMALIS WEHMER	5	72.64	70.47	71.56	143.11
	10	50.79	49.05	49.92	99.84
	15	71.77	71.77	71.77	143.54
	20	78.69	80.76	79.73	159.45
	25	59.75	63.21	61.48	122.96
	30	68.41	61.16	64.79	129.57
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-
M.HIEMALIS WEHMER F. HIEMALIS	5	52.93	55.83	54.38	108.76
	10	44.96	42.57	43.77	87.53
	15	62.21	55.18	58.70	117.39
	20	45.68	48.15	46.92	93.83
	25	74.56	72.17	73.37	146.73
	30	-	-	-	-
	40	-	-	-	-
	50	-	-	-	-
	60	-	-	-	-

MOLAR GROWTH YIELD IN mg DRY WEIGHT MOL^{-1} GLUCOSE.
- No Growth.

APPENDIX 2:4 (Continued) TWO WAY ANOVAR TABLE

SPECIES	5C	10C	15C	20C	25C	30C	40C	50C	60C	SPECIES TOTAL
M. PUSILLUS "ASTON"	-	-	-	-	140.47	139.20	159.70	151.15	-	590.52
M. PUSILLUS "LACEY"	-	-	-	-	126.77	135.65	137.94	159.05	-	559.41
M. PSYCHROPHILUS	41.45	77.68	102.73	94.09	-	-	-	-	-	315.95
M. HIEMALIS WEHMER	143.11	99.84	143.54	159.45	122.96	129.57	-	-	-	798.47
M. HIEMALIS WEHMER F. HIEMALIS	108.76	87.53	117.39	93.83	146.73	-	-	-	-	554.24
TEMPERATURE TOTAL:	293.32	265.05	363.66	347.37	536.93	404.24	297.64	310.20	-	

APPENDIX 2:4 (Continued) ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	94920.58601	89	1066.523438	114.46595
TREATMENTS (Tr)	94429.30366			
SPECIES (Sp)	6518.01232	4	1629.50308	*** 174.88843
TEMPERATURE (Te)	16314.89874	8	2039.362342	*** 218.87709
Sp x Te (Tr-(Te+Sp))	71596.3926	32	2237.387268	*** 240.13036
ERROR (T - Tr)	419.28235	45	9.3173855	

LEAST SIGNIFICANT DIFFERENCE = 2.55 (TEMPERATURE)

APPENDIX 3:1 THE EFFECT OF GROWTH TEMPERATURE ON SPORANGIOSPOROGENESIS

(I) M. PUSILLUS

		TEMPERATURE (C)		
TIME (HOURS)		25	30	40
M. PUSILLUS "ASTON"	1	72	40	24
	2	72	40	24
	$\bar{x}$	72	40	24
M. PUSILLUS "LACEY"	1	96	52	24
	2	96	52	24
	$\bar{x}$	96	52	24

FIGURES REFER TO THE TIME TAKEN IN HOURS FOR SPORANGIOSPOROGENESIS

APPENDIX 3:1 THE EFFECT OF GROWTH TEMPERATURE ON SPORANGIOSPOROGENESIS

(II) M. HIEMALIS WEHMER

	TEMPERATURE (C)				
TIME (HOURS)	5	10	15	20	25
1	168	166	96	75	48
2	168	168	96	75	48
$\bar{x}$	168	167	96	75	48

FIGURES REFER TO THE TIME TAKEN IN HOURS FOR SPORANGIOSPOROGENESIS

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	23756.0	9		
TEMPERATURE (Te)	23749.6	4	5937.4	14843.5***
ERROR (T-Te)	2.0	5	0.4	

LEAST SIGNIFICANT DIFFERENCE = 4.2

APPENDIX 3:1 THE EFFECT OF GROWTH TEMPERATURE ON SPORANGIOSPOROGENESIS

(III) M. HIEMALIS WEHMER F. HIEMALIS

	TEMPERATURE (C)				
TIME (HOURS)	5	10	15	20	25
1	240	166	96	67	48
2	240	166	96	72	48
$\bar{x}$	240	166	96	69.5	48

FIGURES REFER TO THE TIME TAKEN IN HOURS FOR SPORANGIOSPOROGENESIS

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	49512.9	9		
TEMPERATURE (Te)	49500.4	4	12375.1	4950.04***
ERROR (T-Te)	12.5	5	2.5	

LEAST SIGNIFICANT DIFFERENCE = 4.1

APPENDIX 3:1 THE EFFECT OF GROWTH TEMPERATURE ON SPORANGIOSPOROGENESIS

(IV) M. PSYCHROPHILUS

PERCENTAGE SPORULATION	TEMPERATURE (C) 5	10	15	20
1	38.8	34.0	47.4	1.0
2	39.1	38.6	48.6	1.0
$\bar{x}$	38.95	36.3	48.0	1.0

FIGURES REFER TO PERCENTAGE SPORANGIOSPOROGENESIS AFTER 7 DAYS INCUBATION

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	2571.89875	7		
TEMPERATURE (Te)	2560.53575	3	853.17916	300.8123987***
ERROR (T-Te)	11.345	4	2.83625	

LEAST SIGNIFICANT DIFFERENCE = 4.7

APPENDIX 3:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON SPORANGIOSPORE GERMINATION

SPECIES	TEMPERATURE (C) 5	10	15	20	25	30	40	50
M. PUSILLUS "ASTON"	-	-	-	-	5.2✡	29.0±4.2	4.0±0.0	11.3±0.4
M. PUSILLUS "LACEY"	-	-	-	-	11.8✡	13.0±0.7	3.5±0.0	24.5±0.7
M. HIEMALIS WEHMER	51.8±0.4	44.5±2.1	21.8±0.4	15.5±0.7	5.5±0.0	44.2✡	-	-
M. HIEMALIS WEHMER F. HIEMALIS	12.5✡	40.5±0.5	28.5±1.5	30.0✡	16.5✡	6.4✡	-	-
M. PSYCHRO-PHILUS	47.4✡	61.0±1.4	66.5±2.1	33.8✡	-	-	-	-

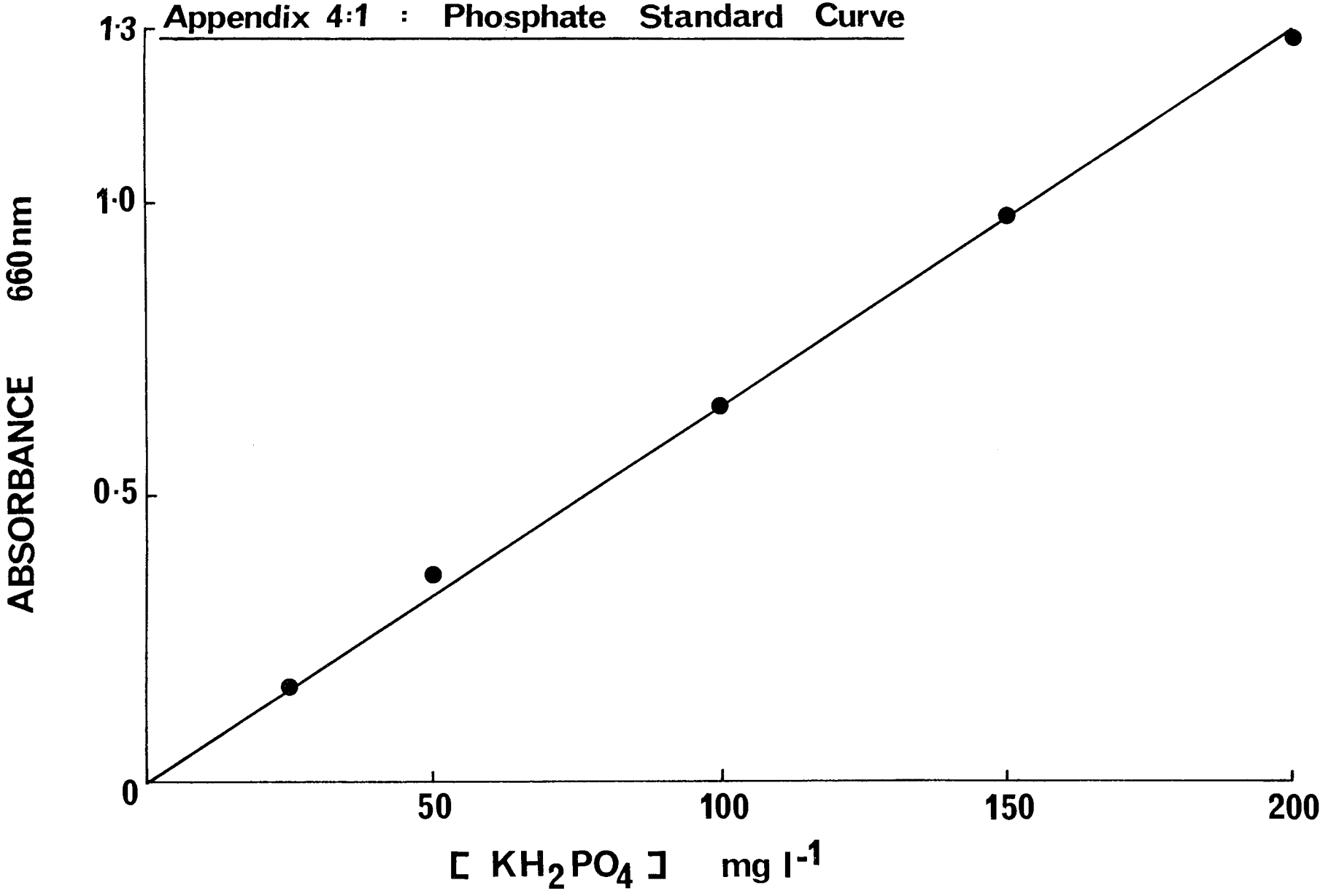
- NO GERMINATION FIGURES REFER TO LP_{50} IN HOURS (MEAN 2 REPS. ± S.D. (6n-1))

✡ MAXIMUM PERCENTAGE GERMINATION

APPENDIX 4:1

PHOSPHATE STANDARD CURVE

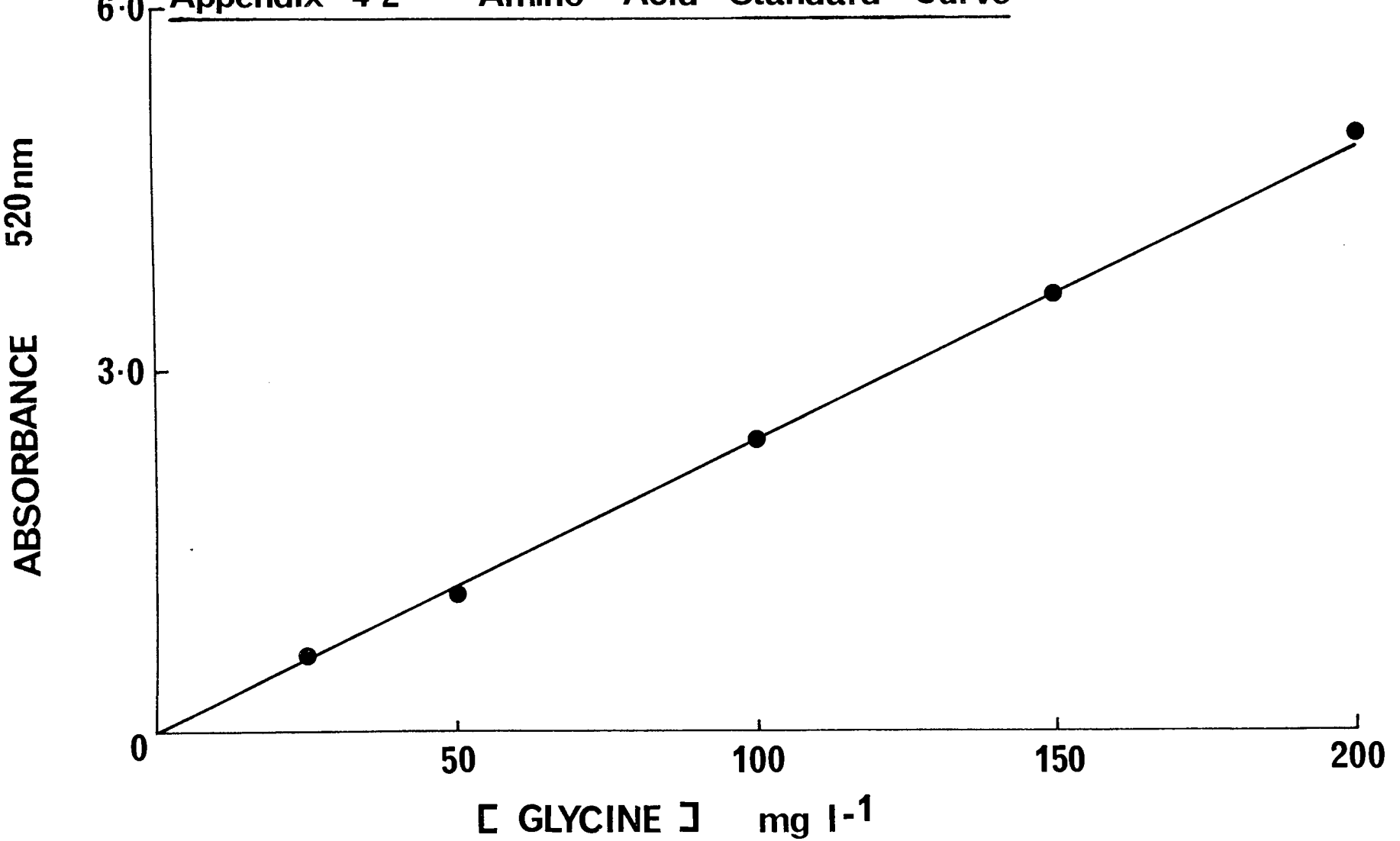
Appendix 4:1 : Phosphate Standard Curve



APPENDIX 4:2

AMINO ACID STANDARD CURVE

Appendix 4:2 : Amino Acid Standard Curve



APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(I) M. PSYCHROPHILUS (a) PHOSPHATE

TEMPERATURE (C)		(PHOSPHATE) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	2.073	0.000	2.073
	2	2.379	0.000	2.379
	3	2.142	0.000	2.142
10	1	2.678	0.000	2.678
	2	1.918	0.000	1.918
	3	2.130	0.000	2.130
15	1	1.826	0.161	1.987
	2	1.813	0.326	2.139
	3	1.759	0.224	1.983
25	1	0.803	1.034	1.837
	2	0.744	0.986	1.730
	3	0.998	0.863	1.861
30	1	0.374	1.802	2.176
	2	0.362	2.060	2.422
	3	0.415	1.836	2.251
40	1	0.559	2.364	2.923
	2	0.601	1.842	2.443
	3	0.431	1.963	2.394
50	1	0.316	1.605	1.921
	2	0.527	2.245	2.772
	3	0.339	1.736	2.075
60	1	0.412	1.537	1.949
	2	0.463	2.099	2.562
	3	0.361	1.476	1.837
i/e Total		26.423	26.159	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (I) M. PSYCHROPHILUS

(a) PHOSPHATE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	32.79176992	47	0.697697232	19.779305
TREATMENTS (Tr)	31.66299592			
TEMPERATURE (Te)	1.17228492	7	0.167469274	4.7476533***
i/e (i/e)	1.452×10^{-3}	1	1.452×10^{-3}	0.0411066
Te x i/e (Tr-(Te+i/e))	30.489259	7	4.355608428	123.47893***
ERROR (T - Tr)	1.128774	32	0.0352741	

LEAST SIGNIFICANT DIFFERENCE = 0.281 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(I) M. PSYCHROPHILUS (b) AMINO ACIDS

TEMPERATURE (C)		(AMINO ACIDS) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	3.165	0.389	3.554
	2	2.552	0.260	2.812
	3	2.068	0.182	2.250
10	1	2.587	0.263	2.850
	2	1.352	0.150	1.502
	3	2.119	0.272	2.391
15	1	2.786	0.714	3.500
	2	2.541	0.430	2.971
	3	2.463	0.452	2.915
25	1	0.967	1.983	2.950
	2	1.031	1.734	2.765
	3	1.122	2.160	3.282
30	1	0.482	2.584	3.066
	2	0.881	1.936	2.817
	3	0.943	2.232	3.175
40	1	0.801	2.478	3.279
	2	1.019	2.673	3.692
	3	1.227	2.815	4.042
50	1	0.985	2.582	3.567
	2	1.222	2.844	4.066
	3	1.022	2.673	3.695
60	1	1.062	2.687	3.749
	2	0.883	2.031	2.914
	3	0.991	2.222	3.213
i/e Total		36.271	38.746	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (I) M. PSYCHROPHILUS

(b) AMINO ACIDS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	39.32652898	47	0.836734659	12.143095
TREATMENTS (Tr)	37.12152833			
TEMPERATURE (Te)	3.8931315	7	0.556161642	8.0712853***
i/e (i/e)	0.1088622	1	0.1088622	1.5798607
Te x i/e (Tr-(Te+i/e))	33.11953463	7	4.73136209	68.663806***
ERROR (T - Tr)	2.20500065	32	0.0689062	

LEAST SIGNIFICANT DIFFERENCE = 0.392 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(II) M. HIEMALIS WEHMER (a) PHOSPHATE

TEMPERATURE (C)		(PHOSPHATE) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	3.847	0.052	3.539
	2	2.390	0.057	2.447
	3	3.167	0.111	3.278
10	1	2.363	0.165	2.528
	2	2.648	1.120	2.768
	3	2.564	0.119	2.683
15	1	2.917	0.088	3.005
	2	2.147	0.000	2.147
	3	2.889	0.125	3.014
25	1	2.768	0.014	2.782
	2	4.093	0.000	4.093
	3	3.490	0.000	3.490
30	1	2.472	0.300	2.772
	2	2.701	0.207	2.908
	3	2.113	0.283	2.396
40	1	1.566	2.023	3.589
	2	1.422	2.095	3.517
	3	1.120	1.843	2.963
50	1	0.676	2.376	3.052
	2	0.643	2.365	3.008
	3	0.807	3.121	2.928
60	1	0.951	1.942	2.893
	2	0.647	1.511	2.158
	3	0.515	1.374	1.889
i/e Total		50.556	20.291	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (II) M. HIEMALIS WEHMER

(a) PHOSPHATE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	68.38169498	47	1.45492968	15.851565
TREATMENTS (Tr)	65.44458766			
TEMPERATURE (Te)	3.5324755	7	0.504639357	*** 5.4980824
i/e (i/e)	19.08271304	1	19.08271304	*** 216.01804
Te x i/e (Tr-(Te+i/e))	42.82939912	7	6.118485588	*** 66.661351
ERROR (T - Tr)	2.93710732	32	0.0917846	

LEAST SIGNIFICANT DIFFERENCE = 0.450 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(II) M. HIEMALIS WEHMER (b) AMINO ACIDS

TEMPERATURE (C)		(AMINO ACIDS) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	3.585	0.094	3.679
	2	2.630	0.192	2.759
	3	5.337	0.554	5.891
10	1	2.966	0.357	3.323
	2	3.716	0.341	4.057
	3	3.377	0.189	3.566
15	1	3.755	0.157	3.912
	2	4.151	0.000	4.151
	3	2.464	0.085	2.549
25	1	7.219	0.102	7.321
	2	5.094	0.025	5.119
	3	5.814	0.050	5.864
30	1	2.698	0.630	3.328
	2	2.878	0.398	3.276
	3	1.934	0.537	2.471
40	1	1.916	5.322	7.238
	2	1.772	5.316	7.088
	3	1.411	6.064	7.475
50	1	0.891	3.973	4.864
	2	0.715	3.046	3.761
	3	0.577	6.158	6.735
60	1	1.137	5.501	6.638
	2	1.434	5.216	6.650
	3	0.934	4.668	5.602
i/e Total		68.405	48.912	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (II) M. HIEMALIS WEHMER

(b) AMINO ACIDS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	215.8573485	47	4.592709542	10.597313
TREATMENTS (Tr)	201.9890495			
TEMPERATURE (Te)	32.534024	7	4.647717714	10.724241***
i/e (i/e)	7.91618854	1	7.91618854	18.265978***
Te x i/e (Tr-(Te+i/e))	161.5388369	7	23.0769767	53.248297***
ERROR (T - Tr)	13.868299	32	0.4333843	

LEAST SIGNIFICANT DIFFERENCE = 0.97 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(III) M. HIEMALIS WEHMER F. HIEMALIS (a) PHOSPHATE

TEMPERATURE (C)		(PHOSPHATE) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	3.402	0.200	3.602
	2	3.045	0.021	3.066
	3	4.078	0.069	4.147
10	1	2.838	0.182	3.020
	2	1.852	0.277	2.129
	3	1.549	0.316	1.865
15	1	2.436	0.227	2.663
	2	2.306	0.111	2.417
	3	1.935	0.199	2.134
25	1	1.337	0.489	1.826
	2	2.003	0.259	2.262
	3	1.881	0.427	2.308
30	1	1.391	0.317	1.708
	2	1.938	0.356	2.294
	3	1.398	0.266	1.664
40	1	0.735	1.943	2.678
	2	0.466	2.199	2.665
	3	0.957	2.900	3.857
50	1	0.443	1.397	1.840
	2	0.747	1.716	2.463
	3	0.310	1.269	1.579
60	1	0.342	1.195	1.537
	2	0.343	1.768	2.111
	3	0.074	1.487	1.561
i/e Total		37.806	19.59	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (III) M. HIEMALIS WEHMER F. HIEMALIS

(a) PHOSPHATE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	48.907865	47	1.040592872	10.606713
TREATMENTS (Tr)	45.768439			
TEMPERATURE (Te)	6.002937	7	0.857562428	*** 8.7410928
i/e (i/e)	6.912972	1	6.912972	*** 70.463595
Te x i/e (Tr-(Te+i/e))	32.85253	7	4.693218571	*** 47.837753
ERROR (T - Tr)	3.139426	32	0.098107	

LEAST SIGNIFICANT DIFFERENCE = 0.470 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(III) M. HIEMALIS WEHMER F. HIEMALIS (b) AMINO ACIDS

TEMPERATURE (C)		(AMINO ACIDS) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	3.063	0.161	3.224
	2	2.798	0.268	3.066
	3	2.940	0.210	3.150
10	1	2.879	0.000	2.879
	2	1.231	0.000	1.231
	3	1.196	0.000	1.196
15	1	3.068	0.187	3.255
	2	3.210	0.134	3.344
	3	2.753	0.060	2.813
25	1	1.957	0.233	2.190
	2	3.374	0.342	3.716
	3	1.601	0.103	1.704
30	1	2.490	0.543	3.033
	2	2.584	0.686	3.270
	3	2.414	0.151	2.565
40	1	0.730	2.792	3.522
	2	1.032	1.829	2.861
	3	1.566	3.021	4.587
50	1	1.182	3.673	4.855
	2	2.026	5.657	7.683
	3	0.767	3.424	4.191
60	1	0.387	2.128	2.515
	2	0.534	3.249	3.783
	3	0.089	1.976	2.065
i/e Total		45.871	30.827	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (III) M. HIEMALIS WEHMER F. HIEMALIS

(b) AMINO ACIDS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	86.51772	47	1.840802553	5.8848018
TREATMENTS (Tr)	76.50792066			
TEMPERATURE (Te)	20.085145	7	2.869306428	*** 9.1727926
i/e (i/e)	4.69586508	1	4.69586508	*** 15.012058
Te x i/e (Tr-(Te+i/e))	51.72691058	7	7.389558654	*** 23.62344
ERROR (T - Tr)	10.00979934	32	0.3128062	

LEAST SIGNIFICANT DIFFERENCE = 0.84 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(IV) M. PUSILLUS "ASTON" (a) PHOSPHATE

TEMPERATURE (C)		(PHOSPHATE) $\mu M\ h^{-1}\ mg^{-1}$ i	e	TEMPERATURE TOTAL
5	1	0.725	0.152	0.877
	2	1.056	0.220	1.276
	3	0.687	0.202	0.889
10	1	1.357	0.060	1.417
	2	1.327	0.073	1.400
	3	0.849	0.095	0.944
15	1	1.115	0.446	1.561
	2	0.999	0.323	1.322
	3	0.957	0.252	1.209
25	1	0.975	0.409	1.384
	2	0.639	0.452	1.091
	3	1.001	0.446	1.447
30	1	0.683	0.280	0.963
	2	0.817	0.540	1.357
	3	1.101	0.799	1.900
40	1	0.373	0.778	1.151
	2	0.449	1.153	1.602
	3	0.484	0.808	1.292
50	1	0.163	0.581	0.744
	2	0.148	0.658	0.806
	3	0.103	0.517	0.620
60	1	0.245	1.255	1.500
	2	0.333	1.632	1.965
	3	0.261	1.515	1.776
i/e Total		16.487	13.646	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (IV) M. PUSILLUS "ASTON"

(a) PHOSPHATE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	8.93685748	47	0.170996967	7.0775397
TREATMENTS (Tr)	7.26372148			
TEMPERATURE (Te)	1.47095798	7	0.210136854	8.6975352 ***
i/e (i/e)	0.213466688	1	0.213466688	8.8353917 ***
Te x i/e (Tr-(Te+i/e))	5.579296812	7	0.797042401	32.989482 ***
ERROR (T - Tr)	0.773136	32	0.0241605	

LEAST SIGNIFICANT DIFFERENCE = 0.230 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(IV) M. PUSILLUS "ASTON" (b) AMINO ACIDS

TEMPERATURE (C)		(AMINO ACIDS) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	2.820	0.392	3.212
	2	2.977	0.644	3.621
	3	2.182	0.426	2.608
10	1	3.114	0.093	3.207
	2	2.570	0.083	2.653
	3	1.693	0.077	1.770
15	1	3.869	1.300	5.169
	2	3.144	1.331	4.475
	3	3.130	1.132	4.262
25	1	2.794	0.884	3.678
	2	2.120	1.187	3.307
	3	2.631	1.101	3.732
30	1	2.276	0.752	3.028
	2	2.459	0.670	3.129
	3	3.165	1.307	4.472
40	1	1.391	1.968	3.359
	2	1.506	3.073	4.579
	3	1.669	3.220	4.889
50	1	0.896	2.046	2.942
	2	1.357	3.291	4.648
	3	1.161	2.487	3.648
60	1	0.278	2.996	3.274
	2	0.522	4.507	5.029
	3	0.446	3.508	3.954
i/e Total		50.170	38.475	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (IV) M. PUSILLUS "ASTON"

(b) AMINO ACIDS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	61.80587648	47	1.315018648	7.1693887
TREATMENTS (Tr)	55.93639183			
TEMPERATURE (Te)	8.447425	7	1.206775	*** 6.5792522
i/e (i/e)	2.84943804	1	2.84943804	*** 15.534935
Te x i/e (Tr-(Te+i/e))	44.63952879	7	6.377075541	*** 34.767366
ERROR (T - Tr)	5.86948465	32	0.1834213	

LEAST SIGNIFICANT DIFFERENCE = 0.640 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(V) M. PUSILLUS "LACEY" (a) PHOSPHATE

TEMPERATURE (C)		(PHOSPHATE) μM h^{-1} mg^{-1} i	e	TEMPERATURE TOTAL
5	1	1.257	0.110	1.347
	2	0.907	0.062	0.969
	3	1.010	0.077	1.087
10	1	0.865	0.194	1.059
	2	1.286	0.115	1.401
	3	1.344	0.085	1.429
15	1	1.088	0.199	1.287
	2	1.193	0.187	1.380
	3	0.660	0.071	0.731
25	1	1.370	0.098	1.468
	2	1.482	0.124	1.606
	3	1.419	0.105	1.524
30	1	2.456	0.352	2.808
	2	2.838	0.291	3.129
	3	3.069	0.422	3.491
40	1	0.571	0.571	1.142
	2	0.562	0.639	1.201
	3	0.490	0.626	1.116
50	1	0.113	0.856	0.969
	2	0.199	0.918	1.117
	3	0.138	0.967	1.105
60	1	0.273	1.314	1.587
	2	0.200	1.431	1.631
	3	0.208	1.172	1.380
i/e Total		24.978	10.986	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (V) M. PUSILLUS "LACEY"

(a) PHOSPHATE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	28.747731	47	0.505270872	26.456046
TREATMENTS (Tr)	23.11661566			
TEMPERATURE (Te)	5.333293	7	0.761899	*** 39.893132
i/e (i/e)	4.078668	1	4.078668	*** 213.55959
Te x i/e (Tr-(Te+i/e))	13.70465466	7	1.957807808	*** 102.51107
ERROR (T - Tr)	0.63111534	32	0.0190985	

LEAST SIGNIFICANT DIFFERENCE = 0.210 (TEMPERATURE)

APPENDIX 4:3 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON METABOLITE EFFLUX

(V) M. PUSILLUS "LACEY" (b) AMINO ACIDS

TEMPERATURE (C)		(AMINO ACIDS) $\mu M\ h^{-1}\ mg^{-1}$ i	e	TEMPERATURE TOTAL
5	1	2.658	0.321	2.979
	2	2.678	0.292	2.970
	3	2.547	0.209	2.756
10	1	2.487	0.540	3.027
	2	3.165	0.517	3.682
	3	3.204	0.511	3.715
15	1	3.366	0.457	3.823
	2	3.792	0.341	4.133
	3	2.103	0.288	2.391
25	1	3.740	0.314	4.054
	2	3.247	0.370	3.617
	3	4.241	0.417	4.658
30	1	4.642	1.125	5.767
	2	4.937	0.977	5.914
	3	5.320	1.146	6.466
40	1	1.700	1.836	3.536
	2	1.758	2.179	3.937
	3	1.030	1.562	2.592
50	1	1.141	4.655	5.796
	2	1.283	4.008	5.291
	3	1.144	4.160	5.304
60	1	0.391	5.059	5.450
	2	0.571	3.839	4.410
	3	0.269	3.011	3.280
i/e Total		61.414	38.134	

i = intracellular e = extracellular

APPENDIX 4:3 (Continued) ANOVAR SUMMARY TABLE (V) M. PUSILLUS "LACEY"

(b) AMINO ACIDS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	119.8580336	47	2.550170927	14.631575
TREATMENTS (Tr)	114.2806797			
TEMPERATURE (Te)	15.9630967	7	2.280442385	*** 13.08401
i/e (i/e)	11.29080003	1	11.29080003	*** 64.78083
Te x i/e (Tr-(Te+i/e))	87.02678297	7	12.43239756	*** 71.33073
ERROR (T - Tr)	5.5773539	32	0.1742923	

LEAST SIGNIFICANT DIFFERENCE = 0.620 (TEMPERATURE)

APPENDIX 4:3 MEAN PHOSPHATE EFFLUX

TEMPERATURE (C)	M. PSYCHROPHILUS	M. HIEMALIS WEHMER	M. HIEMALIS WEHMER F. HIEMALIS	M. PUSILLUS "ASTON"	M. PUSILLUS "LACEY"
5	0.0	7.9	2.6	20.8	15.3
10	0.0	12.8	12.0	6.5	18.6
15	11.6	7.2	7.5	24.6	21.3
25	53.1	5.7	18.9	33.9	15.6
30	83.2	18.3	16.7	36.3	19.6
40	79.5	50.4	76.8	67.4	46.7
50	82.5	62.5	75.3	81.0	67.8
60	80.5	56.8	85.4	84.0	70.8

FIGURES ARE MEAN PERCENTAGE EFFLUX

APPENDIX 4:3 MEAN AMINO ACID EFFLUX

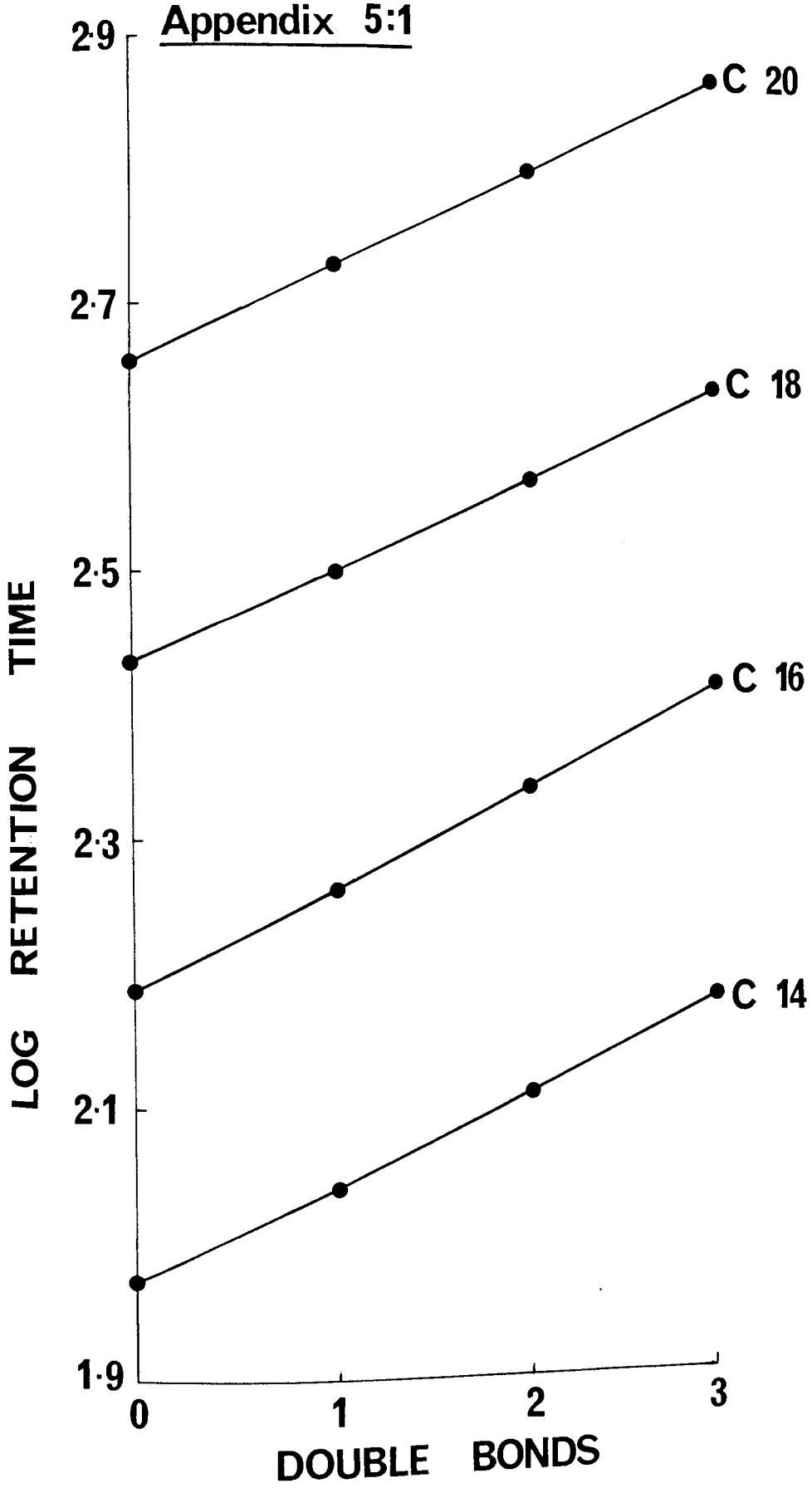
TEMPERATURE (C)	M. PSYCHROPHILUS	M. HIEMALIS WEHMER	M. HIEMALIS WEHMER F. HIEMALIS	M. PUSILLUS "ASTON"	M. PUSILLUS "LACEY"
5	9.6	12.8	6.8	15.4	18.1
10	10.1	14.5	0.0	3.5	23.0
15	17.0	7.2	4.0	27.2	19.0
25	65.3	5.7	8.6	29.8	17.4
30	74.5	24.7	14.9	23.6	25.4
40	72.3	61.4	69.7	63.7	48.3
50	71.6	67.2	77.0	69.5	62.0
60	70.3	61.5	88.6	89.9	68.4

FIGURES ARE MEAN PERCENTAGE EFFLUX

APPENDIX 5:1 FATTY ACID DATA AND GC CALIBRATION CURVE (190C)

SYSTEMATIC NAME	TRIVIAL NAME	SHORTHAND DESIGNATION	RETENTION TIME RELATIVE TO 16:0 (10% DEGS, 190C)
TETRADECENOIC	MYRISTIC	14:0	0.59
CIS, 9-TETRADECENOIC	MYRISTOLEIC	14:1	0.66
CIS,CIS,9,12-TETRADECADIENOIC	-	14:2	0.82
HEXADECENOIC	PALMITIC	16:0	1:00
CIS,9-HEXADECENOIC	PALMITOLEIC	16:1	1:15
CIS,CIS,7,12-HEXADECADIENOIC	-	16:2	1:39
OCTADECENEOIC	STEARIC	18:0	1:70
CIS,9-OCTADECENOIC	OLEIC	18:1	1:96
CIS,CIS,9,12-OCTADECADIENOIC	LINOLEIC	18:2	2:35
EICOSENOIC	ARACHIDIC	20:0	2:89
CIS,CIS,CIS,9,12,15-OCTADECATRIENOIC	α-LINOLENIC	18:3	3:25
CIS,9-EICOSENOIC	GLADOLEIC	20:1	3:32
CIS,CIS,11,14-EICOSADIENOIC	-	20:2	3.99
CIS,CIS,CIS,8,11,14-EICOSATRIENOIC	HOMO-α-LINOLENIC	20:3	5.39

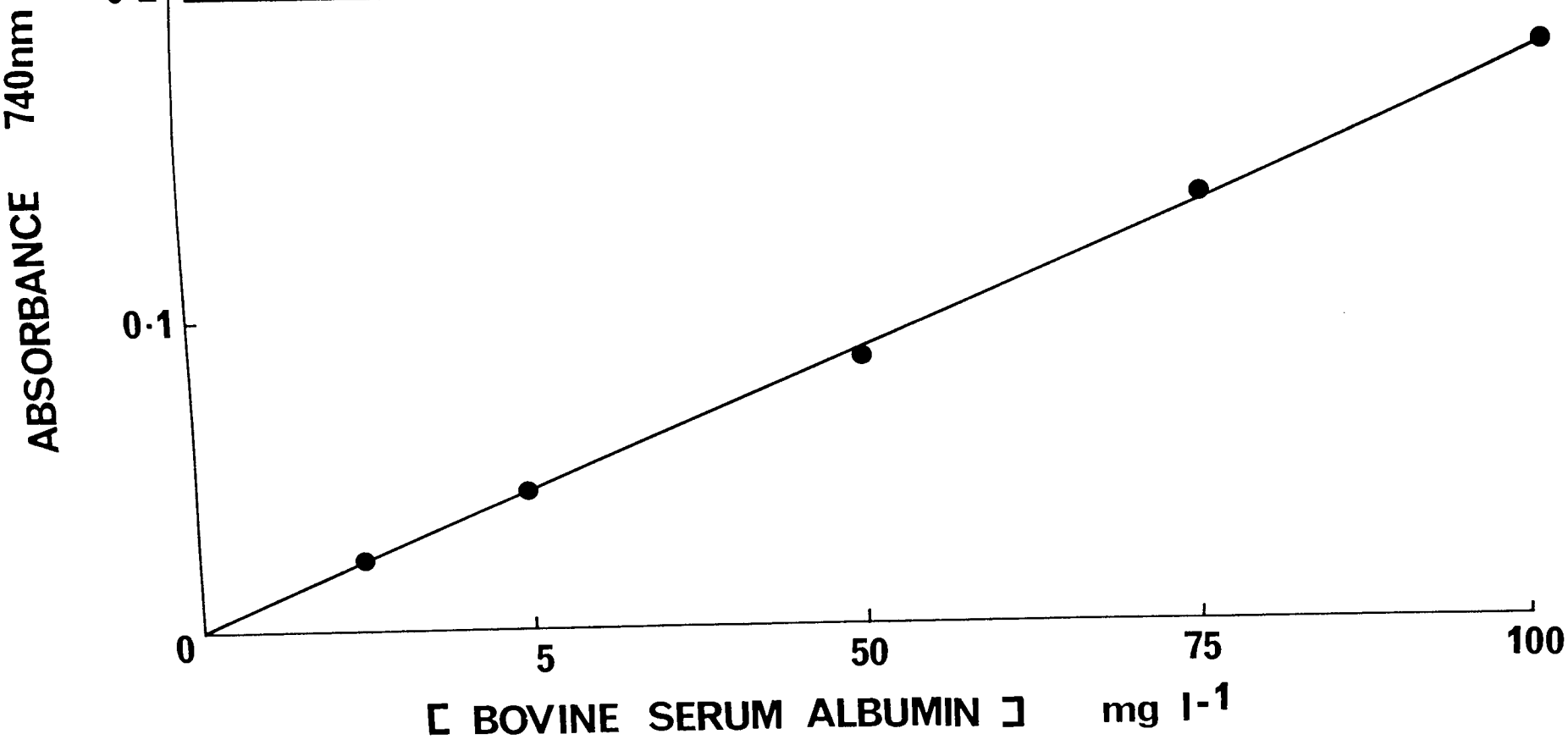
Appendix 5:1


2·9
2·7
2·5
2·3
2·1
1·9
LOG RETENTION TIME
C 20
C 18
C 16
C 14
0
1
2
3
DOUBLE BONDS

APPENDIX 5:2

PROTEIN STANDARD CURVE

Appendix 5:2 : Protein Standard Curve



APPENDIX 5:3 METHYL FATTY ACID COMPOSITION AT OPTIMUM GROWTH TEMPERATURE - TOTAL FATTY ACIDS

FATTY ACID	M. PUSILLUS "ASTON"	M. PUSILLUS "LACEY"	M. HIEMALIS WEHMER	M. H. W. F HIEMALIS	M. PSYCHROPHILUS
14:0	6.0 ± 5.3	+	+	+	+
14:1	5.7 ± 0.0	5.7 ± 0.0	10.0 ± 0.0	5.7 ± 0.0	12.0 ± 0.8
14:2	+	1.9 ± 3.3	+	+	+
16:0	30.2 ± 0.8	30.6 ± 1.2	26.6 ± 0.8	17.8 ± 0.5	19.9 ± 1.4
16:1	8.7 ± 1.1	6.0 ± 5.3	8.7 ± 1.1	5.7 ± 0.0	+
16:2	+	+	+	+	+
18:0	13.3 ± 0.8	12.4 ± 0.8	15.3 ± 1.1	9.9 ± 1.7	11.0 ± 1.7
18:1	44.0 ± 2.2	44.6 ± 1.3	37.7 ± 1.2	44.2 ± 1.2	35.9 ± 0.7
18:2	17.4 ± 1.0	17.7 ± 1.2	22.2 ± 1.3	23.3 ± 1.2	24.9 ± 0.4
20:0	12.0 ± 0.8	12.4 ± 4.8	18.7 ± 0.6	25.6 ± 0.4	22.7 ± 2.4
18:3	+	+	5.7 ± 0.0	+	+
20:1	+	+	9.4 ± 1.1	7.9 ± 2.2	19.0 ± 1.1
20:2	+	+	1.9 ± 3.3	3.8 ± 3.3	+
20:3	+	+	+	+	+

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6n - 1)

+ MINOR COMPONENT (LESS THAN 1%)

APPENDIX 5:4 THE EFFECT OF GROWTH TEMPERATURE ON TOTAL LIPID UNSATURATION

(I) M. PUSILLUS

TEMPERATURE (C)		(Δ MOLE) LIPID UNSATURATION 1	2	3	TREATMENT TOTAL
M. PUSILLUS "ASTON"	25	1.031	1.053	1.042	3.126
	30	0.964	0.952	0.952	2.868
	40	0.938	0.922	0.997	2.857
	50	1.073	1.165	1.076	3.314
M.PUSILLUS "LACEY"	25	1.042	1.048	1.060	3.150
	30	0.952	0.958	0.964	2.874
	40	0.886	0.944	1.094	2.924
	50	1.178	0.802	0.816	2.796

TWO WAY ANOVAR TABLE

SPECIES	TEMPERATURE (C) 25	30	40	50	SPECIES TOTAL
M. PUSILLUS "ASTON"	3.126	2.868	2.857	3.314	12.165
M. PUSILLUS "LACEY"	3.150	2.874	2.924	2.796	11.744
TEMPERATURE TOTAL	6.276	5.742	5.781	6.110	

APPENDIX 5:4 (Continued) ANOVAR SUMMARY TABLE (I) M. PUSILLUS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	0.202103959	23	8.7871286×10^{-3}	1.1423189
TREATMENTS (Tr)	0.07902596			
SPECIES (Sp)	7.38504×10^{-3}	1	7.38504×10^{-3}	0.9600486
TEMPERATURE (Te)	0.03345512	3	0.011151706	1.4497111
Sp x Te (Tr-(Te+Sp))	0.0381858	3	0.0127286	1.6547067
ERROR (T - Tr)	0.123077999	16	7.69236×10^{-3}	

APPENDIX 5:4 THE EFFECT OF GROWTH TEMPERATURE ON TOTAL LIPID UNSATURATION (II) M. HIEMALIS WEHMER

LIPID(Δ MOLE) UNSATURATION	TEMPERATURE (C) 5	10	15	20	25	30
1	1.317	1.345	1.355	1.401	1.498	0.919
2	1.360	1.387	1.377	1.286	1.278	0.955
3	1.267	1.443	1.393	1.416	1.401	0.941
TEMPERATURE TOTAL	3.944	4.175	4.125	4.103	4.177	2.795

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F
TOTAL (T)	0.519226278	17		
TEMPERATURE (Te)	0.436842946	5	0.087368589	*** 12.72615531
ERROR (T-Te)	0.082383332	12	6.8652776×10^{-3}	

LEAST SIGNIFICANT DIFFERENCE = 0.147

APPENDIX 5:4 THE EFFECT OF GROWTH TEMPERATURE ON TOTAL LIPID UNSATURATION

(III) M. HIEMALIS WEHMER F. HIEMALIS

LIPID(Δ MOLE) UNSATURATION	TEMPERATURE (C) 5	10	15	20	25
1	1.248	1.171	1.233	1.177	1.035
2	1.350	1.102	1.211	1.147	1.150
3	1.194	0.960	1.091	1.195	1.127
TEMPERATURE TOTAL	3.792	3.232	3.536	3.519	3.312

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F
TOTAL (T)	0.1196676	14		
TEMPERATURE (Te)	0.063904266	4	0.015976066	2.864976832
ERROR (T-Te)	0.055763334	10	0.0055763334	

APPENDIX 5:4 THE EFFECT OF GROWTH TEMPERATURE ON TOTAL LIPID UNSATURATION

(IV) M. PSYCHROPHILUS

LIPID(Δ MOLE) UNSATURATION	TEMPERATURE (C) 5	10	15	20
1	1.232	1.164	1.109	0.960
2	1.306	1.382	1.109	0.990
3	1.496	1.197	1.129	0.984
TEMPERATURE TOTAL	4.234	3.499	3.347	2.934

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F
TOTAL (T)	0.344967667	11		
TEMPERATURE (Te)	0.29415767	3	0.098052556	*** 15.43830933
ERROR (T-Te)	0.050809997	8	6.3512496×10^{-3}	

LEAST SIGNIFICANT DIFFERENCE = 0.150

APPENDIX 5:5 METHYL FATTY ACID COMPOSITION OF PHOSPHOLIPID COMPONENT AT OPTIMUM GROWTH TEMPERATURE

FATTY ACID	M. PUSILLUS "ASTON"	M. PUSILLUS "LACEY"	M. HIEMALIS WEHMER	M. H. W. F. HIEMALIS	M. PSYCHROPHILUS
14:0	5.7 ± 0.0	7.6 ± 3.3	10.0 ± 0.0	8.7 ± 1.1	10.0 ± 0.0
14:1	+	+	+	+	+
14:2	+	11.2 ± 5.4	11.0 ± 0.9	6.5 ± 1.4	7.3 ± 1.4
16:0	27.8 ± 0.8	28.2 ± 1.0	27.1 ± 0.8	20.0 ± 0.5	24.1 ± 0.5
16:1	4.6 ± 4.2	+	+	6.5 ± 1.4	7.3 ± 1.4
16:2	+	5.7 ± 0.0	12.4 ± 0.8	18.1 ± 0.0	5.7 ± 0.0
18:0	6.5 ± 1.4	5.7 ± 0.0	12.0 ± 0.8	10.5 ± 0.9	8.1 ± 0.0
18:1	43.3 ± 0.6	46.7 ± 4.1	23.8 ± 1.2	35.1 ± 0.7	28.9 ± 0.8
18:2	21.7 ± 0.5	19.3 ± 1.6	21.7 ± 0.5	31.0 ± 2.9	25.1 ± 0.0
20:0	12.9 ± 0.0	11.9 ± 1.7	23.1 ± 0.5	33.2 ± 0.0	22.3 ± 0.5
18:3	+	+	+	+	12.4 ± 0.8
20:1	+	+	24.9 ± 0.4	8.7 ± 1.1	22.5 ± 1.7
20:2	5.7 ± 0.0	7.3 ± 1.4	+	5.7 ± 0.0	+
20:3	5.7 ± 0.0	+	+	+	+

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6n - 1) + MINOR COMPONENT (LESS THAN 1%)

APPENDIX 5:6 METHYL FATTY ACID COMPOSITION OF PLASMA MEMBRANE PHOSPHOLIPID AT OPTIMUM GROWTH TEMPERATURE

FATTY ACID	M. PUSILLUS "ASTON"	M. PUSILLUS "LACEY"	M. HIEMALIS WEHMER	M. H. W. F. HIEMALIS	M. PSYCHROPHILUS
14:0	11.5 ± 0.0	11.5 ± 0.0	13.6 ± 0.9	+	10.0 ± 0.0
14:1	+	+	+	+	+
14:2	12.2 ± 0.8	10.8 ± 1.1	19.4 ± 1.3	9.9 ± 0.6	5.7 ± 0.0
16:0	29.4 ± 1.0	28.7 ± 0.9	32.0 ± 1.9	32.6 ± 0.0	22.4 ± 0.6
16:1	+	+	+	+	+
16:2	+	+	19.9 ± 0.6	22.4 ± 0.6	8.1 ± 0.0
18:0	12.9 ± 1.0	10.0 ± 0.0	+	+	10.8 ± 0.0
18:1	41.1 ± 0.8	43.8 ± 0.0	33.2 ± 0.0	33.5 ± 0.4	32.9 ± 0.4
18:2	14.2 ± 0.0	15.9 ± 0.8	17.5 ± 0.0	15.3 ± 0.0	27.7 ± 0.5
20:0	8.1 ± 0.0	10.0 ± 0.0	+	22.2 ± 1.0	26.2 ± 0.6
18:3	+	+	+	+	5.7 ± 0.0
20:1	+	+	10.0 ± 0.0	5.7 ± 0.0	+
20:2	5.7 ± 0.0	5.7 ± 0.0	+	+	+
20:3	5.7 ± 0.0	5.7 ± 0.0	+	+	12.9 ± 0.0

FIGURES REFER TO PERCENTAGE COMPOSITION AND ARE THE MEAN OF THREE REPLICATES

± STANDARD DEVIATION (6n - 1) + MINOR COMPONENT (LESS THAN 1%)

APPENDIX 5:7 A COMPARISON BETWEEN THE PHOSPHOLIPID UNSATURATION OF TOTAL AND PLASMA MEMBRANE PHOSPHOLIPID COMPONENTS AT OPTIMUM GROWTH TEMPERATURE

	PHOSPHOLIPID UNSATURATION (Δ MOLE)	
	TOTAL	PLASMA MEMBRANE
M. PUSILLUS "ASTON"	1.255 ± 0.032	1.290 ± 0.061
M. PUSLLIUS "LACEY"	1.271 ± 0.044	1.257 ± 0.032
M. HIEMALIS WEHMER	1.390 ± 0.038	1.342 ± 0.040
M. HIEMALIS W.F. HIEMALIS	1.410 ± 0.024	1.542 ± 0.024
M. PSYCHROPHILUS	1.649 ± 0.018	1.717 ± 0.006
	MEAN 3 REPS ± S.D.(6n-1)	MEAN 2 REPS ± S.D.(6n-1)

APPENDIX 5:8 THE EFFECT OF GROWTH TEMPERATURE ON PHOSPHOLIPID UNSATURATION

(I) M. PUSILLUS

TEMPERATURE (C)		PHOSPHOLIPID UNSATURATION			TEMPERATURE TOTAL
M. PUSILLUS "ASTON"	25	1.206	1.321	1.278	3.805
	30	1.202	1.285	1.216	3.703
	40	1.218	1.275	1.273	3.766
	50	1.219	1.308	1.213	3.740
M.PUSILLUS "LACEY"	25	1.133	1.300	1.183	3.616
	30	1.236	1.219	1.247	3.702
	40	1.221	1.302	1.291	3.814
	50	1.193	1.199	1.230	3.622

TWO WAY ANOVAR TABLE

SPECIES	TEMPERATURE (C) 25	30	40	50	SPECIES TOTAL
M. PUSILLUS "ASTON"	3.805	3.703	3.766	3.740	15.014
M. PUSILLUS "LACEY"	3.616	3.702	3.814	3.622	14.754
TEMPERATURE TOTAL	7.421	7.405	7.579	7.362	

APPENDIX 5:8 (Continued) ANOVAR SUMMARY TABLE (I) M. PUSILLUS

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	0.051395334	23	2.2345797×10^{-3}	0.9360829
TREATMENT (Tr)	0.01320067			
SPECIES (Sp)	2.816673×10^{-3}	1	2.816673×10^{-3}	1.1799263
TEMPERATURE (Te)	2.01584×10^{-3}	3	6.7194666×10^{-4}	0.2339973
Sp x Te (Tr-(Te+Sp))	8.368157×10^{-3}	3	2.7893856×10^{-3}	1.1684954
ERROR (T-Tr)	0.038194664	16	2.38716×10^{-3}	

APPENDIX 5:8 THE EFFECT OF GROWTH TEMPERATURE ON PHOSPHOLIPID UNSATURATION

(II) M. HIEMALIS WEHMER

PHOSPHOLIPID UNSATURATION (Δ MOLE)	TEMPERATURE (C) 5	10	15	20	25	30
1	1.400	1.374	1.376	1.368	1.423	1.244
2	1.376	1.374	1.366	1.381	1.349	1.277
3	1.384	1.372	1.398	1.392	1.397	1.212
TEMPERATURE TOTAL	4.160	4.120	4.140	4.141	4.169	3.733

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	0.053924925	17	3.1720555×10^{-3}	6.284067863
TEMPERATURE (Te)	0.047867616	5	9.5735232×10^{-3}	*** 18.9658313
ERROR (T-Te)	6.057329×10^{-3}	12	5.0477741×10^{-4}	

LEAST SIGNIFICANT DIFFERENCE = 0.040

APPENDIX 5:8 THE EFFECT OF GROWTH TEMPERATURE ON PHOSPHOLIPID UNSATURATION

(III) M. HIEMALIS WEHMER F. HIEMALIS

PHOSPHOLIPID UNSATURATION (Δ MOLE)	TEMPERATURE (C) 5	10	15	20	25
1	1.446	1.415	1.403	1.419	1.346
2	1.421	1.663	1.437	1.349	1.408
3	1.391	1.389	1.391	1.344	1.366
TEMPERATURE TOTAL	4.258	4.467	4.231	4.112	4.120

ANOVAR SUMMARY TABLE

SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	0.081489734	14	5.8206952×10^{-3}	1.079372446
TEMPERATURE (Te)	0.027563073	4	6.8907682×10^{-3}	1.277803608
ERROR (T-Te)	0.053926661	10	0.0053926661	

APPENDIX 5:8 THE EFFECT OF GROWTH TEMPERATURE ON PHOSPHOLIPID UNSATURATION

(IV) M. PSYCHROPHILUS

PHOSPHOLIPID UNSATURATION (Δ MOLE)	TEMPERATURE (C) 5	10	15	20
1	1.613	1.641	1.481	1.407
2	1.640	1.636	1.375	1.462
3	1.632	1.669	1.433	1.435
TEMPERATURE TOTAL	4.885	4.946	4.289	4.304

ANOVAR SUMMARY TABLE

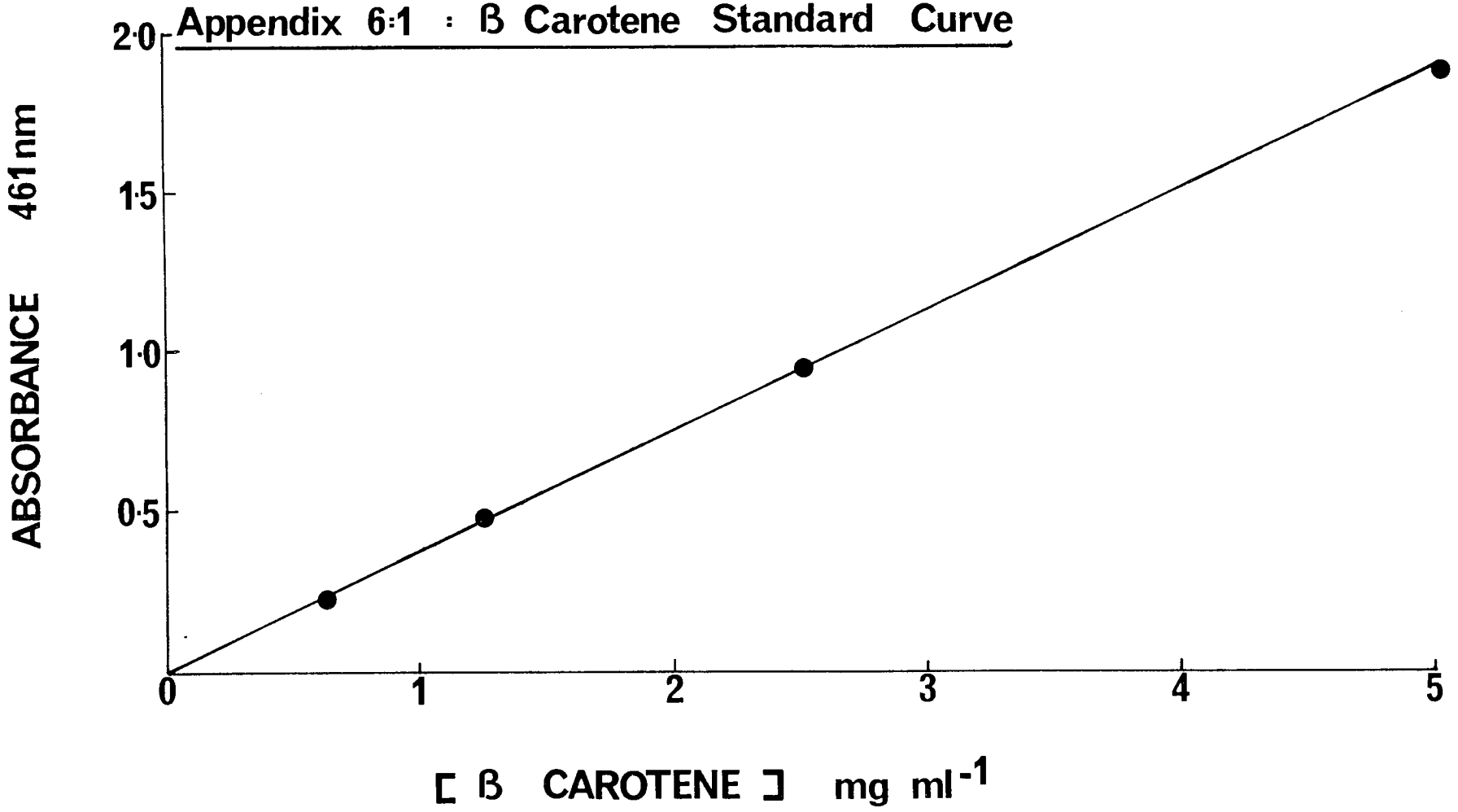
SUM OF VARIATION	SS	Df	MS	F
TOTAL (T)	0.136542667	11	0.012412969	12.16262568 ***
TEMPERATURE (Te)	0.128378003	3	0.042792667	41.92962944
ERROR (T-Te)	8.164664×10^{-3}	8	1.020583×10^{-3}	

LEAST SIGNIFICANT DIFFERENCE = 0.060

APPENDIX 6:1

ß-CAROTENE STANDARD CURVE

Appendix 6:1 : ß Carotene Standard Curve



APPENDIX 7:1 ISOELECTRIC FOCUSING DATA

(1) RUNNING CONDITIONS

CONDITION	INITIAL	FINAL
Voltage,U(v)	300	1500
Current, I(mA)	50	30
Power,P(W)	30	30

Anode Electrode Solution 1M H_3PO_4,
Cathode Electrode Solution 1M NaOH,
Cooling Temperature 10C, Time 1.5 hours.

(2) SILVER STAIN (i) REAGENTS

REAGENT	COMPOSITION
Fixative A	30% Methanol/10% Trichloroacetic Acid (TCA)/3.5% Sulphosalicylic Acid (v/w/w)
Fixative B	30% Methanol/12% TCA (v/w)
Fixative C	10% Methanol/5% Acetic Acid (v/v)
Oxidiser (25C)	0.0034 M Potassium Dichromate/0.0032 M Nitric Acid
Silver Reagent (25C)	0.012 M Silver Nitrate
Developer (25C)	0.28 M Sodium Carbonate/0.5 ml Formalin (Paraformaldehyde) per litre.
Stop	5% TCA (w/v)

All solutions made up in deionised water (less than 1μ mho conductivity). Gels stored in 50% methanol (v/v) to control swelling.

SILVER STAIN (ii) STAINING PROTOCOL

REAGENT	Volume (ml)	Duration
1. Fixative A	400	1 hour
2. Fixative B	400	Overnight (several changes)
3. Fixative C	400	30 minutes
4. Fixative C	400	30 minutes
5. Deionised Water	300	10 minutes
6. Deionised Water	300	10 minutes
7. Deionised Water	300	10 minutes
8. Oxidiser	300	15 minutes
9. Deionised Water	300	10 minutes
10. Deionised Water	300	10 minutes
11. Deionised Water	300	10 minutes
12. Silver Reagent	300	30 minutes
13. Deionised Water	300	10 minutes
14. Deionised Water	300	10 minutes
15. Developer	300	30 seconds
16. Developer	300	5 minutes
17. Developer	300	*5 minutes
18. Stop	300	10 minutes

* May be omitted depending on the desired intensity of the bands

APPENDIX 7:2 THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASE ACTIVITY

SPECIES	TEMPERATURE (C)	ATPase ACTIVITY ($\mu M\ PO_4$ h^{-1} mg^{-1}) 1	2	TOTAL
M. PUSILLUS "ASTON"	10	0.51	0.51	1.02
	20	0.53	0.56	1.09
	30	0.62	0.63	1.25
	40	0.73	0.71	1.44
	50	0.60	0.67	1.27
	60	0.60	0.49	1.09
	70	0.67	0.47	1.14
M. PUSILLUS "LACEY"	10	0.56	0.67	1.23
	20	0.72	0.72	1.44
	30	0.77	0.83	1.60
	40	0.90	0.89	1.79
	50	0.81	0.72	1.53
	60	0.53	0.49	1.02
	70	0.53	0.63	1.16
M. HIEMALIS WEHMER	10	1.05	0.88	1.93
	20	0.88	0.88	1.76
	30	1.75	1.93	3.68
	40	0.88	1.05	1.93
	50	1.05	0.53	1.58
	60	0.88	0.88	1.76
	70	0.88	0.88	1.76
M. HIEMALIS WEHMER F. HIEMALIS	10	1.03	1.61	2.64
	20	1.73	1.73	2.46
	30	2.63	2.05	4.68
	40	1.46	1.76	3.32
	50	1.61	1.76	3.37
	60	1.46	1.61	3.07
	70	1.46	1.46	2.92
M. PSYCHROPHILUS	10	0.19	0.19	0.38
	20	0.40	0.39	0.79
	30	0.32	0.27	0.59
	40	0.44	0.32	0.76
	50	0.21	0.26	0.47
	60	0.18	0.12	0.30
	70	0.14	0.19	0.33

APPENDIX 7:2 TWO WAY ANOVAR TABLE

SPECIES	TEMPERATURE (C) 10	20	30	40	50	60	70	SPECIES TOTAL
M. PUSILLUS "ASTON"	1.02	1.09	1.25	1.44	1.27	1.09	1.14	8.30
M. PUSILLUS "LACEY"	1.23	1.44	1.60	1.79	1.53	1.02	1.16	9.77
M. HIEMALIS WEHMER	1.93	1.76	3.68	1.93	1.58	1.76	1.76	14.40
M. HIEMALIS WEHMER F. HIEMALIS	2.64	1.46	4.68	3.22	3.37	3.07	2.92	21.36
M. PSYCHROPHILUS	0.38	0.79	0.59	0.76	0.47	0.30	0.33	3.62
TEMPERATURE TOTAL	7.20	6.54	11.80	9.14	8.22	7.24	7.31	

APPENDIX 7.2 ANOVAR SUMMARY TABLE

SUM OF VARIATION	SUM OF SQUARES (SS)	DEGREES OF FREEDOM (Df)	MEAN SQUARE (MS)	F
TOTAL (T)	18.30306429	69	0.265261801	14.417521
TREATMENT (Tr)	17.65911429			
SPECIES (Sp)	12.92545714	4	3.231364285	*** 175.63126
TEMPERATURE (Te)	1.93129429	6	0.321882381	*** 17.494961
Sp x Te (Tr-(Te+Sp))	2.80236286	24	0.116765119	*** 6.3464275
ERROR (T-Tr)	0.64395	35	0.0183985	

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