STUDIES ON THERMOPHILIC FUNGI WITH PARTICULAR REFERENCE TO THEIR NITROGEN NUTRITION AND LIPID COMPOSITION.

by

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CONTAINS PULLOUTS

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ABSTRACT.

Two aspects of the physiology of thermophilic fungi have been investigated, their nitrogen nutrition and lipid composition. The main experimental material was the phycomycete fungus Mucor pusillus.

Preliminary experiments indicated that M. pusillus grew and spored well in still culture at 45°-50°, on a synthetic medium containing a nitrogen source at a concentration of 250mg. N/l. and a carbon source at 20g./l. These culture conditions were used in a study of the effect of different nitrogen sources on the growth and sporulation of this fungus. Nutritionally, M. pusillus proved extremely versatile, being able to grow and spore on a wide range of inorganic and organic nitrogen sources.

The fatty acid composition of the mycelial lipids of psychrophilic, mesophilic, thermotolerant and thermophilic fungi in the Mucorales was studied by gas-liquid chromatography (GLC), while the individual fatty acids were identified by combined mass spectrometry and gas chromatography. The lipids of thermophilic and thermotolerant fungi were found to be more saturated than those of psychrophilic and mesophilic fungi.

The effect of various environmental factors on the fatty acid composition of the lipids of M. pusillus were also studied

by GLC. The mycelial lipids were found to be more unsaturated when the fungus was grown at a lower temperature, or when the nitrogen content of the medium was increased; increasing the carbon concentration of the medium resulted in increased synthesis of mycelial lipids. Electrometric determination of the oxygen partial pressure (pO_2) in the medium of cultures of M. pusillus showed that at 50° the fungus grew under almost completely anaerobic conditions, and the possibility that the oxygen concentration may limit the biosynthesis of unsaturated fatty acids was investigated. Increasing the oxygen concentration of the gas phase above growing surface cultures resulted in only small increases in the degree of lipid unsaturation, and it is considered that significant increases in lipid unsaturation would require highly aerated submerged culture conditions.

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INTRODUCTION.

The simplest description of the term "thermophilic" is based on its literal derivation from the Greek, thermos meaning heat, and philos meaning loving, or having an affinity for. A thermophile is therefore an organism which has an affinity for temperatures higher than the normal, as distinct from a mesophile, which prefers a more moderate temperature, and a psychrophile which prefers a low temperature. It is more than one hundred years since the discovery of thermophilic micro-organisms; thermophily is now known to occur in the algae, bacteria, Actinomycetes and fungi.

Historically, the first group of thermophilic micro-organisms to be reported were algae growing in the geysers of California (Brewer 1866). Thermophilic algae, the majority of which are members of the Cyanophyta (blue-green algae), have optimum growth temperatures in the range 50°-75° (Brock and Brock 1966, Brock 1967).

The first report of a thermophilic fungus was by Lindt (1886). In the fungi the thermophilic habit appears to be restricted to members of the Phycomycetes, Ascomycetes and Fungi Imperfecti; no thermophilic basidiomycete fungus has yet been isolated. Thermophily is less well developed in the fungi

than in other micro-organisms, and the maximum growth temperature reported for fungi is 60° (Cooney and Emerson 1964, Sumner, Morgan and Evans in press).

The first discovery of a thermophilic bacterium is attributed to Miquel (1888). The majority of thermophilic bacteria are members of the genus Bacillus, though thermophily also occurs in members of several other groups. Thermophilic bacteria grow well in the temperature range 50°-70° (Allen, 1953), as also do thermophilic actinomycetes (Erikson 1955).

There is some controversy regarding the maximum temperature for the growth of micro-organisms. Working with blue-green algae, Kempner (1963) reported an upper temperature limit of 73°. There is evidence however that the upper temperature for bacterial growth is considerably in excess of this figure. Copeland (1936) has reported profuse bacterial growth in hot springs of the Yellowstone National Park at 85-88°, and Zobell and Johnson (1949) consider that bacteria in oil well brines may grow at temperatures around 100°.

Since thermophily has developed more strongly in the algae, bacteria and Actinomycetes than in the fungi, it is necessary to use different temperature criteria in order to define a thermophilic fungus. The most widely accepted definition is that of Cooney and Emerson (1964), which states that "a thermophilic

fungus is one that has a maximum temperature for growth at or above 50° and a minimum temperature for growth at or above 20°". Cooney and Emerson consider that a thermotolerant fungus is one which has a maximum growth temperature around 50°, but has a minimum temperature for growth below 20°.

There have been a large number of studies directed towards discovering the physiological bases of thermophily; the subject has been extensively reviewed by Gaughran (1947), Allen (1953), Koffler (1957), Ingraham (1962), Langridge (1963), Cooney and Emerson (1964), Farrell and Rose (1967) and Campbell and Pace (1968). In general, studies have been concentrated on the temperature-labile molecules of the cell, e.g. nucleic acids, proteins and lipids. Much of the available data have been obtained from studies with thermophilic bacteria, in particular with Bacillus stearothermophilus and these results are broadly applicable to other micro-organisms.

Several comparisons of the heat stability of nucleic acids from thermophilic and mesophilic bacteria have been made. The "melting" temperatures (T_m) and the base compositions of the DNA of thermophilic bacteria have been found to be similar to those of mesophilic bacteria (Marmur 1960, Walker and Campbell 1965, Saunders and Campbell 1966, Pace and Campbell 1967). In addition a comparison of the heat stability of the RNA of the

mesophile Escherichia coli and the thermophile Bacillus stearothermophilus revealed no significant differences; the similarities in thermal stability of the RNA of these organisms applied both to the soluble RNA (S-RNA) (Arca 1964, Saunders and Campbell 1966, Friedman and Weinstein 1966) and to the ribosomal RNA (r-RNA)(Mangiatini et al. 1962, 1965, Saunders and Campbell 1966).

However it is interesting to note that in B. stearothermophilus the ribosomes themselves were more heat stable than the ribosomes of E. coli; a fact supported by experiments on the effect of temperature on amino acid incorporation (Friedman and Weinstein 1966, and Friedman et al. 1967), and also by thermal denaturation experiments (Mangiatini et al. 1962, 1965, Saunders and Campbell 1966, Pace and Campbell 1967, Stenesh and Yang 1967, Stenesh and Holazo 1967, Friedman et al. 1967). In a study involving 19 selected organisms which included psychrophiles, mesophiles and thermophiles, Pace and Campbell (1967) found a positive relationship between melting temperature of the ribosomes and the maximum growth temperature of the organism.

The reasons for the increased heat stability of the ribosomes of thermophiles are not known, though in view of the fact that the r-RNA species from B. stearothermophilus and

E. coli are almost equally thermostable, enhanced stability of thermophile ribosomes is not attributable to the nucleic acid portion of the organelle. Moreover, since a comparison of the total amino acid composition of B. stearothermophilus ribosomes with that reported for E. coli ribosomes (Spahr 1962) showed no significant differences, the protein composition of the ribosome is unlikely to be responsible for the heat stability of the organelle.

Campbell and Pace (1968) consider that in the intact ribosome, thermal stability may depend on the fine structure of the RNA - protein interaction, and suggest also that the primary structure of the ribosome proteins may be important in this regard.

A great deal of controversy has resulted from the view of Setchell (1903) that the existence of thermophilic micro-organisms at the extremely high temperatures of hot springs must involve some intrinsic difference between their proteins and the proteins of mesophiles. There is a considerable amount of evidence in support of this view, and an impressive number of enzymes have been extracted which are more heat stable when derived from thermophiles than when derived from mesophiles. However, as the majority of these experiments were carried out

with crude cell extracts it was not possible to state unequivocally that the enzymes examined were intrinsically heat stable; for heat stability might have been due to the presence of protective factors in the cell extract. In this connection a number of substrate-protected enzymes have been isolated which are inactivated at their temperature of production in the absence of substrate, e.g. isocitrate lyase (Daron 1967) and certain membrane bound enzymes (Downey, Georgi and Militzer 1962).

An intensive study has been carried out involving purification and crystallisation, of an extracellular ∝-amylase from B. stearothermophilus (Manning and Campbell 1961, Manning, Campbell and Foster 1961, Campbell and Manning 1961, Campbell and Cleveland 1961). Crystalline ∝-amylase was found to be extremely heat stable, having an optimum temperature for starch hydrolysis between 55° and 70°. Crystalline glyceraldehyde-3-phosphate dehydrogenase isolated from B. stearothermophilus has also been found to be thermostable (Amelunxen 1966, 1967). This is convincing evidence that the pure enzyme is heat-stable, since there is little possibility of it being protected by extraneous material.

The flagella of bacteria, since they consist almost entirely of pure proteins (flagellins) have proved useful

material for studies on thermostability of non-enzymic proteins (Koffler 1957, Koffler, Mallett and Adye 1957). Koffler *et al.* (1957) found that flagellins were more heat resistant when derived from a thermophilic *Bacillus* sp. than from the mesophile *E. coli*. These investigators consider that the greater stability of thermophile flagellins may be due to more numerous and more strategically sited hydrogen bonds, compared with the mesophile flagellins. A similar conclusion was reached by Amelunxen (1967) who demonstrated greater resistance to structural change (as caused by disruption of hydrogen bonding in 8.0 M urea) of glyceraldehyde-3-phosphate dehydrogenase obtained from *B. stearothermophilus* compared with the same enzyme crystallised from rabbit muscle.

There is, therefore, an abundance of evidence to support the view that the cell proteins of thermophiles are relatively more heat stable than their counterparts in mesophiles and psychrophiles. On the other hand there is some evidence to support an alternate hypothesis of thermophily, the "dynamic" hypothesis, in which thermophily is dependent on a capacity for rapid resynthesis of inactivated cell components (Gaughran 1947, Allen 1953).

The "dynamic" or "rapid resynthesis" hypothesis is based upon two pieces of evidence. The first is the finding that certain enzymes of thermophiles are not heat resistant. Secondly, the fact that at high temperatures thermophiles tend to require increasingly complex nutrients is taken as evidence that at high temperatures thermophiles require more ready-made building blocks for rapid enzyme resynthesis (Allen 1950, Baker et al. 1953, 1955).

Rapid resynthesis depends on a high "turnover" of those cellular components which are thermolabile - proteins and nucleic acids. In an investigation of the turnover of protein and nucleic acid in B. stearothermophilus and E. Coli (Bubela and Holdsworth 1966) it was found that at 40° B. stearothermophilus had a more rapid protein and nucleic acid turnover than E. coli. In the thermophile, protein turnover became more rapid as the temperature was increased, being 5-10 times more rapid at 63° than at 40°; the rate of nucleic acid turnover did not increase as the temperature was raised. This investigation also showed that in B. stearothermophilus, amino acid accumulation, and incorporation of uracil into RNA proceeded very slowly below 40°. It may be

therefore, that thermophiles are unable to grow at lower temperatures because the low rates of RNA synthesis and consequentially of protein synthesis.

It is possible that the ability to grow at high temperatures is influenced not only by the enhanced thermostability of certain cell components but also by the rapid turnover of cellular protein.

Temperature has a marked effect on the physical and chemical properties of the cellular lipids, which has led to the idea that the lipid composition may influence the temperature range for growth of micro-organisms. Gaughran (1947a) has suggested that cells cannot grow at temperatures below the solidification point of their lipids (lipid solidification theory) and Heilbrunn (1924) and Belehradek (1931) proposed that at high temperatures melting of lipids destroys essential cell structures. Therefore according to the 'lipid' concepts the minimum growth temperature of the organism is decided by the temperature at which some lipid components solidify and its maximum by that temperature at which other lipid components melt.

Apart from storage lipids, which may have only a temporary presence, cellular lipids are located mainly in the

membrane systems. Membranes, besides providing an internal framework for the spatial arrangement of organelles within the cell, are also active in the transport of solutes into and out of the cell. The most important lipids of bacterial cell membranes are phospholipids (Lennarz 1966, Chapman 1967). Phospholipids can exist in either of two physical states, liquid or crystalline, depending on the freezing points of their fatty acid side chains (Byrne and Chapman 1964). Unsaturated fatty acids have a lower freezing point than saturated fatty acids, and the presence of unsaturated fatty acid side chains lowers the freezing point of phospholipids (Byrne and Chapman 1964).

These findings can be linked with the observations that in poikilothermic organisms, the lipids formed at low temperatures are more unsaturated and therefore presumably more liquid than lipids formed at higher temperatures (Terroine et al.1927, 1930; Pearson and Raper 1927, Gaughran 1947a, Johnson 1957, Kates and Baxter 1962 and Marr and Ingraham 1962). However, Marr and Ingraham found that the fatty acid composition of E. Coli could also be influenced by changes in the concentration of nutrients in the culture medium. These

investigators concluded that there was no direct relation between the fatty acid composition of the bacterium and the incubation temperature, and therefore that the fatty acid composition does not determine the minimum temperature for growth. Investigations of the lipid composition of thermophilic bacteria have tended to support the conclusions of Marr and Ingraham. An extension of the 'lipid solidification' theory might lead one to predict that thermophiles would have high proportions of saturated fatty acids and a low degree of lipid unsaturation. However Gaughran (1947a) found that increasing the temperature had little effect on the degree of unsaturation of the lipids of a thermophilic Bacillus sp. and Long and Williams (1960) found that the lipids of spores of B. stearothermophilus became more unsaturated at higher temperatures.

It is possible to sum up the evidence presented above by listing a number of factors which may enable thermophiles to live at high temperatures, e.g. high levels of thermostability of ribosomes and enzymic and non-enzymic proteins; increased rates of protein and nucleic acid turnover which may or may not be associated with a loss of metabolic activity as seen by

increased nutrient requirements at high temperature. No information could be found in the literature to indicate whether evolution of the thermophilic habit has involved adaptive changes in the fatty acid composition of the lipids.

It must be emphasised that almost without exception the investigations into the biochemical basis of thermophily so far mentioned have been performed with thermophilic bacteria. Comparable information for thermophilic fungi is restricted to the investigations of lipase and acid protease activities of Mucor pusillus (Somkuti and Babel 1968, 1968a, 1968b) in which it was found that both enzymes had an optimum temperature of about 55° though above this temperature loss of activity was extremely rapid.

It was considered that there were two main areas in which research with thermophilic fungi would be profitable, firstly, a study of the effect of nutrition on the growth and sporulation of a thermophilic fungus grown near its maximum temperature, and secondly an investigation of the fatty acid composition of thermophilic fungi.

A study on the nutrition of a thermophilic fungus commended itself for two reasons. Firstly, only recently has it become realised that thermophilic and thermotolerant fungi

are of widespread ecological occurrence. In addition to the habitats listed by Cooney and Emerson (1964, pages 122 and 123) thermophilic fungi have been isolated from the cooling towers of power stations and still-warm coal spoil tips (Sumner, Morgan and Evans in press), city rubbish (Von Klotopek 1962) and birds nests (Apinis and Pugh, 1967). In addition, thermophilic fungi are of importance economically in composting and the self-heating of stored agricultural products (Fergus 1962, Cooney and Emerson 1964, Hudson and Yung Chang 1967). Indeed, Fergus (1962) has stated of thermophilic fungi "studies of their nutrition and enzyme activities in chemically defined media with single carbon and nitrogen sources would help to elucidate their action and importance in the degradation of complex organic debris in nature".

Secondly it is known that at high temperatures the nutrient requirements of micro-organisms become more complex than at lower temperatures. This is taken as evidence for the loss of metabolic activity due to enzyme inactivation, growth occurring only if the end-product of the inactivated enzyme-catalysed reaction is exogenously supplied. Langridge (1963) cites ample evidence for the loss of metabolic activity in B. stearothermophilus grown at high temperatures; no

comparable date could be found in the literature concerning thermophilic fungi.

As mentioned earlier, very little is known about the composition of the lipids of thermophiles and for this reason it was considered that a study of the fatty acid composition in psychrophiles, mesophiles and thermophiles growing at their optimum temperatures would be useful.

The main experimental material used throughout this investigation was Mucor pusillus Lindt. Taxonomically this fungus is a member of the zygomycete family in the Class Phycomycetes; a complete taxonomic account is given by Emerson and Cooney (1964). Physiologically, M. pusillus is a true thermophile, based on the definition by Cooney and Emerson, and it grows well in both shake and still culture in a simple, chemically defined medium without growth factors. In addition the nutrition and lipid composition of several mesophilic fungi in the Mucoraceae have also been studied, thus providing useful data for comparative purposes.

CHAPTER I. MATERIALS AND METHODS.

1.1 Chemicals.

Wherever possible chemicals used were of A.R. grade, or of the highest purity available commercially. Solvents were not redistilled except where specifically stated. Amino acids were Biochemical grade (chromatographically homogeneous) supplied by British Drug Houses Ltd., Poole, Dorset; BF_3/methanol reagent, Florisil, glycerol tristearate, glycerol monostearate, cholesterol stearate and cholesterol were also supplied by B.D.H. Ltd. Fatty acid methyl esters (99% pure by GLC) were purchased from Fluka A.G., Buchs, Switz. Diethyleneglycolsuccinate and chromosorb G were supplied by Varian Aerograph Ltd.,Wythenshawe, Manchester, England. Davison's silica was obtained from Koch-Lite Laboratories Ltd., Colnbrook, Bucks., and Kieselgel PF_{254} from Merck A.G., Darmstadt.

1.2 Organisms.

Of the organisms used in this investigation the following were obtained from the Commonwealth Mycological Institute, Kew, England:

Mucor pusillus	Lindt.	(IMI. 71629).
Mucor ramannianus	Moller.	(IMI. 35044 a).
Mucor racemosus	Fresen.	(IMI. 103730).
Mucor hiemalis (+)	Wehm.	(IMI. 21216).
Mucor hiemalis (-)	Wehm.	(IMI. 21217).
Mucor mucedo	Auct.	(IMI. 103731).

Three isolates of Mucor strictus and two strains of Mucor oblongisporus were obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands:

Mucor strictus Hagem.	(CBS. 100.66)
" " "	(CBS. 575.66)
" " "	(CBS. 576.66)
Mucor oblongisporus Naoumoff.	(CBS. 173.27)
" " "	(CBS. 220.29)

In addition a number of thermophilic and thermotolerant fungi were isolated and made available by Mr. H.C. Evans of this department. These species were isolated in the locality of The University of Keele, Newcastle, Staffs:

Mucor sp. 1 (isolated from coal waste tip).

Mucor sp. 2 (isolated from cow dung).

Mucor sp. 3 (isolated from coal waste tip).

Rhizopus sp. 1 (isolated from horse dung).

Rhizopus sp. 2 (isolated from cooling tower of a power station).

Rhizopus sp. 3 (isolated from coal waste tip).

All species were maintained on potato-dextrose agar slopes and subcultured monthly.

1.3 Methods of Culture.

The culture medium was similar to that of Brian et al. (1946). The basic medium contained per litre of deionised water:

KH_2PO_4	1.0g.
$MgSO_4.7H_2O$	0.5g.
Minor element solution	1.0ml.

The minor element solution contained, per litre of deionised water:

$FeSO_4.7H_2O$	0.100g.
$CuSO_4.5H_2O$	0.015g.
$ZnSO_4.7H_2O$	0.100g.
$MnSO_4.4H_2O$	0.100g.
$KMoO_4$	0.010g.

Various nitrogen and carbon sources were added in specific concentrations to the basic medium; full details of these additions will be given in the appropriate experimental sections.

The initial pH of culture media was adjusted to 6.0-6.5 with IN.NaOH. In some experiments sodium succinate (5g./l.) was included to increase the buffering capacity of the culture medium (Morton and MacMillan 1954).

Generally, media were sterilised by autoclaving at 15 lb./in.^{2} for 15 min. However, where proteins, peptides, tryptophan, cystine or urea were used as sources of nitrogen,

these media were sterilised by filtration through a sterile membrane (0.2 μ diameter pores) to prevent thermal decomposition.

Inocula were grown for 7-21 days on potato-dextrose agar slopes dispensed in flat medicine bottles. Spore suspensions were obtained by washing the cultures in ice-cold sterile deionised water and diluting with sterile water to a concentration of $1.5\text{-}2.0 \times 10^{-6}$ spores/ml. (by haemacytometer count).

1.4 Types of Experiment.

1.41 Still Culture.

1.411 Nutrition Experiments. Culture medium (20 ml.) was dispensed into wide-necked 100 ml. Erlenmeyer flasks, plugged with non-absorbent cotton wool and sterilised. The flasks were inoculated with 1 ml. of standard spore suspension and incubated at the required temperature. Replicate flasks (usually 5) were harvested at suitable time intervals, the mycelium filtered off, washed with deionised water, dried and weighed. The culture filtrate was analysed for residual nitrogen content, and the pH determined.

1.412 Lipid Experiments. Culture medium (200 ml.) was dispensed into 750 ml. Erlenmeyer flasks and sterilised. The flasks were each inoculated with 5 ml. of standard spore suspension and incubated at the required temperatures. Replicate flasks (usually 3) were harvested at suitable time intervals, the mycelium filtered off, washed and dried before extraction of the lipid.

1.42 Shake Culture. Inoculated flasks were incubated on a reciprocating shaker which gave 90 strokes/min. each stroke comprising a 4 in. movement. Cultures were placed in a compartment constructed of "Marinite" asbestos sheet and insulated by a laminate of 1 in. polystyrene. This compartment could be heated to any temperature from ambient to 55°. The nichrome wire heaters were situated in the floor of the incubation cabinet and alloy paddles hanging immediately above the heaters effectively dispersed heat as the cabinet moved back and forth. A heat gradient of 1-2° existed between the floor and the roof of the cabinet. The incubation cabinet was designed to receive up to one hundred 100 ml. conical flasks or thirty 750 ml. conical flasks.

1.43 Solid Culture. Sterile culture medium containing 2% agar was poured into sterile plastic petri dishes (8.5 cm. diameter). A small amount of inoculum was transferred by sterile wire and the plates incubated at the selected temperature.

1.5 Measurement of Growth.

1.51 Dry weight. The dry weight was determined by filtering mycelium through a tared sintered glass disc (A.G. Gallenkamp Ltd. Grade I). The resulting mycelial pad was washed with deionised water, dried overnight in an oven at 80° and weighed after cooling to room temperature in a desiccator. The mycelial weight was expressed as mg. dry weight/ml. of culture medium.

In certain circumstances it proved more convenient to describe growth in terms of "cumulative growth". A value for "cumulative growth" was obtained by summing the dry weight measurements obtained at individual harvests of a time-course experiment. e.g. the following dry weight values for the growth of M. pusillus after various incubation periods are summed and growth expressed as "cumulative growth":

time of harvest (hours)	24, 48, 72, 96, 120.
dry weight (mg./ml.).	1.0, 2.0, 3.0, 4.0, 4.0.
"cumulative growth"	= 14.0

1.52 Colony Diameter. On petri dishes, linear growth in cm. was measured as the colony diameter. Where the colony was not circular in shape the long and short axes were measured and the average taken as the measure of growth.

1.6 Measurement of Sporulation.

In some experiments the number of spores produced by the organism was measured. The mycelial mats were carefully removed from the harvested Erlenmeyer flask, transferred to a 250 ml. round-bottom flask containing 50 ml. of 0.5% (v.v.) aqueous solution of "Teepol" and shaken for 15 minutes at high speed on a microid wrist-action shaker. The mycelial mat was removed and retained for dry weight determination. The spore suspension

was made up to 100 ml. with deionised water and the spore density calculated using a haemacytometer chamber in which 80 small squares were counted. The density of each spore suspension was the average value of three such spore counts. Sporulation was expressed either as the number of spores/ml. medium, or as "sporulation index" which is the number of spores (in millions) mg./dry weight mycelium.

The term "cumulative sporulation" was also used. This value was derived by summing the sporulation counts recorded at individual harvests during a time-course experiment. Where an assessment of the density of sporulation per unit weight of mycelium over the entire incubation period was required, the results were expressed as the "cumulative sporulation index". This value was obtained by summing the "sporulation indices" obtained at individual harvests throughout a time-course experiment.

1.7 Analytical Methods.

1.71 pH Measurements were made with a Pye Model 79 bench pH meter using a glass-calomel electrode system.

1.72 Determination of Partial Pressure of Oxygen (pO_2) of Culture Medium.

the pO_2 of culture medium was determined electrometrically using Astrup micro-equipment (Radiometer, Copenhagen) equipped

with an oxygen monitor (Type PHA.928) and oxygen electrode (Type E 5046). Determinations were carried out either at 25° or 50°, depending on the temperature at which the cultures had been incubated. The instrument was allowed to equilibrate at the required temperature for at least 1 hour before calibration. Calibration was carried out as follows: the oxygen electrode was first zero-ed using an oxygen-free solution of 0.01M borax solution containing a few crystals of sodium sulphite. The electrode was then calibrated using a solution of known pO_2; this solution was water saturated with air at the operating temperature. The pO_2 of the solution was calculated using the formula:

$$pO_2 = (B-a) \times \frac{O_2\%}{100}$$

where B = barometric pressure in mm. Hg.

a = partial pressure of water vapour at the operating temperature.

$O_2\%$ = % volume of oxygen in gas used for aeration (i.e. in air = 20.93%). The calibration of the oxygen electrode was checked periodically during the determinations by using the solution of known pO_2. Samples of culture medium were extracted from beneath the mycelial mat by using a disposable syringe with a fine needle. In each pO_2 determination two samples of culture medium were withdrawn; the first sample was used to dispel air

bubbles from the syringe, and the second sample was introduced into the oxygen electrode and used for the actual pO_2 determination. The electrode was flushed with distilled water between individual determinations.

Water baths heated at 25° or 50° were used to maintain cultures awaiting pO_2 determination at the same temperature at which they had been incubated. On each culture medium, pO_2 determinations were carried out in triplicate; partial pressure values were obtained as pO_2 (mm. Hg).

The concentration of dissolved oxygen (μ mole/litre) was obtained from the pO_2 by use of Henry's Law:

$$X = \frac{pO_2}{K}$$

where X = Dissolved O_2 in water expressed as the mole fraction.

K = Henry's Law Constant.

Values for K at various temperatures were obtained from the Handbook of Chemistry and Physics (1949).

$$K_{25^\circ} = 3.25$$

$$K_{50^\circ} = 4.50$$

Concentration of dissolved O_2 (in μ mole /l.) were then obtained from the mole fraction, assuming water contains 55.5 mole/l.

1.73 Total Nitrogen of both culture filtrates and mycelium was determined by the micro-Kjeldahl method. The sample was digested in 1 ml. Analar H_2SO_4 (nitrogen-free) containing a small amount of selenium dioxide to act as a catalyst. Digestion was continued for two hours after clearing, when the digest was transferred quantitatively to a Markham distillation unit, made alkaline with 50% NaOH solution and steam distilled. The ammonia liberated was absorbed in 10 ml. of 2% boric acid containing Conway's mixed indicator (Conway 1947) and titrated with standard HCl.

Conway's mixed indicator contained bromocresol green (33 mg.) and methyl red (66 mg.) dissolved in 100 ml. 95% ethanol. The boric acid absorbent containing Conway's mixed indicator was prepared by dissolving boric acid (20g.) in 200 ml. absolute ethanol plus 700 ml. deionised water, adding 10 ml. Conway's mixed indicator, and making solution up to 1 litre.

1.74 Ammonia Nitrogen in culture filtrates was determined by the Conway microdiffusion method (Conway 1947). The culture filtrate (1 ml.) was added to the outer annulus of the Conway Unit, and the ammonia liberated by the addition of N/2 KOH (1 ml.) was absorbed in the central well into 2% boric acid (1 ml.) containing Conway mixed indicator. The ammonia in the central well was determined by titration with N/50 HCl. The microdiffusion process

was generally allowed to proceed overnight. A blank determination was carried out containing distilled water (1 ml.) in the outer annulus instead of culture filtrate and subtracted from the total.

To avoid confusion it should be pointed out that throughout this investigation the term "ammonia" is intended as a general term, synonymous with "ammonia-nitrogen"; it is not intended, except where stated, to specify only ammonia in the undissociated form.

1.75 Nitrate Nitrogen was determined by the Conway microdiffusion method (Conway 1947). Nitrate in the culture filtrate was reduced to ammonia by the addition of 0.1-0.2g. powdered Devarda's Alloy. Microdiffusion, absorption and determination of ammonia was carried out exactly as described in Section 1.74. Preformed ammonia was also estimated under the same conditions without Devarda's Alloy, and was subtracted, together with a Devarda's Alloy blank containing distilled water, from the total.

1.76 Nitrite Nitrogen in culture filtrates was determined by the Conway microdiffusion method (Conway 1947), nitrite being reduced to ammonia by the addition of Devarda's alloy. Preformed ammonia and a Devarda's alloy blank were subtracted from the total.

1.77 The Detection of Amino Acids by Paper Chromatography. Where single amino acids were used as nitrogen source their presence in the culture filtrate was determined by ascending paper

chromatography on Whatman Number 20 paper. The solvent system was n-butanol, acetic acid, water (100:25:50 v./v.). After drying the developed chromatograms, the amino acids were detected by spraying the papers with a ninhydrin solution (0.1% in 95% ethanol) and heating in an oven at 60° for 5 minutes.

1.8 Lipid Techniques.

1.81 Extraction and Saponification of Lipids. The dried mycelial mats were reduced in bulk by powdering in a small grinder. The grinding process was extremely rapid, contact time between mycelium and cutting surfaces being less than 1 sec. The powdered mycelium was immediately weighed into a tared extraction thimble, and extracted in a Soxhlet apparatus for 8 hr. with benzene and ethanol (594:257 v/v) (Shaw 1965). The quantity of crude lipid per sample was obtained by difference between the weights of dried mycelium before and after extraction of the lipid.

After the completion of extraction the solvent was reduced to a small volume by evaporation under reduced pressure. To prevent oxidation of unsaturated fatty acids, care was taken during the handling of all lipid extracts to prevent undue exposure of lipid material to air. In addition, extracts were usually analysed immediately after saponification and methylation; when this was not possible they were stored in the refrigerator.

The crude (unhydrolysed) lipids were saponified by refluxing with 0.6 M methanolic KOH for 1 hr. The resulting potassium salts were acidified with dil. HCl and the fatty acids extracted with diethyl ether. The fatty acids were converted to their methyl esters by boiling with BF_3/methanol reagent (2 ml.) for 2 min. (Metcalfe and Schmitz 1961). An equal volume of water was added and the methyl esters extracted with diethyl ether.

1.82 Separation of Lipids.

1.821 Column Chromatography. The procedure followed was similar to that of Shaw (1966). A slurry of Davison's Silica in benzene was packed into a chromatography tube (internal diameter 12 mm.) to give a column 10 mm. long and to which was added approximately 300 mg. crude lipid dissolved in benzene. The column was eluted with benzene (2 x 60 ml. fractions) followed by ethanol (2 x 50 ml. fractions). The benzene eluate was found, by thin-layer chromatography, to contain the neutral lipids and the ethanol eluate to contain the compound lipids.

1.822 Thin Layer Chromatography (TLC) was used in one experiment only, to identify the components of the neutral and compound lipid fractions. Thin layer plates were prepared as follows:

silica gel (10g.) was shaken with deionised water (20 ml.) for 1-2 min. and then spread on the glass supports (20 cm. x 20 cm.) to give a layer 0.3mm. thick. Undulations were removed from the silica gel layer by gently rocking and tapping the plate from beneath. The plates were air-dried, then heated in an oven at 110° for 30 min. and allowed to cool in a desiccator, where they were stored until required. Lipid extracts were applied to the thin layer plate with a fine capillary and the plates developed with hexane:diethyl ether:acetic acid (80:20:1 by vol.). After the plates had dried they were placed in a dry chromatography tank containing a few crystals of iodine. The separated substances appeared as yellow-brown spots on a light-yellow background. Individual lipid components were identified by comparison with authentic standards run on the same plates; the standards used were glycerol tristearate, glycerol monostearate, cholesterol stearate, cholesterol and palmitoleic acid.

1.83 Gas-Liquid Chromatography (GLC). Methyl esters were fractionated by GLC using glass columns (9ft. x ¼in.) packed with diethyleneglycolsuccinate (5% w/w) on Chromosorb G (100-120 mesh). The columns were operated isothermally at 175° in

a Pye Model 64 gas chromatograph with a flame ionisation detector. The flame ionisation detector was operated at 300° with a mixture of hydrogen (flowrate 35 ml./min.) and compressed air (flowrate 550 ml./min.).* The analyser amplification unit was used at sensitivities of $5 \times 10^{-3} - 50 \times 10^{-3}$. The detector signal was recorded on a potentiometer recorder (Kelvin Servoscribe Recorder 10 mA) with a chart speed of 12 cm./hr.

The peak area of each component fatty acid was calculated from the formula, 0.9 Area = height x width at half the height; measurements were made to the centre of the chart line (Condal-Bosch 1964). Areas of all peaks were added and percentages of this total were calculated for each fatty acid. Prior to complete identification fatty acids were designated as X:Y, where X represents the number of carbon atoms and Y represents the number of double bonds/molecule.

1.84 Separation of Saturated and Unsaturated Fatty Acid Esters.

Saturated and unsaturated esters were separated by treating the mixed esters with mercuric acetate and chromatographing on a small Florisil column (Goldfine and Bloch 1961). The saturated esters were eluted with benzene, and the mercuric acetate adducts of the unsaturated esters were eluted with 5% acetic acid in ethanol. The adducts were decomposed with a mixture of 1 ml. conc. HCl and 5 ml. water, the

* The carrier gas was nitrogen, passing at 50 ml./min.

regenerated esters extracted with light petroleum, and gas chromatographed as an additional check on the identification of peaks due to unsaturated acids.

1.85 Determination of Degree of Unsaturation of Fatty Acid Esters was carried out by chromatography of the complete mixture of fatty acid methyl esters on a small column (internal diameter 12 mm.) of Florisil impregnated with silver nitrate (Willner 1965). The column was coated with alumunium foil to prevent oxidation of $AgNO_3$, and care was taken to ensure that the column was always immersed in solvent, in order to prevent oxidation of unsaturated fatty acid esters. Collection of eluate (5ml. fractions) was facilitated by using a Towers automatic fraction collector (Model A). The fatty acid composition of each 5th fraction was determined by GLC. Saturated esters were eluted in hexane, mono-unsaturated esters in 0.5%-5.0% (v./v.) ether in hexane, di-unsaturated esters in 7.5% (v./v.) ether in hexane, and tri-unsaturated esters in 15% (v./v.) ether in hexane.

1.86 Identification of Fatty Acid Esters. The peaks were provisionally identified by comparison of retention times with those of authentic fatty acid methyl esters. Complete identification of fatty acid esters was obtained by combined GLC-mass spectrometry (see Section 6.12).

1.9 Replication and Accuracy of Results. With the exception of GLC all analytical procedures were carried out using triplicate samples. Individual results were usually within 3% of the mean; repeat analyses were made when results were more varied. In the case of GLC, each sample was analysed on one occasion. The accuracy of the GLC methods employed in this investigation was tested by making six replicate analyses of the methyl esters of a mixture of fatty acids extracted from Mucor pusillus. The results, expressed as means $\pm$ S.D. are as follows:

Fatty acid	
14:0	1.3 ($\pm$ 0.1)
16:0	25.6 ($\pm$ 1.1)
16:1	2.6 ($\pm$ 0.3)
18:0	8.1 ($\pm$ 0.8)
18:1	46.4 ($\pm$ 2.1)
18:2	13.8 ($\pm$ 0.9)
18.3	2.2 ($\pm$ 0.3)

Degree of unsaturation 0.83

The degree of unsaturation (number of double bonds/mole) was calculated as follows:

$$\Delta/\text{mole} = 1.0 \times \left(\frac{\% \text{ monoenes}}{100}\right) + 2.0 \times \left(\frac{\% \text{ dienes}}{100}\right) + 3.0 \times \left(\frac{\% \text{ trienes}}{100}\right).$$

A suggestion that the extraction and saponification procedures used in this investigation might lead to extensive oxidation of unsaturated fatty acids was investigated as follows: cultures of M. pusillus were grown for 6 days on a glucose-ammonia medium when the mycelial mats from 20 culture flasks were removed and placed on paper towels to remove excess liquid. The mycelial mats were sorted at random into two groups, each containing 10 mats.

One group of mycelial mats was dried, and the lipids extracted, saponified and the fatty acid methylated exactly as described in Section 1.81. This procedure was designated Method A.

The other group of mycelial mats were extracted and the lipids processed by Method B. In Method B the mycelium was frozen in liquid nitrogen and freeze-dried; freeze-dried mycelium was ground with a pestle and mortar, grinding being carried out beneath solvent (benzene:ethanol, 594:257 v./v.). The ground mycelium plus solvent was transferred to an extraction thimble in a Soxhlet apparatus and extracted for 8 hours. The lipids were saponified and methylated as in Method A (see Section 1.81), except that all evaporation of solvents was carried out in a stream of nitrogen gas.

The fatty acid methyl esters obtained by Method A and Method B were analysed by GLC. Results are expressed as means $\pm$ S.D. of three replicate analyses of each sample:

Fatty Acid.	Method A.	Method B.
14:0	1.0 (± 0.1)	1.0 (± 0.0)
16:0	22.6 (± 1.6)	23.5 (± 1.0)
16:1	1.9 (± 0.6)	1.9 (± 0.5)
18:0	5.1 (± 1.0)	6.2 (± 0.2)
18:1	56.1 (± 1.9)	52.1 (± 0.9)
18:2	11.9 (± 0.2)	13.1 (± 1.5)
18:3	1.2 (± 0.1)	1.7 (± 0.1)
Degree of Unsaturation	0.85	0.85

The fatty acid compositions of samples prepared by methods A and B were very similar and these results indicate that under the conditions specified in Section 1.81, oxidation of unsaturated fatty acids did not reach a significant level.

CHAPTER 2. GROWTH RATES OF TEMPERATURE-ADAPTED FUNGI IN THE MUCORALES.

The growth rates of some newly-isolated high temperature fungi were compared with those of mesophilic and psychrophilic species. Rate of growth was assessed by linear spread of the colony.

2.1. Experimental procedure. Triplicate potato dextrose agar plates were inoculated and incubated at various temperatures. Colonies were measured as described in Section 1.52.

2.2. Results. Growth rate curves are shown in Figure 1 and the temperature relations presented in Table 1.

At their optimum temperatures thermophiles and thermotolerants covered the agar plate in 1-2 days whilst mesophiles required 4-9 days; psychrophiles had still not grown over the plate after 14 days.

The definition of Cooney and Emerson (1964) that "a thermophilic fungus is one that has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C" is used to distinguish between thermophilic and thermotolerant fungi; a thermotolerant fungus has a minimum growth temperature below 20°C. A psychrophilic fungus is defined as one which grows well at 0°C within one week (Stokes 1963).

TABLE 1. Temperature relations of Psychrophilic, Mesophilic and Thermophilic Fungi.

(Growth temperature °C).

Organism	Minimum	Optimum	Maximum	Temperature status
Mucor miehei	25	42-45	57	thermophilic
Mucor sp.I	25	45	56	"
Mucor sp.II	25	42-45	55	"
Rhizopus sp.III	28	48-50	60	"
Rhizopus sp.I	16	40-45	55	thermotolerant
Rhizopus sp.II	16	40	50	"
M. racemosus	8	20-25	35	mesophilic
M. ramannianus	10	23-25	35	"
M. mucedo	8	20	30	"
M. hiemalis	8	20-25	35	"
M. strictus (100.66)	0	15-20	20	psychrophilic
M. strictus (575.66)	0	10-20	20	"
M. strictus (575.66)	0	10-20	20	"
M. oblongisporus(220.29)	0	10-20	25	"
M. oblongisporus(173.27)	0	15-20	25	"

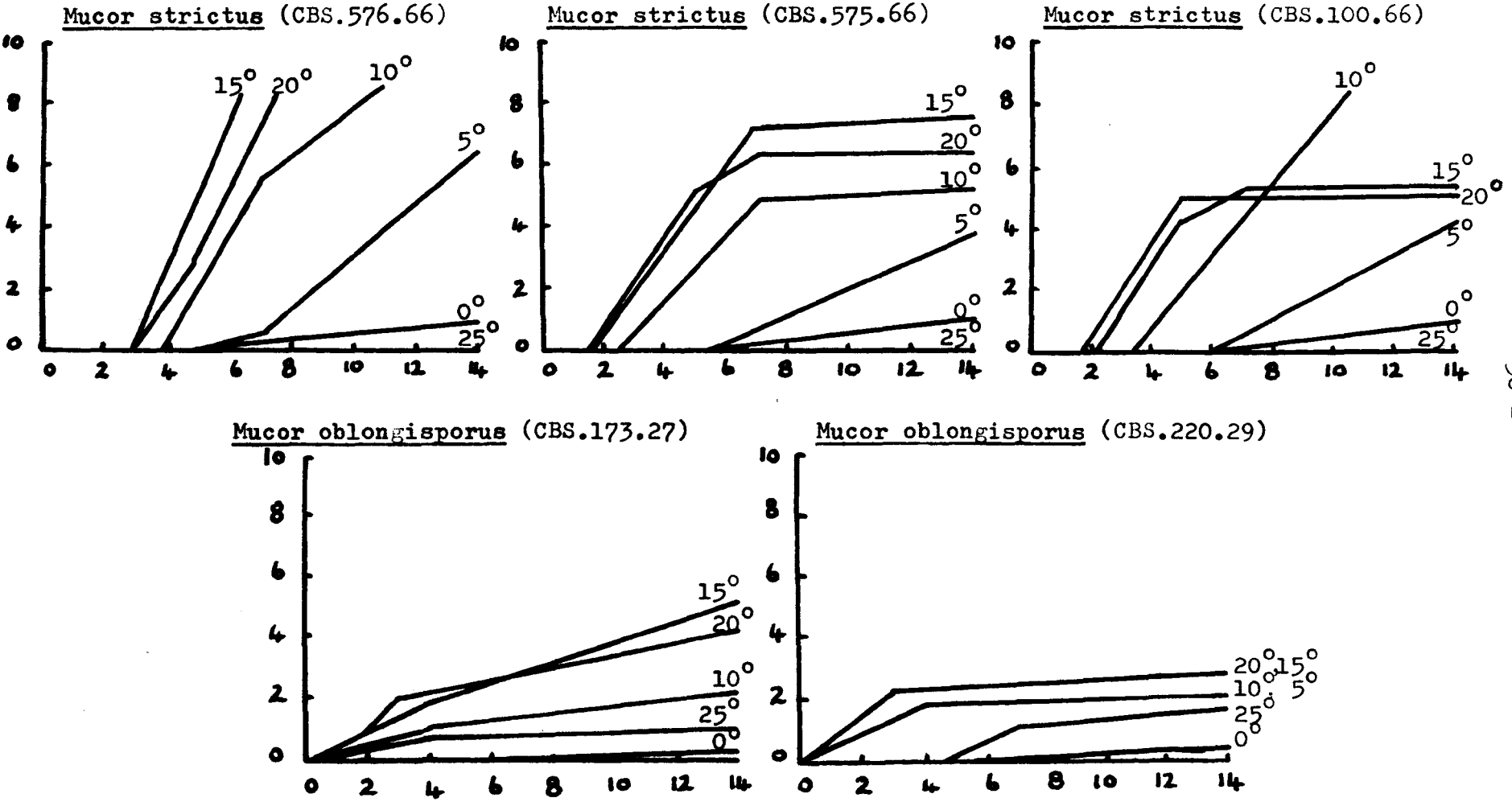


Fig.1. Growth rate curves showing increase in diametric growth at various incubation temperatures.

(x axis : time (days); y axis : colony diameter (cm.))

(a) PSYCHROPHILIC FUNGI.

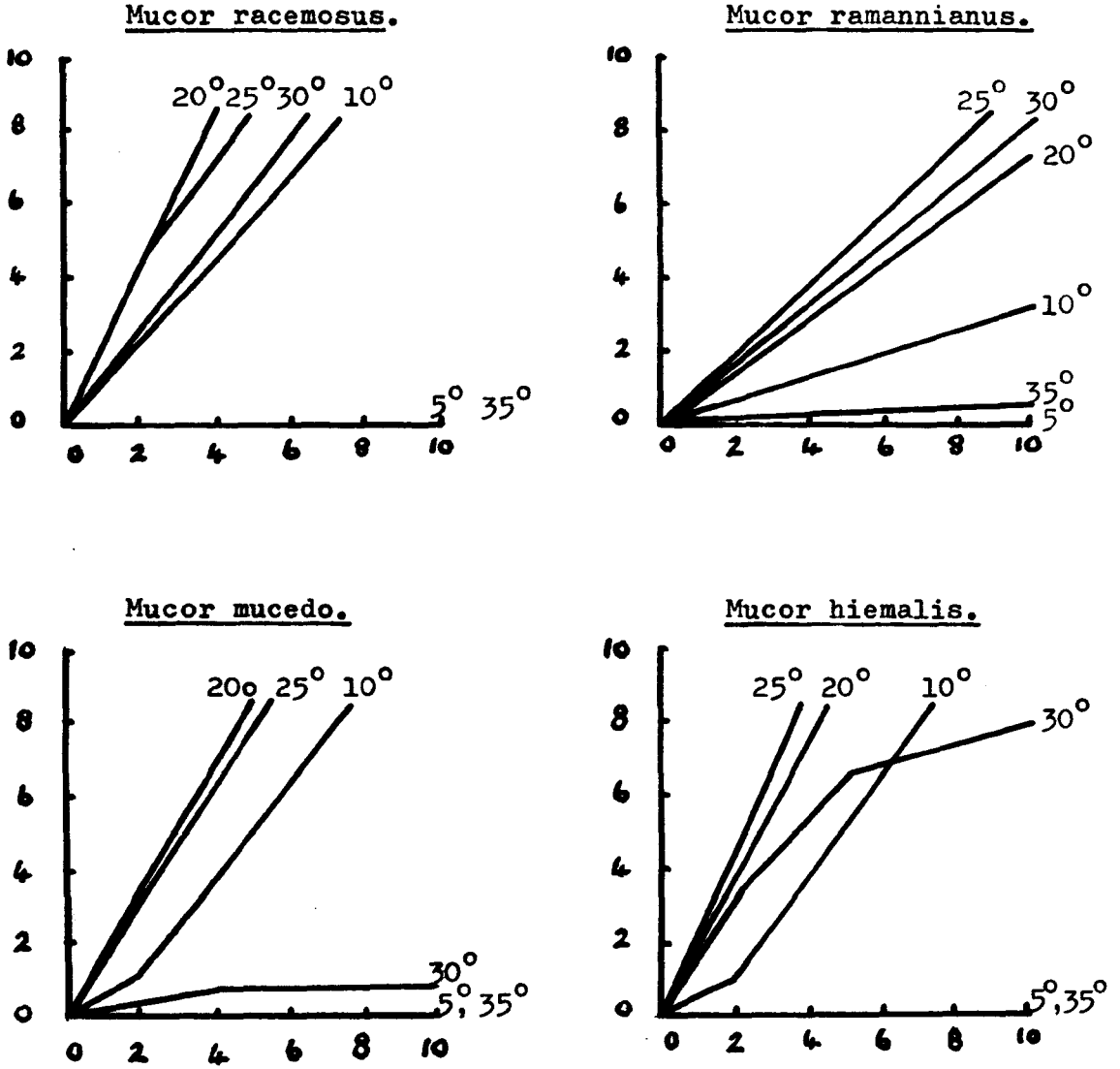


Fig. 1(b). MESOPHILIC FUNGI.

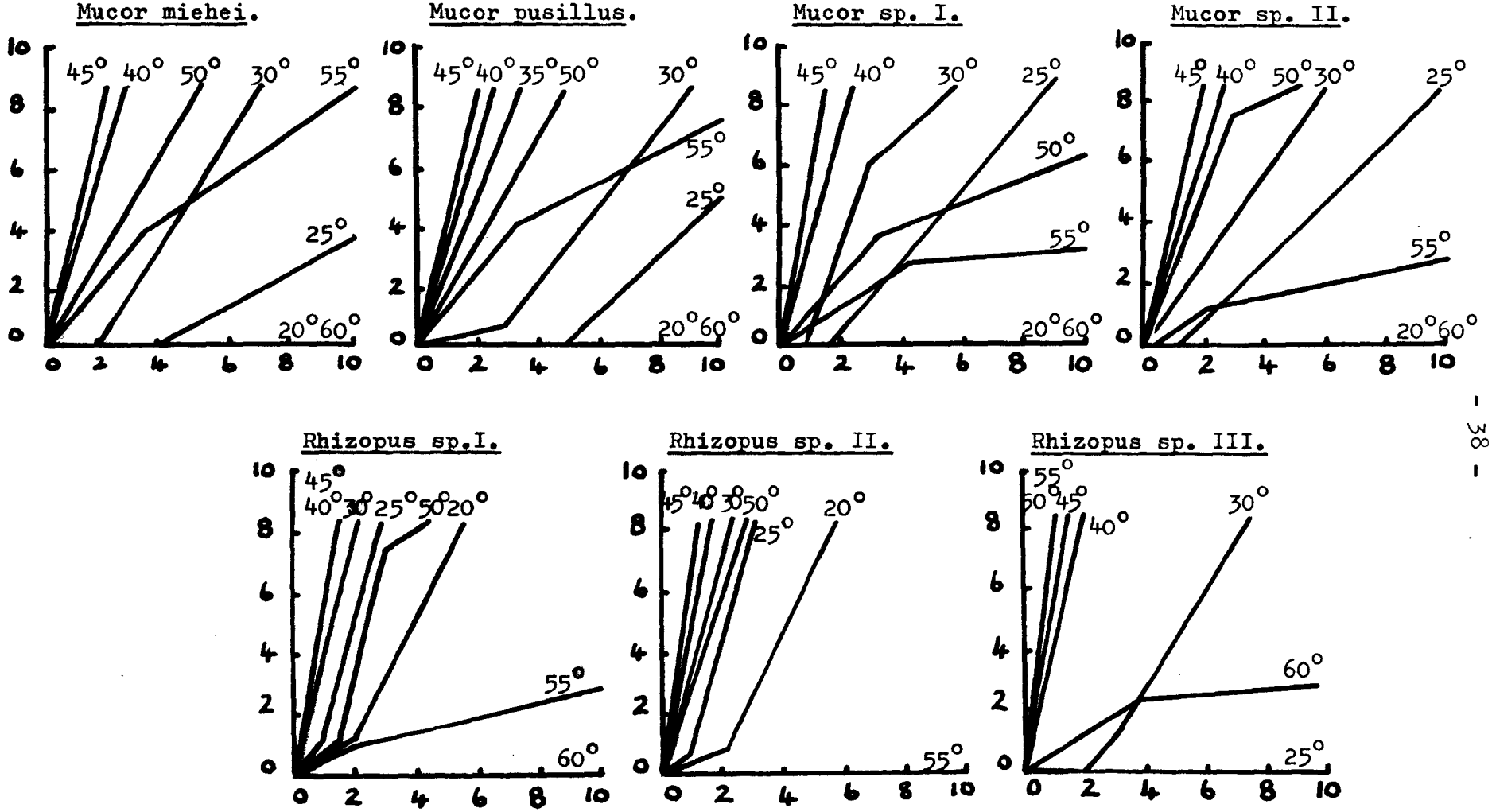


Fig. 1 (c). THERMOTOLERANT AND THERMOPHILIC FUNGI.

CHAPTER 3. ENVIRONMENTAL FACTORS AFFECTING GROWTH AND SPORULATION OF MUCOR PUSILLUS.

A number of preliminary experiments were carried out, the aim of which was to decide on suitable environmental conditions for the study of growth and sporulation of Mucor pusillus. Accordingly, the organism was grown on different concentrations of carbon and nitrogen source, at different temperatures and pH, and in still and shake culture.

3.1 The Effect of Concentration of Nitrogen Source.

3.11 Experimental Procedure. A spore suspension of M. pusillus was prepared by the procedure described earlier, and used for the inoculation of flasks containing one of the following media:

(a) basic medium containing glucose (50 g./l), sodium succinate (5g./l.) and ammonium sulphate present at the following concentrations, 125, 250, 500, 1000 mg.N/l. medium.

(b) basic medium containing glucose (50 g./l.) and casein hydrolysate at the following concentrations, 40, 60, 120, 250, 450 mg.N/l. medium.

Flasks were incubated at 48° and harvested at suitable time intervals. The mycelial mats were removed, dried at 80° overnight, cooled to room temperature in a desiccator, and weighed. The total nitrogen content of culture filtrate was determined by the micro Kjeldahl technique; the pH of culture filtrate was also determined.

3.12 Results.

3.121 Ammonium Sulphate. The results (Tables 2, 3, 4,5 and Fig 2) indicate that all four concentrations of nitrogen source supported good growth. A visual assessment of sporulation revealed satisfactory spore production at all four concentrations. However, at concentrations of 500 and 1000 mg.N/l. the ammonia was not fully assimilated from the medium. It was concluded that a suitable concentration of nitrogen source would involve less than 500 mg. ammonia N/l. medium.

TABLE 2. The effect of ammonium sulphate concentration on growth of M. pusillus. (Growth expressed mg.dry weight/ml.medium).

Time in days.	Concentration of ammonium sulphate (mg.N/l.)			
	125	250	500	1000
2	1.52	1.52	1.70	2.14
3	2.19	1.70	2.30	3.82
4	2.52	2.52	3.85	5.42
6	1.70	2.03	3.49	4.64
8	2.68	2.74	4.18	5.10

TABLE 3. The effect of ammonium sulphate concentration on sporulation of M. pusillus.

Time in days	Concentration of ammonium sulphate (mg.N/l.)			
	125	250	500	1000
6	+	+++	++	+

Sporulation o = no sporulation.
+ = fair "
++ = good "
+++ = excellent "

TABLE 4. Nitrogen content of media originally containing different concentrations of ammonium sulphate. (Total nitrogen remaining in the medium expressed in mg.N/l.).

Time in days	Concentration of ammonium sulphate (mg.N/l.).			
	125	250	500	1000
0	131	239	479	1063
2	30	70	276	647
3	19	44	213	453
4	18	52	135	314
5	21	20	102	325
8	23	23	110	365

TABLE 5. pH drift in buffered media containing different concentrations of ammonium sulphate. (pH units).

Time in Days	Concentration of ammonium sulphate (mg. N/l.)			
	125	250	500	1000
0	6.5	6.5	6.5	6.5
2	4.4	4.0	3.7	3.7
3	4.5	3.9	3.0	3.1
4	4.5	4.0	3.2	2.2
6	5.3	4.8	3.7	3.0
8	5.8	4.9	4.0	2.8

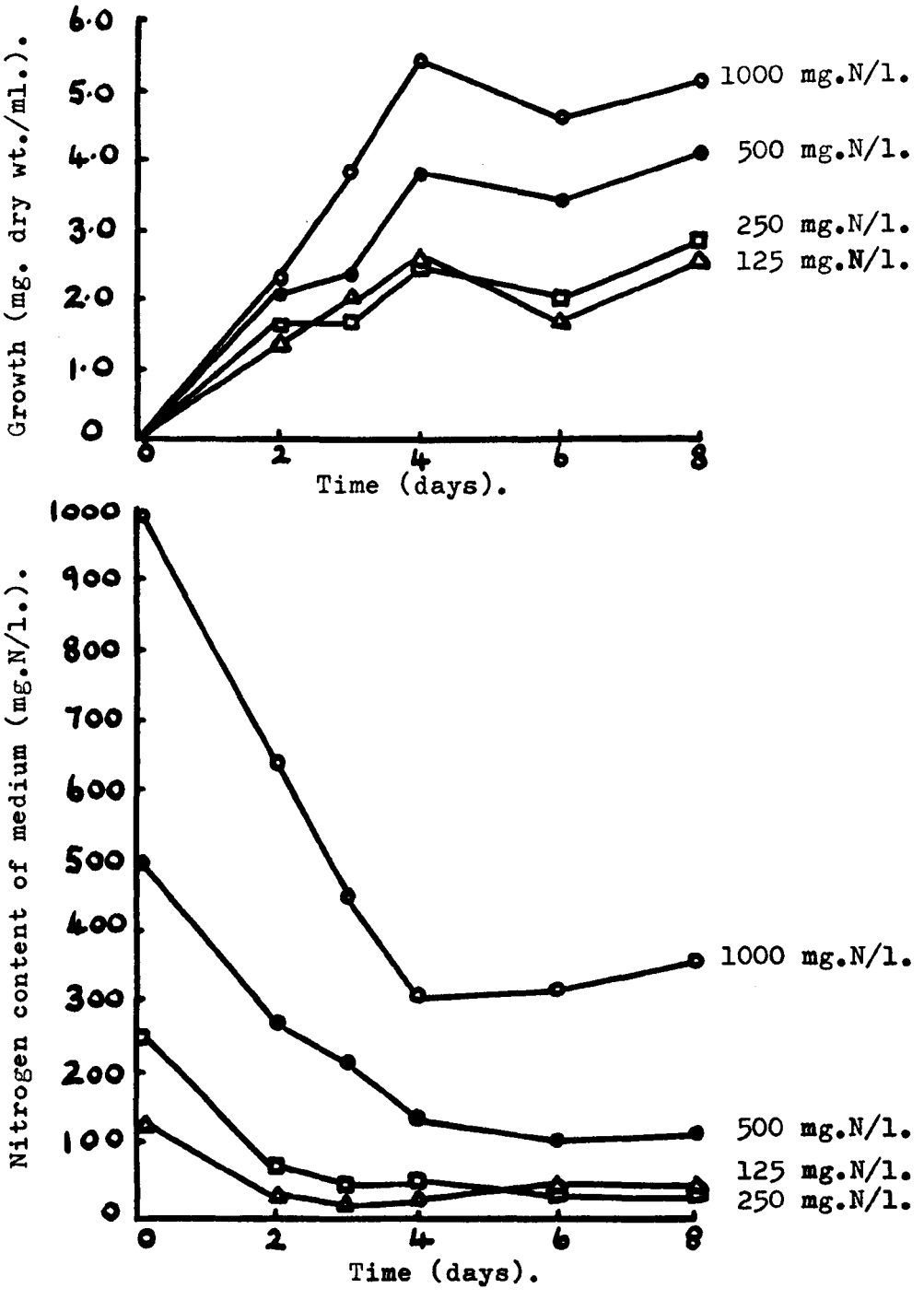


Fig. 2. Effect of different concentrations of ammonium sulphate on growth and nitrogen utilisation by M. pusillus.

3.122 Casein hydrolysate. The results presented in Tables 6 - 9 and Figure 3 indicate that only at concentrations of 250 and 450 mg.N/l. did the organism grow and spore well. At 450 mg.N/l. however, there was incomplete assimilation of nitrogen from the medium, and in the later stages of culture at this concentration a fall in dry weight and a pH drift to alkalinity suggested mycelial autolysis.

A concentration of 250 mg. N/l. was chosen for future experiments since at this concentration the organism grew and spored well, without autolysis.

TABLE 6. Growth of M. pusillus on media containing different concentrations of casein hydrolysate.
(Growth measured as dry weight in mg/ml. medium).

Time in days	Concentration of casein hydrolysate (mg N./l.).				
	40	60	120	250	450
1	0.10	0.28	0.50	0.89	1.67
2	0.22	0.51	0.95	2.42	4.77
3	0.27	0.60	1.25	3.50	6.30
5	0.31	0.70	1.34	3.64	6.76
7	0.35	0.74	1.45	4.75	4.80

TABLE 7. Sporulation of *M. pusillus* on media containing different concentrations of casein hydrolysate. (Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Time in days	Concentration of casein hydrolysate (mg. N/l.)				
	40	60	120	250	450
1	0.15	0.27	0.56	0.60	0.78
2	0.42	0.62	1.55	2.00	2.90
3.	0.61	0.90	1.69	3.12	2.12
5	0.46	0.56	0.95	1.71	0.98
7	0.45	0.43	0.92	1.57	0.71

TABLE 8. Nitrogen content of media originally containing different concentrations of casein hydrolysate.

(Total nitrogen remaining in medium expressed as mg. N/l.)

Time in Days	Original concentration of casein hydrolysate (mg. N/l.)				
	40	60	120	250	450
0	51	69	142	280	460
1	35	60	134	248	306
2	21	28	66	89	123
3	16	21	24	40	77
5	13	19	38	67	144
7	21	19	25	46	165

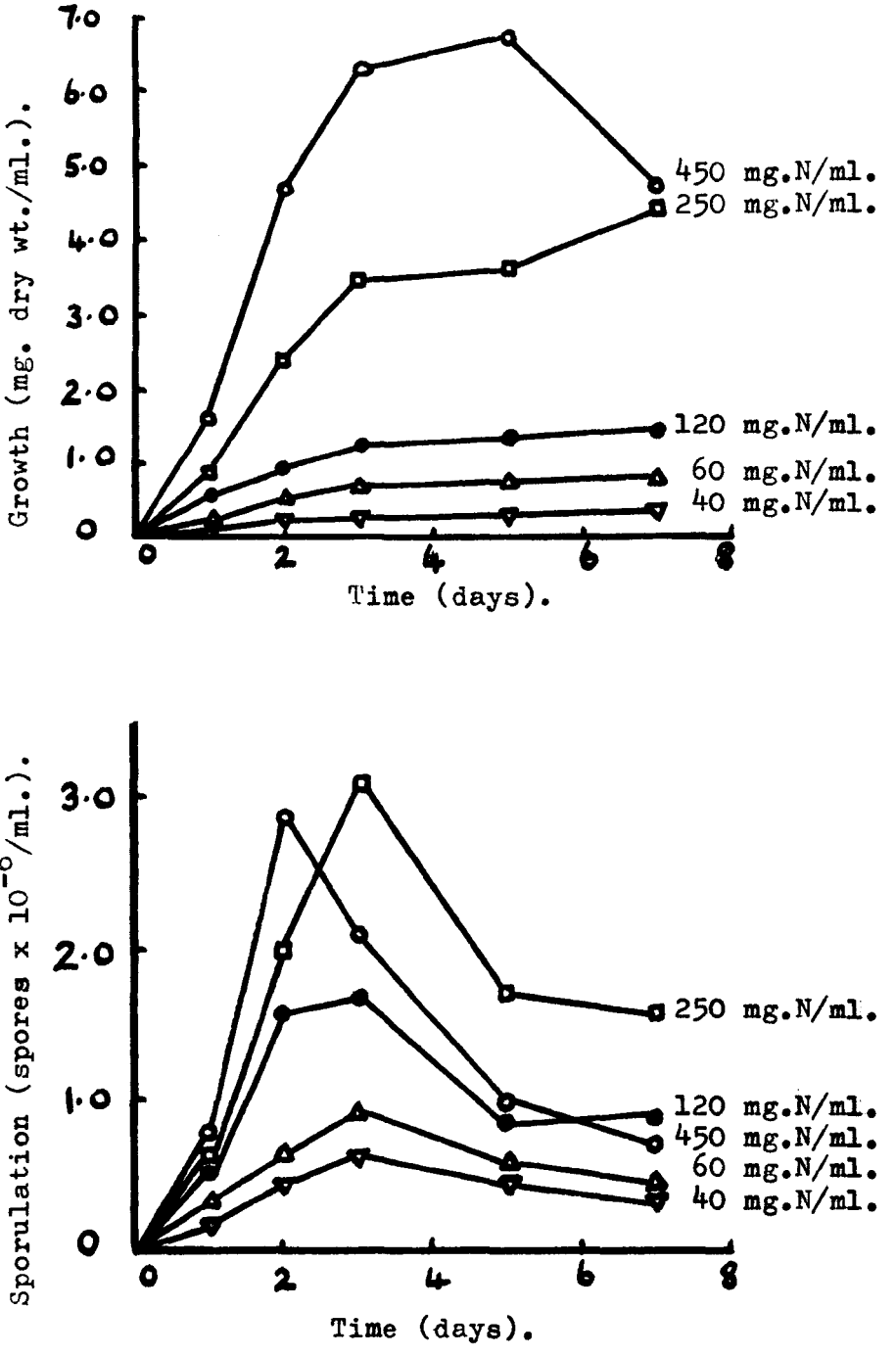


Fig. 3. Effect of different concentrations of casein hydrolysate on growth and sporulation of M. pusillus.

TABLE 9. pH drift in media containing different concentrations of casein hydrolysate.

Time in days	Concentration of casein hydrolysate (mg. N/l.)				
	40	60	120	250	450
0	6.4	6.4	6.4	6.4	6.4
1	5.6	5.4	5.0	5.0	4.9
2	5.5	5.3	5.0	4.3	4.4
3	5.9	5.9	5.7	5.5	4.8
5	6.0	5.8	5.5	5.6	6.3
7	6.0	5.8	5.4	4.9	7.7

3.2 The Effect of Concentration of Carbon Source.

3.21 Experimental Procedure. A standardised spore inoculum of M. pusillus was added to flasks containing the basic medium with ammonium sulphate (250 mg. N/l.) as nitrogen source and one of the following carbon sources:

(i) Glucose (AR grade). 20, 50, 100, 200 g./l.
(ii) Glucose (crude). 100, 200 g./l.
(iii) Sucrose (AR grade). 100 g./l.

Flasks were incubated at 48° and harvested at appropriate time intervals. Sporulation was assessed visually, after which the mycelial mats were removed from the culture flasks, dried and weighed. The total nitrogen content of the culture filtrate was determined by the micro Kjeldahl technique; the pH of the culture filtrate was also determined.

3.22 Results. The results, presented in Tables 10 - 13 showed that at all the concentrations tested the fungus grew satisfactorily. A visual examination of the cultures indicated sporulation at all concentrations. At higher concentrations of carbon-source mycelial autolysis occurred and for this reason a level of 20 to 50 g./l. glucose was selected for future experiments.

TABLE 10. Growth of M. pusillus on media containing different concentrations of carbon source. (Growth expressed as dry weight mg./ml. medium).

Time in Days	Concentration of glucose (g./l.)						Concentration of sucrose(g./l.)
	20g./l. (A.R.)	50g./l. (A.R.)	100g./l. (A.R.)	200g./l. (A.R.)	100g./l. (crude)	200g./l. (crude)	100 g./l. (A.R.)
2	1.25	1.64	1.95	1.70	1.35	2.40	1.87
3	2.11	2.43	3.13	3.30	2.45	3.63	2.81
4	2.68	2.82	3.48	3.06	2.18	3.25	2.75
6	2.85	2.62	3.51	3.13	2.28	2.84	2.90
8	2.46	2.62	3.40	2.81	1.86	2.75	2.50

TABLE 11. Sporulation of M. pusillus on media containing different concentrations of carbon source.

Concentration of glucose (g./l.).	Sporulation (Visual examination, arbitrary units).
20 g./l. A.R.	++
50 g./l. A.R.	+++
100 g./l. A.R.	+++
200 g./l. A.R.	++
100 g./l. Crude	+++
200 g./l. Crude	++
100 g./l. Sucrose	+++

TABLE 12. Nitrogen content of media containing different concentrations of carbon source.
(Total nitrogen remaining in medium expressed as mg. N/l.)

Time in Days	Concentration of glucose (g./l.)						Concentration of sucrose (g/l.)
	20g/l. (A.R.)	50g/l. (A.R.)	100g/l. (A.R.)	200g/l. (A.R.)	100g/l. (Crude)	200g/l. (Crude)	100g/l. (A.R.)
0	256	256	256	256	256	256	256
2	167	150	98	63	139	118	76
3	104	44	53	36	91	86	45
4	33	33	27	32	41	60	24
6	29	25	27	47	43	57	29
8	36	27	33	46	53	59	36

TABLE 13. pH drift on media containing different concentrations of carbon source.

Time in Days	Concentration of glucose (g./l.)						Concentration of sucrose (g./l.)
	20g/l. (A.R.)	50g/l. (A.R.)	100g/l. (A.R.)	200g/l. (A.R.)	100g/l. (Crude)	200g/l. (Crude)	100g./l. (A.R.)
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	2.1	2.0	1.9	2.7	3.1	3.0	2.8
3	2.2	2.1	2.0	1.9	2.0	2.0	1.9
4	2.2	2.1	2.1	1.9	2.1	2.1	2.0
6	2.2	2.1	2.1	1.9	2.0	2.2	2.0
8	2.2	2.1	2.1	1.9	2.1	2.2	2.5

3.3 The Effect of Temperature.

3.31 Experimental Procedure. Spores of M. pusillus were inoculated into flasks containing the basic medium with glucose (50 g./l.) and ammonium sulphate 250 mg. N/l.). The incubation temperatures were 25°, 35°, 47°, 53°C. After specific periods of incubation sufficient flasks were harvested to give adequate amounts of mycelium for determination of sporulation and of dry weight. The pH and total nitrogen content of the spent culture filtrates were determined.

3.32 Results. At 53° the organism did not produce aerial hyphae, neither did it spore. Aerial mycelium was produced at all other temperatures. At 25° the mycelial mat was initially white, but by the 5th day had become grey in colour. At 45° and 35° young mycelium was white in colour, becoming brown by the 3rd and 4th days respectively. The organism barely grew at 53°. The optimum growth temperature varied throughout the incubation period. In the early stages the organism grew best at 47°. After 3 days incubation 35° was the optimum growth temperature, whilst after two weeks the fungus was growing best at 25° (Table 14, figure 4).

Sporulation followed essentially the same course as growth, and after the initial stages of culture, spore production was more profuse at 35° and 25° (Table 15, figure 4).

The rate of assimilation of ammonia from the medium is given in Table 16 and figure 4. At 47° the nitrogen-source was completely exhausted from the medium by the 3rd day. The process occurred at a slightly slower rate at 35°, but at 25° the ammonia was not completely assimilated until the 8th day of incubation. At 53° only a small proportion of the nitrogen-source was utilised.

As a consequence of assimilation of ammonia from the medium the pH became progressively more acid. (Table 17, figure 4). At 25°, 35° and 47° the pH of the medium was approximately pH 2. At 53° the medium was less acid.

A suitable temperature for future experiments was considered to be about 47° - the highest temperature at which the fungus produced adequate amounts of mycelium and spores.

TABLE 14. Effect of temperature on growth of M. pusillus (Growth measured as dry weight mg./ml. medium).

Time in days	Temperature.			
	25°	35°	47°	53°
1	0	trace	0.28	trace
1.5	trace	0.24	0.85	0.10
2	trace	0.68	1.55	0.14
3	0.23	2.12	2.27	0.30
4	0.29	2.96	1.95	0.32
5	0.98	-	-	-
6	1.15	3.22	2.15	0.32
8	2.71	3.76	2.28	0.35
10	3.58	4.36	2.12	0.31
12	3.86	4.10	2.00	0.23
14	3.86	3.86	2.02	0.25

TABLE 15. Effect of temperature on sporulation of M. pusillus (Sporulation expressed as number of spores x 10^{-6}/ml.medium).

Time in days	Temperature °C.			
	25°	35°	47°	53°
3	0.21	1.97	1.05	0
6	0.14	3.72	0.99	0
8	1.24	2.92	1.08	0
10	2.10	2.29	0.94	0
14	1.68	3.00	0.83	0

TABLE 16. The nitrogen content of the medium of cultures grown at different temperatures.
(Total nitrogen content of the medium expressed as mg.N/ml.).

Time in Days	Amount of nitrogen remaining in medium at following temperatures.			
	25°	35°	47°	53°
0	253	253	253	253
1	-	-	152	-
1.5	-	172	131	201
2	-	122	82	189
3	185	36	31	216
4	184	47	59	214
5	135	-	-	-
6	128	36	41	197
8	42	23	50	232
10	42	33	50	199
12	40	41	52	205
14	38	39	71	217

TABLE 17. The effect of temperature on pH drift of the medium.

Time in days	Temperature °C.			
	25°	35°	47°	53°
0	6.5	6.5	6.5	6.5
1	6.4	4.9	3.2	5.0
1.5	6.1	3.6	2.0	4.8
2	5.8	2.3	1.8	3.2
3	3.5	1.7	1.8	3.0
4	3.4	1.8	1.8	3.2
5	2.5	1.9	1.9	3.2
6	2.5	2.0	2.2	3.5
8	2.0	1.9	1.9	3.2
10	2.0	2.0	2.0	3.5
12	1.9	1.9	2.1	3.4
14	1.9	2.0	2.3	3.5

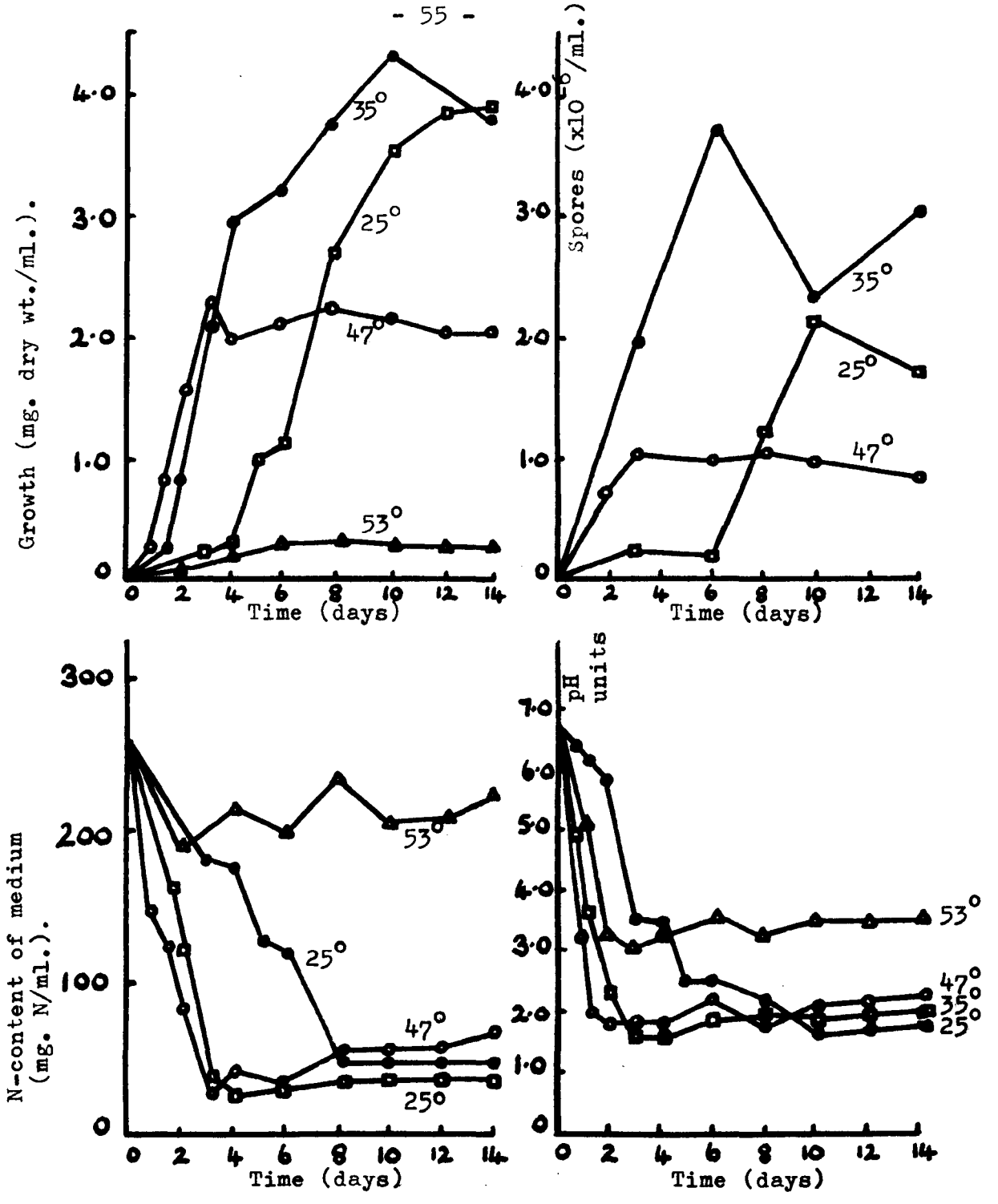


Fig. 4. Effect of incubation temperature on the growth and sporulation of M. pusillus, and the nitrogen content and pH drift of the medium.

3.4 The Effect of Aeration.

3.41 Experimental Procedure. The basic medium containing glucose (50g./l.) and ammonium sulphate (250 mg.N/l.) was dispensed into 750 ml. conical flasks as follows:-

(i) 100 ml. medium, grown in shake culture.

(ii) 200 ml. medium, grown in shake culture.

(iii) 100 ml. medium, grown in still culture.

(iv) 200 ml. medium, grown in still culture.

The flasks were inoculated with a standardised suspension of spores incubated at 48° and harvested after suitable periods of incubation. The mycelium was filtered off, and the nitrogen-content and pH of the culture filtrate determined.

3.42 Results. Initially the fungus grew more slowly in still culture than shake culture (Table 18, figure 5) though in the later stages a loss in dry weight occurred in the aerated media. No sporulation occurred in shake culture. As indicated in Tables 19 and 20 the nitrogen source was utilised more rapidly in the shake-culture media. The early stages of growth in shake culture are described more fully in Section 35.

TABLE 18. The effect of aeration on the growth of M. pusillus. (Growth expressed in mg. dry weight/ml. medium).

Time in Days	Still culture.		Shake culture.	
	100 ml.	200 ml.	100 ml.	200 ml.
2	1.31	0.99	1.76	2.01
3	2.08	1.86	1.67	1.96
4	2.30	2.02	1.85	1.80
6	2.23	2.20	1.50	2.28
8	2.20	2.02	1.53	1.86

TABLE 19. The effect of aeration on the nitrogen content of the medium during growth of M. pusillus under different aeration conditions.
(Total Nitrogen remaining in medium expressed as mg. N/l.).

Time in Days	Still culture.		Shake culture.	
	100 ml.	200 ml.	100 ml.	200 ml.
0	241	247	241	247
2	100	96	30	34
3	48	63	31	29
4	32	35	30	31
6	29	30	38	33
8	33	28	42	39

TABLE 20. The effect of aeration on the pH drift of the medium.

Time in Days	Still culture		Shake culture	
	100 ml.	200 ml.	100 ml.	200 ml.
0	6.1	6.1	6.1	6.1
2	2.4	2.3	2.4	1.9
3	2.0	2.0	2.4	1.9
4	2.0	1.9	2.0	2.0
6	1.9	2.1	1.9	2.1
8	2.1	2.1	2.3	2.2

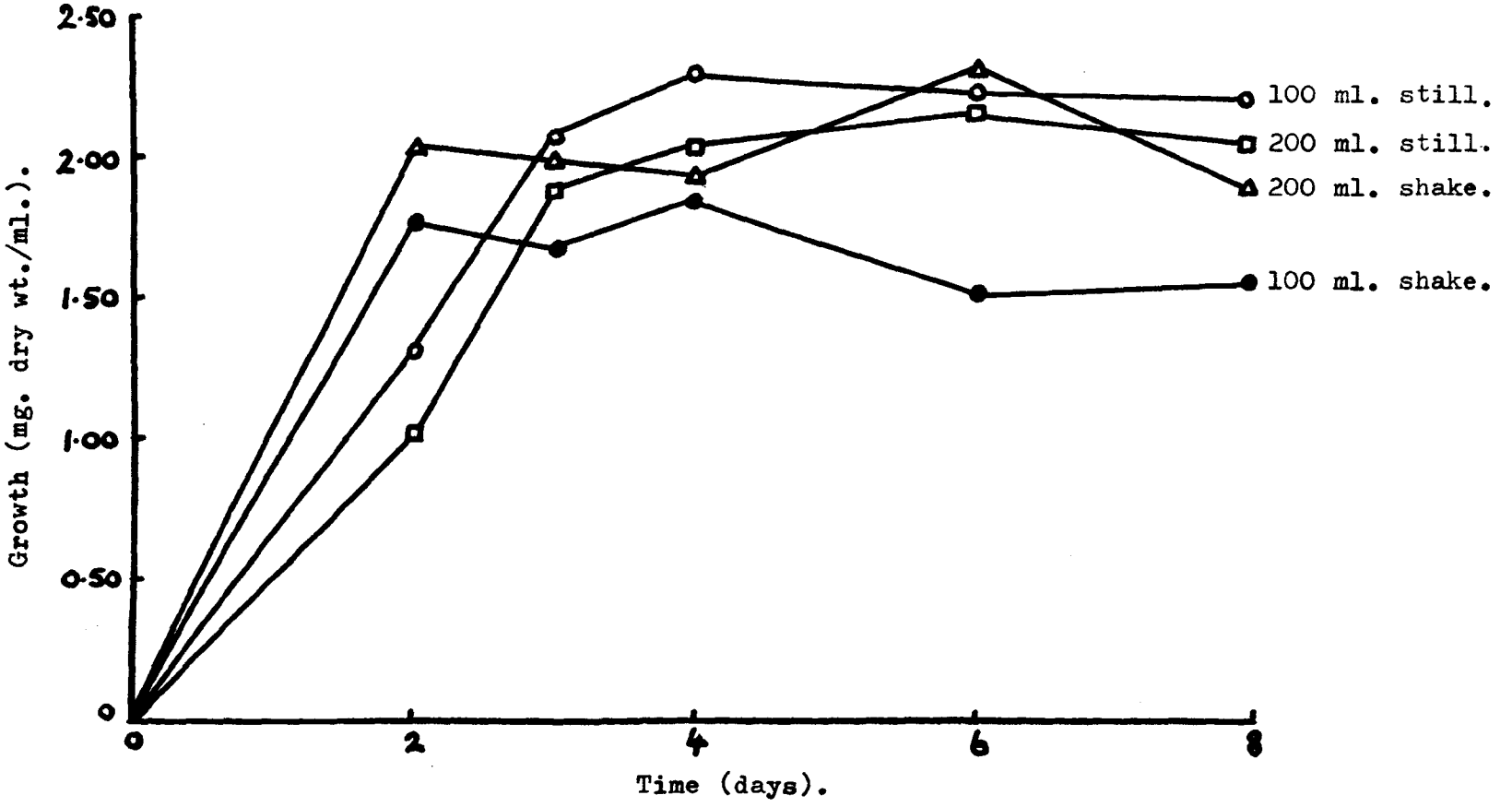


Fig. 5. Effect of aeration on the growth of M. pusillus.

3.5 Spore Germination and Hyphal Growth in a Shaken Medium.

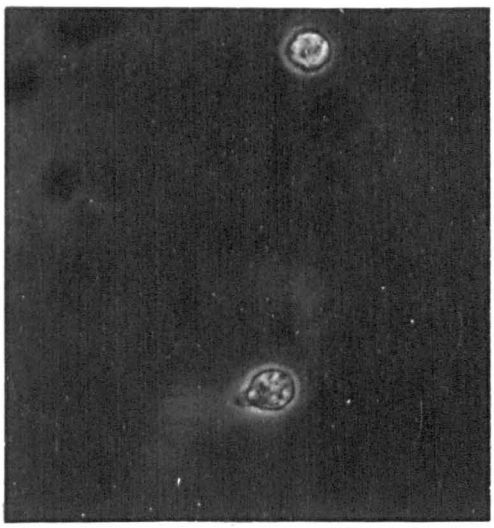
3.51 Experimental Procedure. The basal medium containing glucose (30 g./l.) and ammonium sulphate (230 mg. N/l.) was dispensed in 200 ml. aliquots in 750 ml. conical flasks. The inoculum was grown on potato destrose agar slopes in flat medicine bottles. Spores were removed from 90 medicine bottles which had been incubated 14 days at 48°, by shaking with ice-cold sterile deionised water. The spore suspension was filtered through 3 layers of sterile muslin to remove mycelial fragments, and then centrifuged. The supernatant was discarded and the spore pellet resuspended in sterile deionised water. Each flask was inoculated with 10 ml. spore suspension (equivalent to 50 mg. dry weight of spores). Inoculated flasks were shaken at 48° and harvested at appropriate intervals. The mycelium was examined microscopically prior to removal from the culture medium by filtration. The total nitrogen content and the pH of the spent culture filtrate were determined.

3.52. Results. Data for the total nitrogen content and pH drift of the medium, and growth of the sporelings are given in Table 21 and Figure 6.

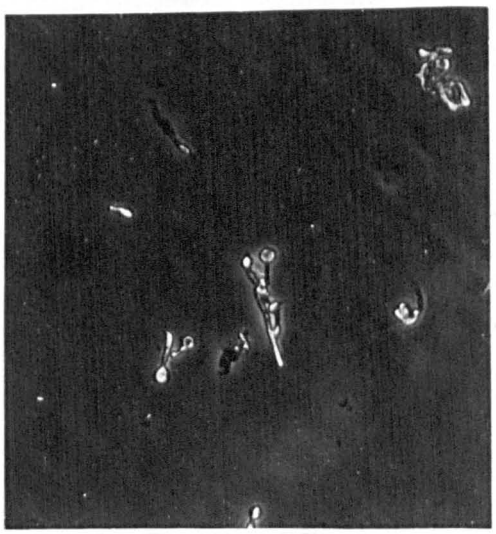
After incubation for 4 - 5 hours most of the spores (> 75%) were swollen, and 6.5 hours after inoculation small hyphal buds had been produced. At this stage 50% of the ammonia had been assimilated

TABLE 21. Spore germination and hyphae extension of M. pusillus in shake culture at 48°.

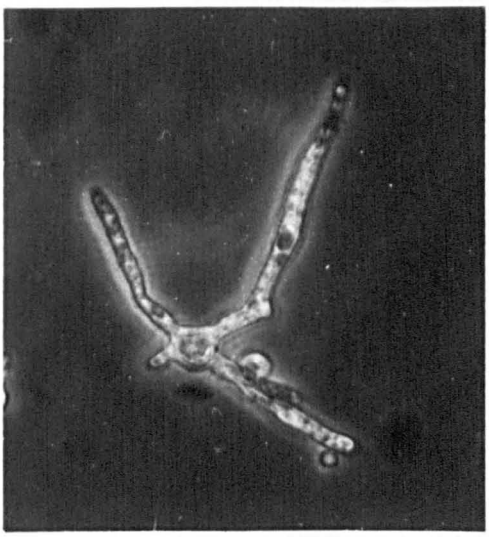
Time in hours	Growth (mg.dry weight/ ml. medium).	Nitrogen assimilation (Ammonia remaining in medium µg. N/ml.)	pH of medium.
0		227	6.0
6.5	0.60	114	2.8
8	1.01	91	2.5
10	1.18	52	2.4
12	1.28	63	2.3
15	1.58	41	2.1
18	1.63	43	2.0
21	1.68	40	2.0
24	1.81	61	2.0
27	1.74	43	1.9
30	1.78	24	2.1
33	1.70	53	2.1
36	1.59	67	2.1
39	1.53	61	2.0



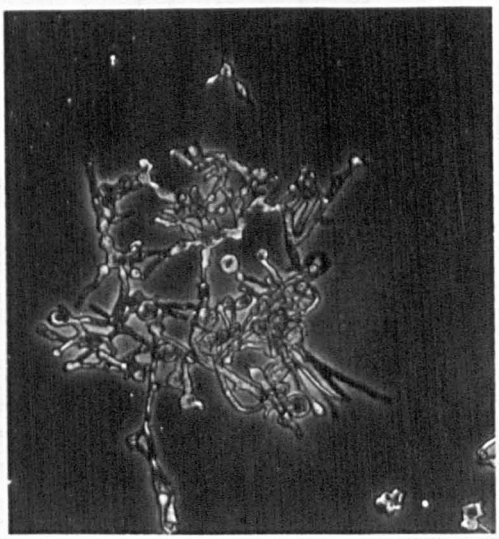
(a) 6.5 hr. incubation (x 176)



(b) 10 hr. incubation (x 52)



(c) 12 hr. incubation (x 176)



(d) 18 hr. incubation (x 52)

Plate 1. Stages in the germination of spores and growth of hyphae of M. pusillus in shake culture. (phase contrast microscopy).

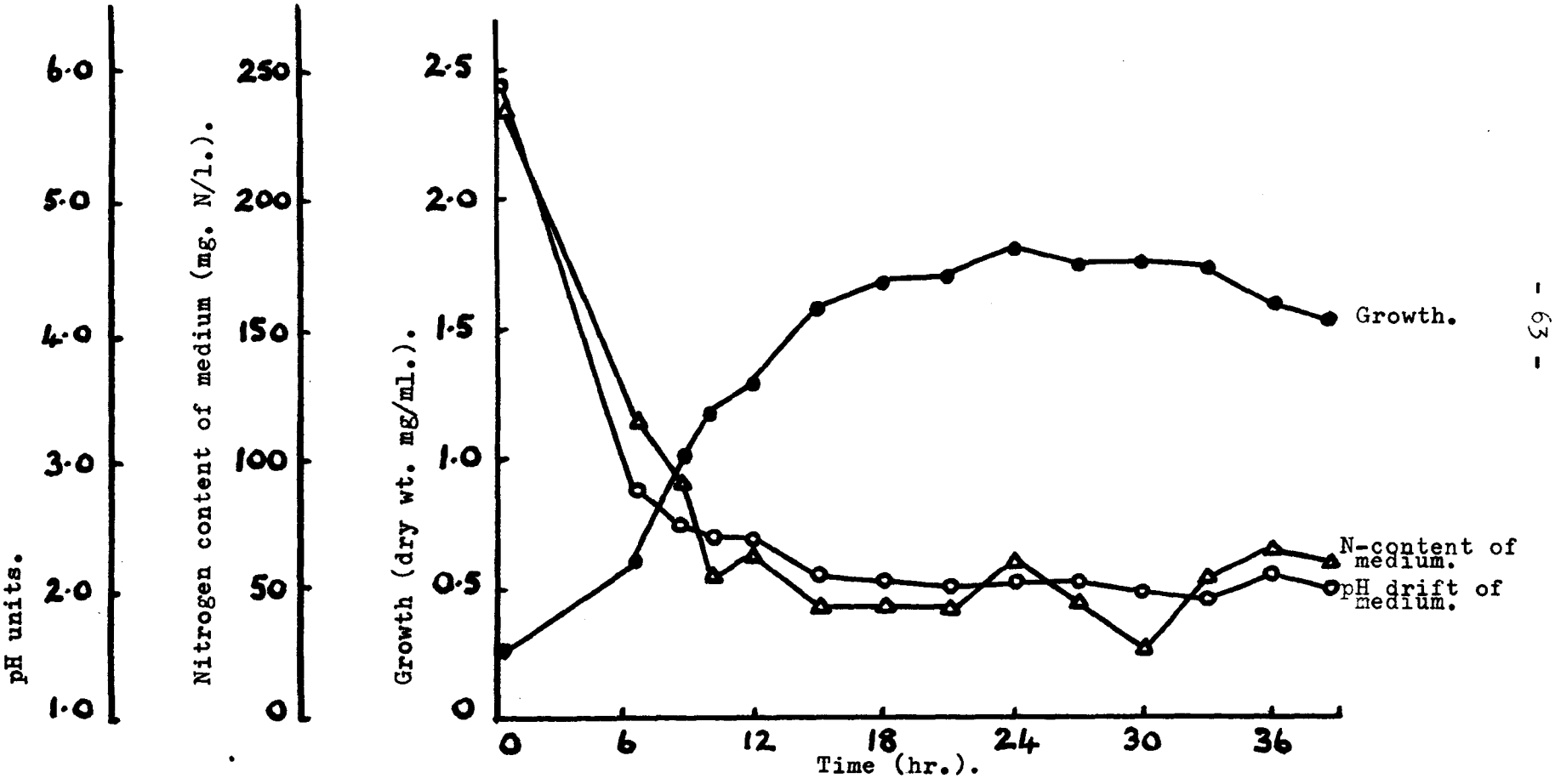


Fig. 6. Medium analysis during the early stages of growth of M. pusillus in shake culture.

from the medium, with a commensurate fall in pH. A phase of rapid growth followed and microscopic examination (Plate 1) showed that the mycelium consisted of thin branched hyphae. As growth proceeded, and mycelial density increased, the culture became composed of loose tangles of hyphae.

3.6. The Effect of Buffering the Medium with Organic Acids on the Growth of M. pusillus.

3.61 Experimental Procedure. The basic medium plus glucose (20 g./l.) was dispensed in 25 ml. aliquots into 100 ml. conical flasks and supplemented as follows:-

Medium A - ammonium sulphate (250 mg. N/l.).

Medium AT - ammonium tartrate (250 mg. N/l.).

Medium A+M - ammonium sulphate (250 mg. N/l.).
+ DL - malic acid (5g./l.).

Medium A+C - ammonium sulphate (250 mg. N/l.).
+ sodium citrate (11 g./l.).

After inoculation with a standard spore suspension and incubation at 48° for appropriate time intervals, 5 replicate flasks of each medium were harvested. The mycelium was filtered, dried and weighed. The culture filtrates were analysed for residual ammonia and total nitrogen content and the pH determined.

3.62 Results. The fungus grew better in buffered media, and the organic acids tested promoted growth more or less equally (Table 22, figure 7). In medium A there was no increase in dry weight after the ammonia was exhausted from the medium. In the buffered media however, considerable increase of dry weight occurred after the medium was fully depleted of ammonia.

A visual examination of the cultures indicated that on the buffered media the fungus spored less well than on Medium A. Studies are described later (Chapter 4) in which this observation receives further attention.

TABLE 22. The effect of buffering the medium with organic acids on the growth of M. pusillus.
(Growth measured as dry weight in mg./ml. medium).

Age of harvest. (hours).	Ammonium Sulphate	Ammonium Tartrate	Ammonium Sulphate & Malic Acid.	Ammonium Sulphate & Sodium citrate
15	0.20	0.20		
24	1.04	0.64		
39	1.80	1.72		
48	2.02	1.84	2.20	2.71
68	2.12	2.08		
112	2.16	2.48		
136	2.12	2.72	3.92	3.64
160	2.08	2.76		
184	2.00	3.40		
208	1.96	3.76	2.88	3.24

Table 23. The effect of organic acids on the pH drift of the medium.

Time in Hours	Ammonium Sulphate	Ammonium Tartrate	Ammonium Sulphate + Malic Acid	Ammonium Sulphate + Sodium Citrate.
0	6.3	6.3	6.4	6.5
15	5.7	5.8		
24	2.9	4.7		
39	2.4	3.5		
48	2.2	3.4	5.0	5.8
68	2.2	3.1		
112	2.2	3.1		
136	2.2	3.1	7.5	7.6
160	2.3	3.1		
184	2.4	3.0		
208	2.4	3.0	7.6	7.6

TABLE 24. The nitrogen content of buffered and unbuffered media after different periods of growth of M. pusillus.

Time in Hours	Ammonium Sulphate		Ammonium Tartrate		Ammonium Sulphate + Malic Acid		Ammonium Sulphate + Sodium Citrate.	
	A	B	A	B	A	B	A	B
0	232	233	214	210	212	210	220	225
15	220	217	204	180				
24	174	149	161	140				
39	106	48	78	31				
48	85	71	73	47	67	22	66	38
68	73	0	32	0				
112	78	0	30	0				
136	74	0	36	0	49	0	48	0
159	66	0	34	0				
183	78	0	23	0				
208	84	0	31	0	98	0	86	0

Figures above refer to - Nitrogen remaining in the medium, expressed in mg. N/l. medium.
A = Total Nitrogen (mg. N/l. medium) as measured by micro-Kjeldahl technique.
B = Ammonia-nitrogen (mg.NH_3-N/l. medium) as measured by Conway's method.

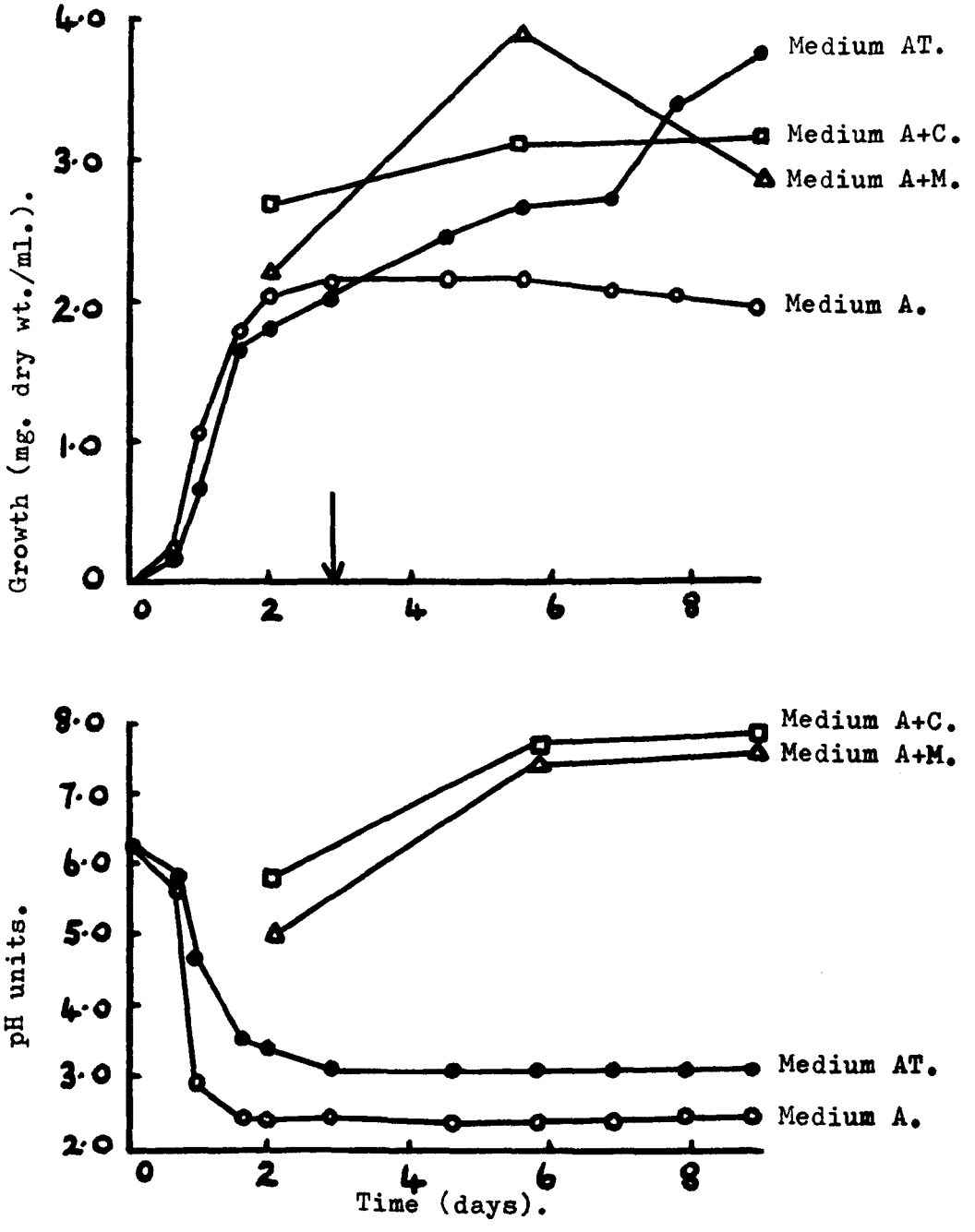


Fig. 7. Effect of buffering the pH drift of the medium on the growth of M. pusillus.
(↓ denotes nitrogen source depleted from medium).

CHAPTER 4. THE EFFECT OF DIFFERENT SOURCES OF NITROGEN ON GROWTH AND SPORULATION OF MUCOR PUSILLUS.

In the previous chapter the ability of M. pusillus to grow and spore on organic and inorganic nitrogen sources was noted. In addition, stabilisation of the pH by the inclusion of an organic acid in the culture medium appeared to retard sporulation. It was decided to study these observations more fully and to assess the effect of different nitrogen sources on growth and sporulation of M. pusillus in buffered and unbuffered media. Particular importance was attached to the utilisation of naturally-occuring nitrogen sources (proteins and the products of protein degradation).

4.1 Inorganic Nitrogen Sources.

4.11 Experimental Procedure. Cultures were grown from a standard spore inoculum, at 48°, in the basic medium containing glucose (20 g./l.) and a nitrogen source (250 mg.N/l. medium). Buffered media contained in addition, sodium succinate (5 g./l. medium). An attempt was made to measure sporulation objectively, as described in Section 1.6. Growth was measured by drying and weighing the mycelial mats obtained from five replicate flasks. The nitrogen content of the culture filtrate was analysed by Conway and microKjeldahl methods, and the pH determined.

4.12 Results. The measurements of growth, spore production and rate of assimilation of nitrogen source for four inorganic sources of nitrogen are given in Table 25. The data show that whilst the fungus grew adequately on all the nitrogen sources supplied, early growth was better on media containing an ammonium nitrogen source. Growth on nitrate and nitrite was apparent after a lag of about 36 hours, due probably to the time taken for the induction of those enzymes involved in the reduction of nitrate and nitrite to ammonia. Where ammonium nitrate was the nitrogen source, ammonia was preferentially assimilated (Table 24 (d)) as indicated by the pH drift of the medium and also by analysis of the ammonia and nitrate contents of the medium by Conway's method. After 8 days incubation most growth was obtained on sodium nitrate; ammonium nitrate, ammonium sulphate and sodium nitrite supporting less growth.

On all the media tested, sporulation reached a peak and then fell away. Maximum sporulation invariably occurred after the nitrogen source had been almost completely assimilated from the medium and the nitrogen level of the mycelium was relatively high.

TABLE 25. The effect of different inorganic nitrogen sources on growth and sporulation of M. pusillus. Analysis of medium and mycelium after different periods of incubation.

(a) Ammonium Sulphate.

Harvest Age of Mycelium in Hours.	Dry Weight of Mycelium (mg./ml.)	Sporulation (Number of Spores x 10^{-6}/ml.)	pH of Medium	Ammonia in Medium (mg.NH_3-N/l)	Total -N in Medium. (mg. N/l.)
Unbuffered Medium.					
0	0	0	6.2	242	248
36	1.04	1.16	2.6	163	175
48	1.76	0.96	2.4	53	71
84	2.64	1.52	2.2	0	27
108	2.74	2.65	2.1	0	33
180	2.48	1.45	2.3	0	32
Buffered Medium Experiment I.					
0	0	0	6.3	238	245
36	0.66	0.12	5.4	173	190
60	1.98	0.24	5.0	0	45
84	2.76	0.16	4.9	0	36
108	3.08	0.16	4.9	0	38
180	3.70	0.12	4.9	0	33
Buffered Medium Experiment II.					
0	0	0	6.2	249	256
36	0.97	0.52	5.4	143	156
60	2.17	0.64	5.0	40	55
84	2.69	0.52	5.0	0	31
108	3.36	0.60	5.4	0	29
180	3.35	0.28	5.8	0	39

(b) Sodium Nitrate.

Harvest Age of Mycelium in Hours.	Dry Weight of Mycelium (mg./ml.)	Sporulation (Number of Spores x 10^{-6}/ml.)	pH	Nitrate in Medium (mg.NO_3-N/l.)	Total - N Medium. (mg. N/l.)
Unbuffered Medium.					
0	0	0	6.5	231	245
36	0.10	0	6.6	209	218
48	0.28	0.32	6.6	152	171
60	1.08	0.49	6.5	73	95
84	3.20	0.93	6.5	35	60
108	3.36	1.46	6.6	0	33
180	4.04	1.33	6.6	0	41
Buffered Medium Experiment I.					
0	0	0	6.4	243	258
48	0.15	0.72	6.4	190	207
60	0.46	0.44	6.4	138	160
84	0.62	0.32	6.3	79	97
108	0.93	0.80	6.1	0	25
180	2.79	0.64	6.1	0	32
Buffered Medium Experiment II.					
0	0	0	6.5	258	273
36	0	0	6.6	247	260
48	0.13	trace	6.6	211	228
60	0.64	0.88	6.6	108	131
84	1.75	1.52	6.3	0	43
108	2.42	1.16	6.3	0	24
132	2.31	1.40	6.4	0	33
180	3.25	0.48	6.2	0	40

(c) Sodium Nitrite.

Harvest Age of Mycelium in Hours.	Dry Weight of Mycelium. (mg./ml.)	Sporulation (Number of Spores x 10^{-6}/ml.)	pH	Nitrite - Nitrogen in Medium. (mg.NO_2- N/l.)	Total - N in Medium. (mg. N/l.)
Unbuffered Medium.					
0	0	0	6.4	232	249
36	0	0	6.4	-	-
48	trace	0	6.4	-	-
60	0.09	0.18	6.6	201	220
84	0.72	0.48	6.7	123	140
108	1.56	0.60	6.7	0	41
132	2.13	0.56	6.9	0	32
180	2.09	0.56	6.8	0	38
Buffered Medium					
0	0	0	6.5	251	270
36	0	0	6.6	-	-
48	trace	0	6.6	-	-
60	0.22	0.56	6.8	191	212
84	0.75	0.80	6.8	131	149
108	1.30	0.84	6.7	0	23
132	3.50	0.76	6.9	0	31
180	3.20	0.24	6.9	0	27

(d) Ammonium Nitrate.

Harvest Age of Mycelium in Hours.	Dry Weight of Mycelium (mg./ml.)	Sporulation (Number of Spores x 10^{-6}/ml.)	pH	Ammonia - Nitrogen in Medium (mg. NH_3-N/l.)	Nitrate - Nitrogen in Medium (mg. NO_3-N/l.)	Total - N in Medium (mg.N/l.)
Unbuffered Medium.						
0	0	0	6.3	120	125	248
36	0.57	0.32	2.9	68	118	193
60	1.48	0.88	3.3	0	47	62
84	2.20	1.36	3.3	0	0	29
108	3.02	1.48	4.9	0	0	37
180	3.10	0.56	5.4	0	0	23
Buffered Medium.						
0	0	0	6.2	118	110	242
36	0.81	0.31	5.6	53	94	171
60	1.91	1.60	5.7	0	31	52
84	2.52	1.70	5.8	0	0	34
108	3.20	1.40	6.2	0	0	21
180	3.30	0.56	6.5	0	0	31

When the medium was buffered with sodium succinate, growth was markedly increased on ammonium sulphate and sodium nitrite, and markedly decreased on sodium nitrate. Where ammonium nitrate was the nitrogen source buffering the medium had little effect on growth.

Stablising the pH of the medium significantly reduced the number of spores produced when ammonium sulphate was the nitrogen source. With the other sources tested, sporulation was not significantly affected by buffering the medium.

4.2 Organic Nitrogen Sources. 4.21 Single Amino Acids.

4.211 Experimental Procedure. Cultures were incubated at 48° in the basic medium containing glucose (20 g./l.) and a single amino acid (250 mg. N/l.) as sole nitrogen source. Amino acid sources were sterilised as described in Section 1.3. The rotatory powers of the amino acids were as follows: racemates (alanine, serine, ∝-aminobutyric acid, valine, leucine, isoleucine, norleucine, norvaline, citrulline, ornithine, threonine, methionine, tryptophan, phenylalanine, dihydroxyphenylalanine); laevorotatory (arginine, lysine, glutamic acid, aspartic acid, cysteine, cystine, histidine, proline, tyrosine); no rotation (glycine). Buffered media contained sodium succinate (5 g./l.).

Sporulation was assessed as described in Section 1.6 and growth was measured by drying and weighing mycelial mats obtained from five replicate flasks. Culture filtrates were analysed for presence or absence of amino acid nitrogen source by paper chromatography; the pH was also determined.

4.212 Results. Date for the growth and sporulation of M. pusillus on unbuffered media containing 29 different amino acids are presented in Tables A, B and C and on buffered media in Tables D, E and F. These data are included in Appendix. A summary is given in Table 26 and Figure 8.

The fungus grew on all but four of the amino acids tested - p-aminobenzoic acid, hydroxyproline, creatine and thyroxine. Glycine, alanine, proline, aspartic acid and glutamic acid supported largest amounts of growth, and cysteine, cystine, dihydroxyphenylalanine (DOPA) tryptophan and histidine the smallest amounts of growth.

Utilisation of the nitrogen source caused an acidic reaction in the culture medium, the pH of which fell to 3-4. Where the medium was buffered with sodium succinate the pH remained in the range 5-6. Generally, the effect of buffering the medium was to reduce the growth of the fungus; except in the cases of methionine, cysteine, cystine and

TABLE 26. A summary of growth and sporulation of M. pusillus on single amino acid nitrogen sources.

Amino Acid.	Cumulative* Growth		Cumulative* Sporulation.		Cumulative* Sporulation Index.	
	Unbuffered Medium	Buffered Medium	Unbuffered Medium	Buffered Medium	Unbuffered Medium	Buffered Medium.
Proline	19.56	11.29	1.86	1.32	0.12	0.11
Glutamic Acid	17.73	12.35	4.48	1.84	0.32	0.15
Glycine	17.45	12.39	3.28	3.00	0.23	0.24
Alanine	15.61	9.90	1.31	2.92	0.10	0.29
Tyrosine	12.42	7.09	4.35	3.31	0.35	0.47
Arginine	12.25	9.39	4.51	1.52	0.37	0.16
Serine	10.98	8.06	4.99	4.52	0.46	0.56
Aspartic Acid	10.36	10.54	2.45	3.56	0.29	0.34
Leucine	10.13	7.16	0.51	3.32	0.00	0.46
Ornithine	9.54	6.68	6.21	5.76	0.66	0.86
Phenylalanine	9.38	9.11	2.23	1.50	0.23	0.16
Valine	9.14	6.47	0.89	2.36	0.01	0.36
-ABA	8.83	7.55	2.02	1.52	0.23	0.20
Isoleucine	8.45	4.20	2.69	1.96	0.31	0.46
Norleucine	8.43	7.30	3.02	1.72	0.28	0.23
Threonine	8.37	5.30	1.07	1.24	0.09	0.28
Citrulline	8.16	5.81	3.48	6.04	0.42	1.04
Norvaline	7.72	6.05	1.85	1.18	0.24	0.20
Methionine	6.88	8.40	6.04	1.92	0.88	0.23
Tryptophan	3.36	2.15	7.69	1.24	2.28	0.58
Cysteine	3.10	4.00	5.02	1.78	1.64	0.44
Lysine	3.00	3.00	3.43	0.60	1.14	0.20
Cystine	1.92	2.10	3.40	2.25	1.77	1.07
Histidine	1.16	1.55	0.57	0.80	0.49	0.50
DOPA	0.32	0.40	0.14	trace	0.44	0.00

* See Sections 1.51 and 1.6

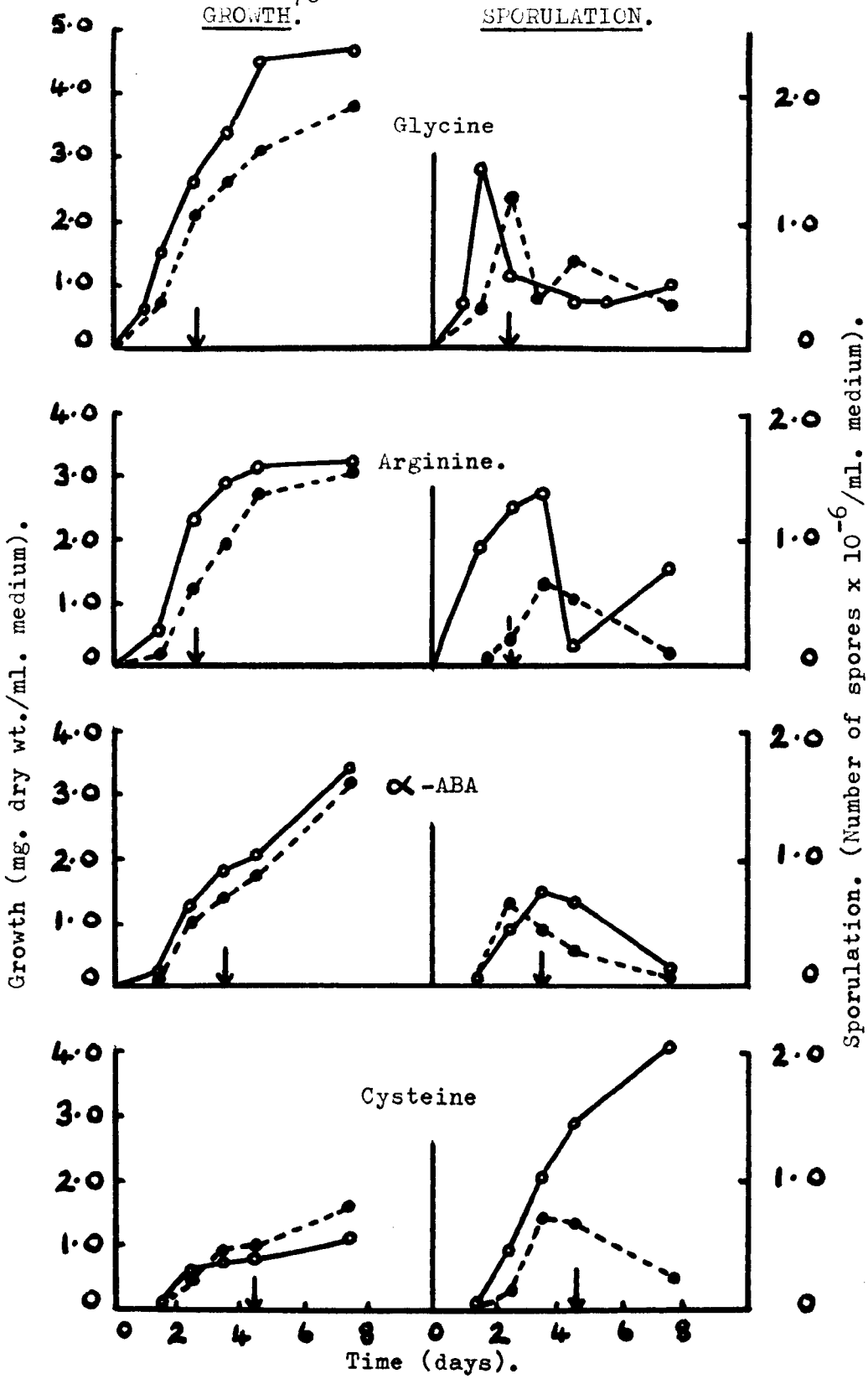


Fig.8. Growth and sporulation of M. pusillus on single amino acid sources in unbuffered (o——o) and buffered (o---o) media. (↓ denotes depletion of amino acid from unbuffered medium).

histidine where growth was better on the buffered medium.

The best sources for sporulation were methionine, cysteine, cystine, tyrosine, tryptophan, ornithine, citrulline and lysine. On alanine, leucine, valine, threonine and histidine sporulation was poor. Addition of sodium succinate to the medium usually reduced sporulation. However, sporulation was increased when media containing alanine, leucine, valine, aspartic acid and citrulline were buffered.

4.22 Mixtures of Amino Acids.

4.221 Experimental Procedure. To the basic medium containing glucose (20g./l.) was added an amino acid mixture; each amino acid being present at a level of 10 mg. N/l. medium. The complete mixture contained 25 amino acids, all of which could be utilised by M. pusillus. Other amino acid mixtures contained 24 amino acids, a different amino acid being omitted from each medium. Buffered media contained sodium succinate (5g./l.). Growth and sporulation were measured as described in Section 4.11.

4.222 Results. The effects of different mixtures of amino acids on the growth and sporulation of M. pusillus are shown in Tables G - L (Appendix).

With one exception growth was very similar irrespective of which amino acid was omitted from the mixture. The organism

grew much more profusely on a medium lacking DOPA. Sporulation was enhanced when norleucine, DOPA, tyrosine or tryptophan were omitted.

The final pH of the medium was in the range 3-4. This drift was mitigated by the addition of sodium succinate, and the pH of cultures was maintained in the range 5.5 - 6.5.

Though growth in the early stages of culture was slower, by the 8th day the organism had grown better on the buffered media. Sporulation was greatly reduced when culture media were buffered.

4.23 Organic Nitrogen Sources other than Amino Acids.

4.231 Experimental Procedure. Nitrogen sources were added at a concentration of 250 mg. N/l. to the basic medium containing glucose (20 g./l.). Buffered media contained sodium succinate (5 g./l.). Casein, gelatin, albumen, glycylglycine, glutathione and urea were sterilised by membrane filtration; all other nitrogen sources were autoclaved.

Growth and sporulation were measured as described in Section 4.11.

4.232 Results. Measurements of growth and sporulation of M. pusillus on a number of different organic nitrogen sources are presented in Tables 27 and 28.

The organism did not grow on glutathione or cytosine, but with the exception of gelatin and albumen all other sources supported moderate to profuse growth and sporulation. Although gelatin and albumen were poor sources for growth they proved excellent sources for sporulation. Buffering of media containing urea or peptone had little effect on growth and sporulation compared with their respective unbuffered media.

TABLE 27. Growth of M. pusillus on unbuffered media containing organic nitrogen sources other than amino acids.
(Growth expressed as mg. dry weight/ml.).

Nitrogen Source.	Age of Mycelial Harvest (in hours)					Cumulative Growth.
	36	60	84	108	180	
Casein	0.26	0.88	1.58	1.89	2.25	6.86
Gelatin	trace	0.13	0.20	0.28	0.24	0.96
Albumen	0	0	trace	0.04	0.08	0.12
Peptone	1.41	1.71	2.45	2.49	2.63	10.69
Glycyl-Glycine	0.25	1.99	3.49	4.90	4.90	15.53
Urea	trace	0.58	1.27	2.86	3.34	8.10
Adenine	0.25	0.25	0.24	0.52	1.83	3.09
Peptone + Buffer	1.10	1.57	2.04	2.40	2.49	9.60
Urea + Buffer	0.63	2.24	2.72	3.43	3.44	12.46
Glutathione	No Growth					
Cytosine	No Growth					

TABLE 28. Sporulation of M. pusillus on unbuffered media containing organic nitrogen sources other than amino acids.

(Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Nitrogen Source.	Age of Mycelial Harvest (in hours)					Cumulative Sporulation.
	36	60	84	108	180	
Casein	0.29	3.76	5.72	7.07	3.96	20.90
Gelatin	0	0.74	2.42	2.47	1.58	7.21
Albumen	0	0	trace	0.97	2.72	3.75
Peptone	2.60	3.92	1.92	4.48	1.92	14.84
Peptone + Buffer	2.16	3.11	3.20	3.24	2.24	13.95
Glycyl - Glycine	0.29	2.30	1.52	1.04	1.18	6.33
Urea	0	0.36	0.80	0.52	0.56	2.24
Urea + Buffer	0.36	2.00	1.56	1.08	0.72	5.72
Adenine	0	0	0.54	0.55	0.66	1.75
Glutathione	No sporulation.					
Cytosine	No sporulation					

CHAPTER 5. DISCUSSION.

The effect of nitrogen-nutrition on the growth and sporulation of Mucor pusillus was investigated. Two methods of assessing growth were used in the present study - measurement of colony diameter, and dry weight of mycelial mass. The measurement of colony diameter as a means of assessing growth rate is based on the assumption that linear extension of the colony is directly proportioned to the amount of growth. In certain cases this assumption is not valid. Stocks and Ward (1962) measured the colony diameter of Rhizopus nigricans on media containing different amino acid mixtures and found that whilst the omission of single amino acids from the mixture had little effect on the colony diameter, such omissions had an effect on the mycelial density and grades of growth were produced which varied from "sparse aerial hyphae" to "heavy opaque aerial mycelium." For a nutritional study the mycelial dry weight method is preferable and was therefore the method used in this investigation. Brancato and Golding (1953), Cochrane (1958) and Crisan (1959) are agreed, however, that the diameter of the fungal colony is a reliable measure of growth for the study of environmental factors if all other variables are kept constant.

For Mucor pusillus a comparison of the optimum temperatures obtained by the colony diameter method and dry weight method showed excellent agreement. Optimum temperature for the former method was defined as that temperature at which the fungus most rapidly completely covered the agar plate, and in the latter as that temperature at which the greatest mycelial weight was produced in the shortest time. The optimum for M. pusillus was 45° by the colony diameter method and 47° by the dry weight method.

In preliminary experiments it was found that growth and sporulation were affected by a number of environmental factors. For example, no sporulation occurred in shake culture, but in still culture the concentration of nutrients and acidity of the medium affected both vegetative and reproductive growth. The concentration of carbon source in the medium was not critical between 20 and 200 g./l. glucose; measureable amounts of mycelium and spores were produced in this range. The concentration of the nitrogen source was much more critical, affecting both growth and sporulation. A concentration of 250 mg. N/l. medium was used because it was completely assimilable and gave a high yield of vegetative and reproductive growth, without significant mycelial autolysis in the later stages of culture.

Fungi can be classified into three groups according to their ability to utilise three different types of nitrogen; nitrate,

ammonia and organic nitrogen (Steinberg 1939). Members of Group I can utilise nitrate, ammonia or organic nitrogen sources and include some species of Mucor as well as many Actinomycetes, Ascomytes and Fungi Imperfecti. Fungi in Group II are unable to use nitrate and are restricted to the use of ammonium salts or organic nitrogen as sole nitrogen source. This group contains many Phycomycetes and Basidiomycetes. Finally, in Group III are those fungi (mainly parasites) which have an absolute requirement for nitrogen in an organic form. A similar form of physiological classification was proposed by Robbins (1937) who included in a fourth group those fungi able to utilise atmospheric nitrogen. The ability to fix nitrogen is less widespread in the fungi than the bacteria, and only the yeasts Pullularia and Rhodotorula appear to have this ability (Metcalfe and Chayen 1954).

Mucor pusillus was found to utilise nitrate, ammonia and various forms of organic nitrogen as sole nitrogen sources and is therefore included in Group I of Steinberg's Classification (equivalent to Group II, Robbins' Classification). The arrangement of fungi into groups based on physiological properties may be significant from an evolutionary standpoint. Progressive evolution is often correlated with a loss of synthetic ability (Cantino and Turian, 1959). More specialised organisms frequently lose the ability to synthesise

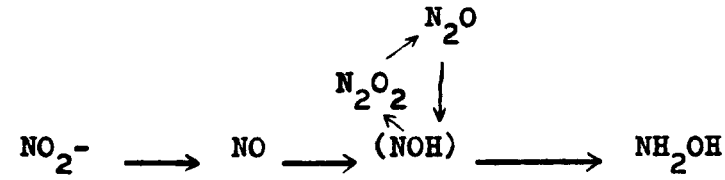
simpler nutrients and require more complex building blocks for their nutrition. The ability of M. pusillus to utilise a full range of nitrogen sources indicates that the adaptations involved in the evolution of thermophily have apparently occurred without any loss of synthetic ability.

Under conditions of still culture in a chemically defined medium M. pusillus used nitrate and nitrite less readily than ammonia, which was completely assimilated after approximately two days growth. When nitrate and nitrite were nitrogen sources they were detected in the culture medium after four days growth. When ammonium nitrate was the nitrogen source, ammonia was preferentially assimilated; nitrate was not utilised until ammonia was completely exhausted from the medium.

The lag period which preceded the assimilation of nitrate and nitrite is probably due to the inductive syntheses of reductase enzymes, and subsequent reduction of these nitrogen sources to ammonia. A number of the enzymes and intermediates are known in the reduction of nitrate to ammonia and the pathway is summarised below:-

NO_3^-	$\longrightarrow$	NO_2^-	$\longrightarrow$	?	$\longrightarrow$	NH_2OH	$\longrightarrow$ NH_4^+
	nitrate reductase.		nitrite reductase				hydroxylamine reductase.
Oxidation/ Reduction State of Nitrogen atom	+ 5		+ 3			- 1	- 3

Little is known about the formation of hydroxylamine from nitrite, though since a change of 4 electrons is involved it is unlikely that this is a single reaction. Nicholas (1965) has proposed the following mechanism for the reduction of nitrite to hydroxylamine using nitric oxide (NO), nitroxyl (NOH), hyponitrite (N_2O_2) and nitrous oxide (N_2O) as intermediates:-



Oxidation/reduction State of nitrogen atom	+ 3	+ 2	+ 1	- 1

Preferential assimilation of ammonia over nitrate has been shown in Neurospora crassa (Kinsky 1961) and Aspergillus nidulans (Cove 1966) to be due to the inhibition of nitrate reductase synthesis by ammonia. The repression of nitrate reductase activity by the reduction end product, ammonia, so-called "feedback inhibition" functions as a means of controlling nitrate utilisation.

Inorganic nitrogen, in the form of ammonia, is ultimately incorporated into organic compounds, an important acceptor molecule being α-ketoglutaric acid. Glutamic acid

dehydrogenase isolated from Neurospora crassa has been shown to catalyse the amination of ∝-ketoglutarate by ammonium ions, forming glutamic acid (Nicholas & Mabey 1960).

In a study of fungus nutrition the most relevant nitrogen compounds are those which occur naturally, namely proteins, peptides and amino acids. M. pusillus was grown on a medium containing individual amino acids as sole nitrogen sources. The fungus grew on all the amino acids tested, with the exception of p-aminobenzoic acid (PABA), hydroxyproline, creatine and thyroxine.

Hydroxyproline has been reported as preventing spore germination in Rhizopus arrhizus (Weber & Ogawa 1965). However among the Ascomycetes this amino acid was a utilisable nitrogen source for Penicillium griseofulvum (Bent & Morton 1964), Aspergillus niger (Steinberg 1942) and Venturia inequalis (Pelletier & Keitt 1954). In the present investigation and the literature, the inhibitory properties of hydroxyproline are limited to M. pusillus and R. arrhizus and it may well be that this inhibition is a property of the Mucorales only, to which both fungi belong. Certainly, hydroxyproline can be utilised by ascomycetes. The non-utilisation of PABA, hydroxyproline, creatine and thyroxine by

M. pusillus may indicate an inability to synthesise the necessary deaminase or transaminase enzymes.

Of the 25 amino acids which were utilised by M. pusillus, glycine, alanine, proline, glutamic acid and aspartic acid supported the largest amounts of growth, while dihydroxyphenylalanine (DOPA) tryptophan, histidine, cysteine and cystine were poor nitrogen sources.

Monoamino, monocarboxylic amino acids with an unbranched carbon chain, e.g. glycine, alanine, serine, α-aminobutyric acid, were better sources than branched chain amino acids, e.g. valine, norvaline, leucine, norleucine and isoleucine. This may be due to differences in permeability, for while glycine, alanine and serine were completely assimilated from the medium within 60 hours, and α-aminobutyric acid within 84 hours, valine, norvaline, leucine, norleucine and isoleucine were not completely assimilated until 108 hours incubation. There seems to be no relation between efficient utilisation of any amino acid and such properties as isoelectric point, length of carbon chain, presence or absence of sulphur, acidity, basicity or aromatic properties.

Deamination of amino acid nitrogen-sources resulted in a fall in pH of the culture medium to pH 3.0 - 4.5. When

buffered with sodium succinate the pH of the medium remained in the range 5.0 - 6.5. On buffered media almost all of the amino acids supported less growth, compared with growth in unbuffered media. Cysteine, cystine, methionine and histidine were exceptions, and M. pusillus grew better when these sources were contained in buffered media.

The uptake of amino acids by fungi is influenced by the pH of the culture medium. Whitaker (1966) found that in Penicillium griseofulvum the optimum pH for the uptake of aspartic acid was pH 5.8, and for leucine and lysine pH 5.8 - 6.6. In Neurospora crassa phenylalanine was accumulated most rapidly at pH 5.5 (DeBusk & DeBusk 1965). A more acid range of pH optima existed for Botrytis fabae (Jones 1963) where uptake of valine was optimal at pH 3.4 - 4.0, glutamic acid pH 4.0 and lysine at pH 3.0. The uptake of glutamic acid at pH 6.0 and of lysine at pH 7.0 were found by Jones to be negligible. The increased growth of M. pusillus on amino acids in unbuffered rather than in buffered media may reflect a pH optimum for amino acid accumulation in the same range as Botrytis fabae.

It has been stated (Cochrane 1958, Nicholas 1965) that fungi usually grow better with a natural or artificial

mixture of amino acids than with any single amino acid. In the present work with M. pusillus, however, growth on an artificial mixture of amino acids (present in equal proportions on a total nitrogen basis) was inferior to that on individual amino acids, with the exception of cysteine, cystine, DOPA, tryptophan and histidine, all of which supported only poor growth. On the other hand a natural amino acid mixture, casein hydrolysate, was a better nitrogen source than all single amino acids with the exception of glycine, alanine, glutamic acid and proline. The amino acid composition of casein - high in leucine, proline and glutamic acid, and low in cysteine, cystine, tryptophan and histidine is evidently a favourable one for the growth of M. pusillus.

In experiments where M. pusillus was grown on an artificial mixture of 25 amino acids from which individual amino acids were omitted, the exclusion of DOPA enhanced the growth of the fungus. No other amino acid significantly affected growth when omitted from the artificial mixture, which is in broad agreement with Foster's view of the non-essentiality of any single amino acid (Foster 1949 p.111).

In a study of the effects of amino acids on the various stages in the life-cycle of Rhizopus nigricans (Stocks & Ward 1962), DOPA, though not utilised as a nitrogen source, appeared on the one hand to hasten spore germination and on the other to delay spore production. The view that DOPA, since it is not a known constituent of the amino acid pools of micro organisms is not a "normal" amino acid, is fully accepted. However, the unexplained effects of this amino acid on growth and sporulation of M. pusillus and spore germination and sporulation of R. nigricans are none the less of interest.

Of three proteins tested as sole nitrogen sources only one, casein, supported satisfactory growth of M. pusillus. That gelatin and egg albumin were very poor nitrogen sources for growth would seem to indicate either that deficiencies exist in the proteinase activity of M. pusillus or that the amino acid constitution of albumin and gelatin are unfavourable for this organism. There is evidence to support both possibilities. With regard to their amino acid compositions albumin is deficient in many amino acids - glycine, alanine, valine, leucine, isoleucine, phenylalanine, proline, serine and threonine, and gelatin, while having a virtually complete

amino acid complement, contains a high proportion of hydroxyproline - a known inhibitor of spore germination in Rhizopus arrhizus (Weber & Ogawa 1965).

There are two pieces of evidence which may be taken to indicate proteinase deficiencies in M. pusillus. Firstly, the growth of this organism on unhydrolysed casein was only 50% of that obtained on casein hydrolysate. Secondly, the inability of this fungus to utilise glutathione (γ-glutamylcysteylglycine) as a nitrogen source, though its components glutamic acid, cysteine and glycine were all potentially useable, points to a specific lack of the peptidases necessary for the hydrolyses of γ-glutamyl - cysteine and cysteylglycine peptide bonds. Inability to utilise glutathione is all the more surprising since this compound is almost ubiquitous in fungi, functioning as a coenzyme for triosephosphate dehydrogenase, an enzyme involved in the production of energy from carbohydrates.

Though there have been a number of studies concerning sporulation of mucoraceous fungi (Muller 1956, Stocks & Ward 1962, Sarbhoy 1965) only qualitative data were reported. In a nutritional study, where the sporulation response may be quantitative, the use of arbitrary descriptions

of sporulation such as "sparse", "moderate" and "profuse" are of limited value. Sporulation data have more application when:-

(1) Sporing is estimated quantitatively by counting or weighing spores.

(2) Time-course experiments are used so that sporulation is assessed at several phases in the life history of the organism.

(3) Spore production is correlated with mycelial production.

In M. pusillus the temperature range of sporulation was narrower than that of growth. In still culture the fungus grew from 22° - 55°, but of four temperatures tested, sporulation occurred at 25°, 35°, 47° and not at 53°. At 53° growth of the organism remained submerged and the usual mycelial mat was not produced. Morton et al (1958) reported that of all the factors inducing sporulation of Penicillium griseofulvum the most influential was the exposure of the fungus to aerial conditions. This is not surprising since these conditions probably approximate those which the fungus encounters in nature and to which it is adapted.

The concentration of nitrogen source which was optimal for growth of M. pusillus (250 mg N/l. medium) was not optimal for sporulation. There was an inverse relationship between sporing and concentration of nitrogen source. Sporing, expressed as the "cumulative sporulation index" was 8.43 at 40 mg.N/l., 4.00 at 250 mg. N/l. and 1.70 at 500 mg. N/l. For a study of the effect of nitrogen nutrition on sporulation, a concentration of 250 mg. N/l. was used since at this level M. pusillus produced high sporulation counts from mycelium which underwent little or no autolysis.

M. pusillus spored well on a wide rangeof inorganic and organic nitrogen sources. Especially high spore counts were obtained when ammonia, glutamic acid, tyrosine, serine, the sulphur-containing and basic amino acids, casein, gelatin, peptone and urea were nitrogen sources. As with growth, buffering the medium tended to reduce sporulation both in actual spore counts, and in the "sporulation index", an effect especially marked on media containing ammonia or artificial mixtures of amino acids.

Exceptionally high "sporulation indices" were obtained with the protein and polypeptide sources tested. Casein and peptone contain a wide range of amino acids and were

good sources both for growth and sporulation. With gelatin and albumin, however, high "sporulation indices" resulted from the production of moderate amounts of spores by minute amounts of mycelium. Gelatin contains hydroxyproline as a major component and this may be responsible for high spore counts by preventing spore germination. Egg albumin is made up almost entirely of those amino acids effective in promoting sporulation - the sulphur-containing and basic amino acids, tyrosine and glutamic acid; while the monoaminocarboxylic acids and proline, which enhanced vegetative growth, are absent.

There have been a number of studies on the external factors affecting sporulation in fungi and these are listed by Morton et al. (1958). Relatively little is known, however, about the ways in which external factors influence the internal changes necessary to cause the change from a vegetative to a reproductive phase.

A number of investigations into biochemical changes associated with induction of sporulation are now discussed. From an intensive study of the induction of sporulation of several Penicillium species (Morton, England and Towler 1958, Morton 1961, Armstrong, England, Morton & Webb 1963) it was found that, for all the species tested, the most influential

and immediate stimulus to conidiation was the emergence of submerged hyphae into the air. Under shake-culture conditions, where the mycelium remained submerged, sporing could only be induced by special factors such as a high carbon : nitrogen ratio, the presence of a "sporing factor" identified as anhydroglucose, or a high concentration of calcium ion. For Pen. notatum a concentration of Ca^{++} of 35 p.p.m. was necessary to induce maximum conidiation; strontium and barium also induced sporulation but were less effective (Hadley & Harnold 1958).

Another factor associated with sporogenesis is changing enzyme activity. In the mitochondria of sporulating, as opposed to non-sporulating, mycelium of Neurospora crassa there is induction of isocitrate lyase and reduction of Krebs Cycle enzymes, causing activation of the glyoxalate cycle (Turian 1960, Weiss & Turian 1966). The activity of isocitrate lyase, coupled with that of glyoxalate-alanine transaminase, leads to increased synthesis of glycine which is necessary for synthesis of the purine ring. The shift from the Krebs to the glyoxalate cycle is thought to be associated with the increased nucleic acid synthesis and nuclear division during sporulation. Increased activity of

proteinase and nuclease enzymes has also been demonstrated in sporogenous mycelium of Pen. griseofulvum (Morton Dickerson & England 1960).

Changes in the rate of respiration may also induce sporulation. In bacteria, a correlation has been suggested between the cutting-off of a supply of energy to the cell, and endospore formation (Foster, 1956). An analagous process may occur in fungi. The induction of zygospore formation in a heterothallic strain of Rhizopus sexualis and of sporangiospore (asexual spore) formation in homothallic strains of Mucor hiemalis and Phycomyces blakesleeanus have been correlated with a check in the rate of respiration of these fungi.(Hawker and Hepden 1962).

The respiration rate, in turn, can be affected by nitrogen supply. In the mucoraceous fungus, Zygorhyncus moelleri, the respiration rate was doubled by the addition of an ammonium nitrogen source; the rate of respiration fell almost to its original level upon exhaustion of the ammonia from the medium (Moses 1954). It seems possible, therefore, that peak sporulation of M. pusillus which usually coincided with, or immediately followed, exhaustion of the nitrogen source, may

be associated with a check in respiration rate due to the absence of an assimilable nitrogen source.

Although there is an enormous amount of information concerning fungal morphogenesis (Ainsworth & Sussman 1966, Vol. 2) the fundamental problems remain unsolved. Of the many metabolic changes known to occur in sporulation not one has been proved unequivocally to have a causal connection with morphogenesis.

CHAPTER 6. THE EFFECT OF GROWTH TEMPERATURE ON THE FATTY ACID COMPOSITION OF FUNGI IN THE ORDER MUCORALES.

There are conflicting reports in the literature on the effect of environmental temperature on the composition of the fatty acids of filamentous fungi. In some fungi increases in lipid unsaturation occur at higher growth temperatures (Pearson and Raper 1927, Gad and Hassan 1964, and Shaw 1966), whilst in others the lipids become more unsaturated at lower growth temperatures (Gregory and Woodbine 1953, Singh and Walker 1956, Salmonowicz and Niewiadomski 1965, and Shaw 1966). In other fungi there appears to be little relationship between temperature and fatty acid composition (Prill, Wenck and Peterson 1935, and Bowman and Mumma 1968).

Since previous investigations were all conducted with mesophilic fungi, the maximum temperature at which lipids have been studied in growing fungi is around 35°. In the present study, by using psychrophilic, mesophilic, thermotolerant and thermophilic species belonging to the same Order it has been possible to investigate the fatty acid composition of filamentous fungi over virtually their entire temperature range. Changes in fatty acid composition during the growth cycle were followed at 10° and 20° in psychrophiles, 25° in mesophiles and 28° and 48° in thermotolerants and thermophiles.

6.1. The Fatty Acid Composition of the Mycelium of Temperature-Adapted Filamentous Fungi in the Mucorales.

6.11 Experimental Procedure. The following species were used, a full record of their provenance is included in Section 1.2.

(i) Psychrophiles. *M. strictus* (3 isolates).
M. oblongisporus (2 isolates).

(ii) Mesophiles. *M. hiemalis* (+ and - strains),
M. mucedo, *M. racemosus*, *M. ramannianus*.

(iii) Thermotolerants. *Rhizopus* sp.I, *Rhizopus* sp.II.

(iv) Thermophiles. *Mucor* sp.I, *Mucor* sp.II, *Mucor* sp.III (*M. miehei*), *Rhizopus* sp.III.

All species were grown on the basic medium plus glucose (20 g./l.), ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.). In the cases of *M. ramannianus*, *M. racemosus*, *M. miehei* and *Mucor* sp.I, the medium had to be supplemented with yeast extract (5 g./l.). Culture medium was dispensed in 200 ml. portions in 750 ml. Erlenmeyer flasks. The pH of all media was adjusted to 6.0-6.5 prior to autoclaving at 15 lbs./in.^{2} for fifteen minutes.

Inocula were grown on the above media solidified with agar (20 g./l.) and dispensed in flat medicine bottles. Psychrophiles were incubated at 10°, mesophiles at 25°, thermo-

tolerants and thermophiles at 48°. After 2-3 weeks growth the spores were removed by shaking with sterile ice-cold distilled water. The resulting spore suspension was diluted with sterile distilled water to a density of 1.5-2.0 x 10^{-6} spores/ml. Each culture flask was inoculated with 5 ml. of standard spore suspension. Flasks were incubated in still culture at the indicated temperatures and harvested in triplicate after suitable incubation periods. The mycelium was removed by filtration on sintered glass discs and washed with distilled water. The mycelium was dried overnight at 80°, cooled in a desiccator, weighed and powdered in a small grinder.

The powdered mycelium was extracted in a Soxhlet apparatus, the lipids hydrolysed and the fatty acids methylated and analysed by gas-liquid chromatography (GLC).

6.12 The Identification of Individual Fatty Acids Extracted in this Investigation.

The peaks were provisionally identified by comparison of retention times with those of authentic standards. The methyl esters of fatty acids used as reference were obtained from Fluka A.G., Buchs, Switz. and included methyl esters of C 12 to C 20 straight chain saturated fatty acids, as well as the methyl esters of palmitoleic, oleic, linoleic and α-linolenic acid. α-Linolenic acid can be clearly separated from the γ-isomer by

GLC on diethyleneglycolsuccinate. The methyl esters of C 18 fatty acids extracted from mucoraceous fungi gave the following retention times relative to methyl stearate: oleate 1.09; linoleate 1.30; γ-linolenate 1.52. The methyl ester of authentic α-linolenic acid had a retention time relative to methyl stearate of 1.69 (c.f. James 1959 and Shaw 1965).

Complete identification of fatty acid esters was obtained by combined gas chromatography - mass spectrometry. A comparison between mass spectra of the methyl esters of the C 18 unsaturated acids extracted in this investigation and mass spectra of methyl esters of authentic fatty acids showed that the C 18 monoene was identical with methyl oleate, the diene with methyl linoleate, and the triene with methyl γ-linolenate. A comparison of their mass spectra showed that the C 18 triene was not methyl α-linolenate.

In early experiments six esters U1-U6 consistently appeared in chromatograms; these esters did not correspond to any known fungal lipid esters and experiments were undertaken to identify them. Reaction of the mixture of fatty acid esters with mercuric acetate (Goldfine and Bloch 1961), which resulted in the formation of mercury adducts with the unsaturated components, followed by chromatography on a Florisil column,

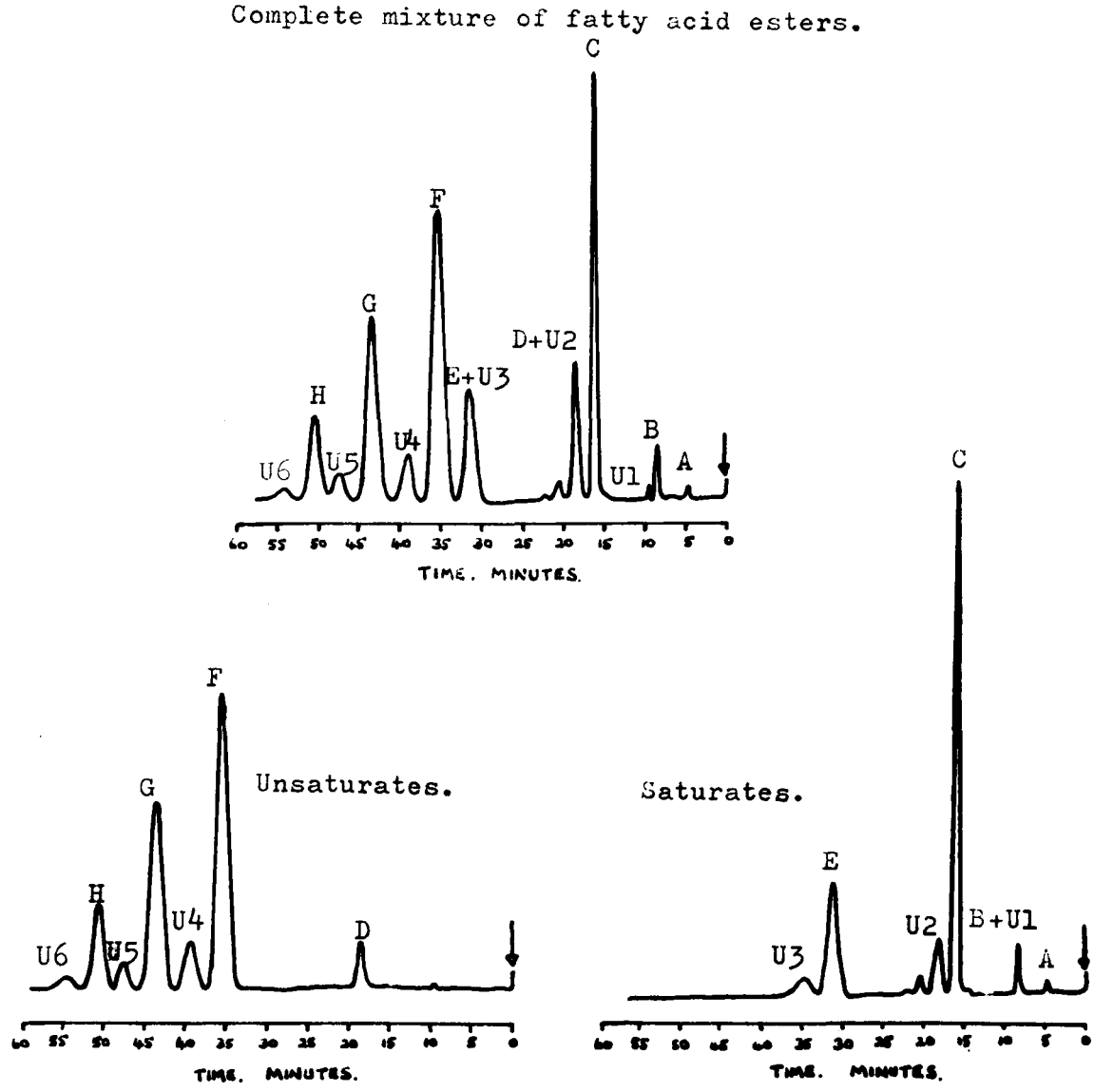


Fig. 9. Gas chromatographs of esters of fatty acids from mucoraceous fungi after separation of the saturated and unsaturated fatty acids, as their mercuric acetate adducts, on a Florisil column. (A = laurate, B = myristate, C = palmitate, D = palmitoleate, E = stearate, F = oleate, G = linoleate, H = linolenate, U1 - U6 = "unknown" esters).

separated the mixture into saturated and unsaturated esters (Fig.9). "Unknown" esters U1, U2 and U3 were found to be saturated, whilst U4, U5 and U6 were unsaturated.

The degree of unsaturation of the "unknown" esters U4, U5 and U6 and of the "known" esters D, F, G and H (see Fig.12) was checked by chromatography of the complete mixture of fatty acid esters on a column of Florisil impregnated with silver nitrate (Willner 1965). The elution pattern is given in Table 29 and illustrated in Fig. 10. Of the "unknown" unsaturated esters, U4 had one double bond and was eluted together with methyl palmitoleate and methyl oleate: U5 had two double bonds and was eluted with methyl linoleate, while U6 had three double bonds and was eluted with methyl linolenate.

The complete mixture of fatty acid esters was investigated by combined gas chromatography - mass spectrometry; the mass spectrum for each component wasobtained on at least two occasions during the elution of the peak. The mass spectra of two fatty acid esters, Component C and Component U2 were considered in detail (Fig.11) since these esters had similar retention times on DEGS and silicone S.E.30 columns, and similar chromatographic behaviour on a column of Florisil impregnated with silver nitrate (Fig.10).

TABLE 29. Elution of fatty acid esters from a Florisil-silver nitrate column.
(10 ml. fractions collected. Each 5th fraction analysed by GLC).

Eluant.	Volume of Eluant (ml.).	Fractions Combined.	Identity of Eluate.
Hexane	100 ml.	1-10	myristate, palmitate, stearate, U1, U2, U3.
0.5% Ether in Hexane	200 ml.		
1.0% " " "	200 ml.		
2.5% " " "	200 ml.	14-102 (combined)	palmitoleate.
4.0% " " "	200 ml.		oleate, U4.
5.0% " " "	200 ml.		
7.5% " " "	400 ml.	117-147	linoleate, U5.
10.0% " " "	300 ml.	148-164	traces of monoenoic esters.
15.0% " " "	100 ml.	179-188	linolenate, U6.

Complete mixture of fatty acid esters.

Saturates.

Monoenes.

Dienes

Trienes.

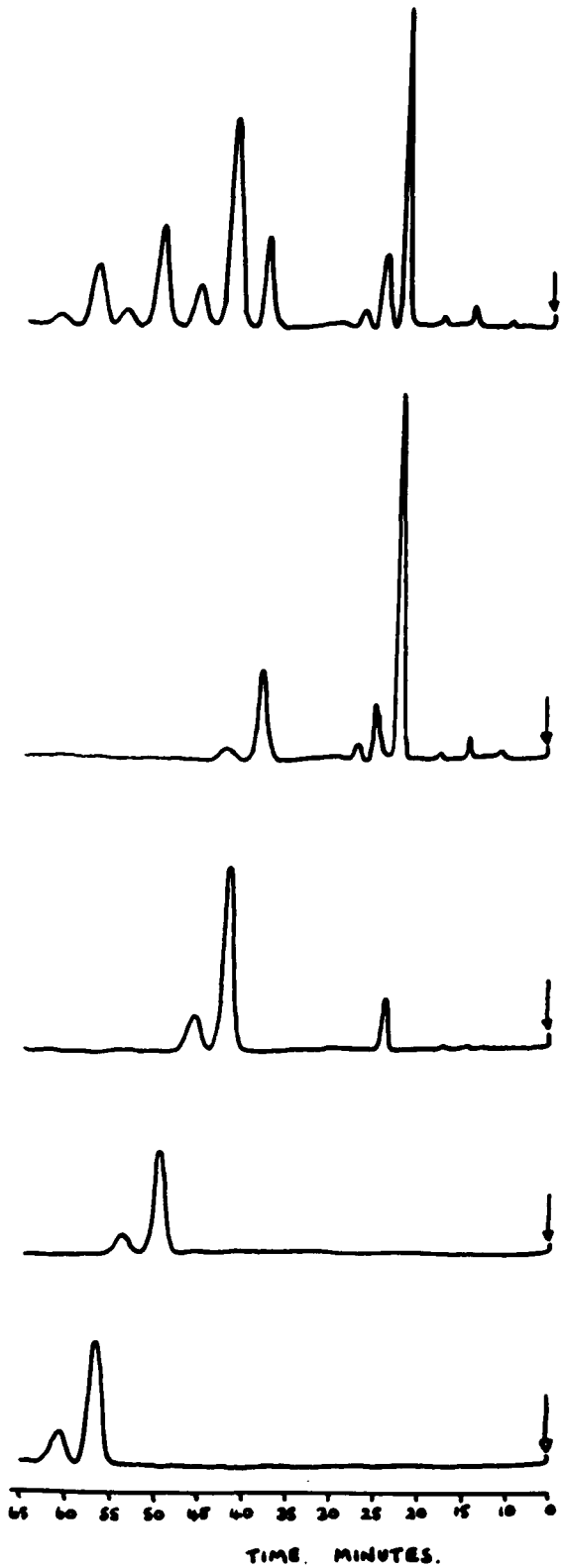


Fig.10. Gas chromatographs of esters of fatty acids from mucoraceous fungi, separated in saturates, mono-, di-, and trienes by chromatography on a Florisil/$AgNO_3$ column.

On the basis of retention time data on DEGS and Silicone S.E.30 columns, and chromatographic behaviour on a Florisil column impregnated with silver nitrate, Component C was tentatively identified as methyl palmitate. Confirmatory evidence was obtained by mass spectrometry; the molecular ion of Component C at m/e 270 corresponding to the mass of methyl palmitate ($C_{15}H_{31}CO_2CH_3$ = 270). In addition, β - cleavage and transfer of a γ-hydrogen atom (McLafferty Rearrangement) resulting in the formation of a strong peak at m/e 74, and loss of $-OCH_3$ (m/e 239) provided further evidence for a straight chain methyl ester.

The retention time of Component U2 on silicone S.E.30 was between that of a C16 methyl ester and a C17 methyl ester, and chromatography on a Florisil column impregnated with silver nitrate indicated that Component U2 was a saturated ester. The mass spectrum of component U2 showed a molecular ion at m/e 284, which suggested a fatty acid ester containing one methylene group more than Component C (methyl palmitate). β -cleavage of Component U2 resulted in a prominent ion at m/e 88 indicating that this component was not a straight chain methyl ester.

Both an α-methyl branched methyl ester (I) and a straight chain ethyl ester (II) would give rise to an ion of m/e 88 by McLafferty rearrangement and cleavage.

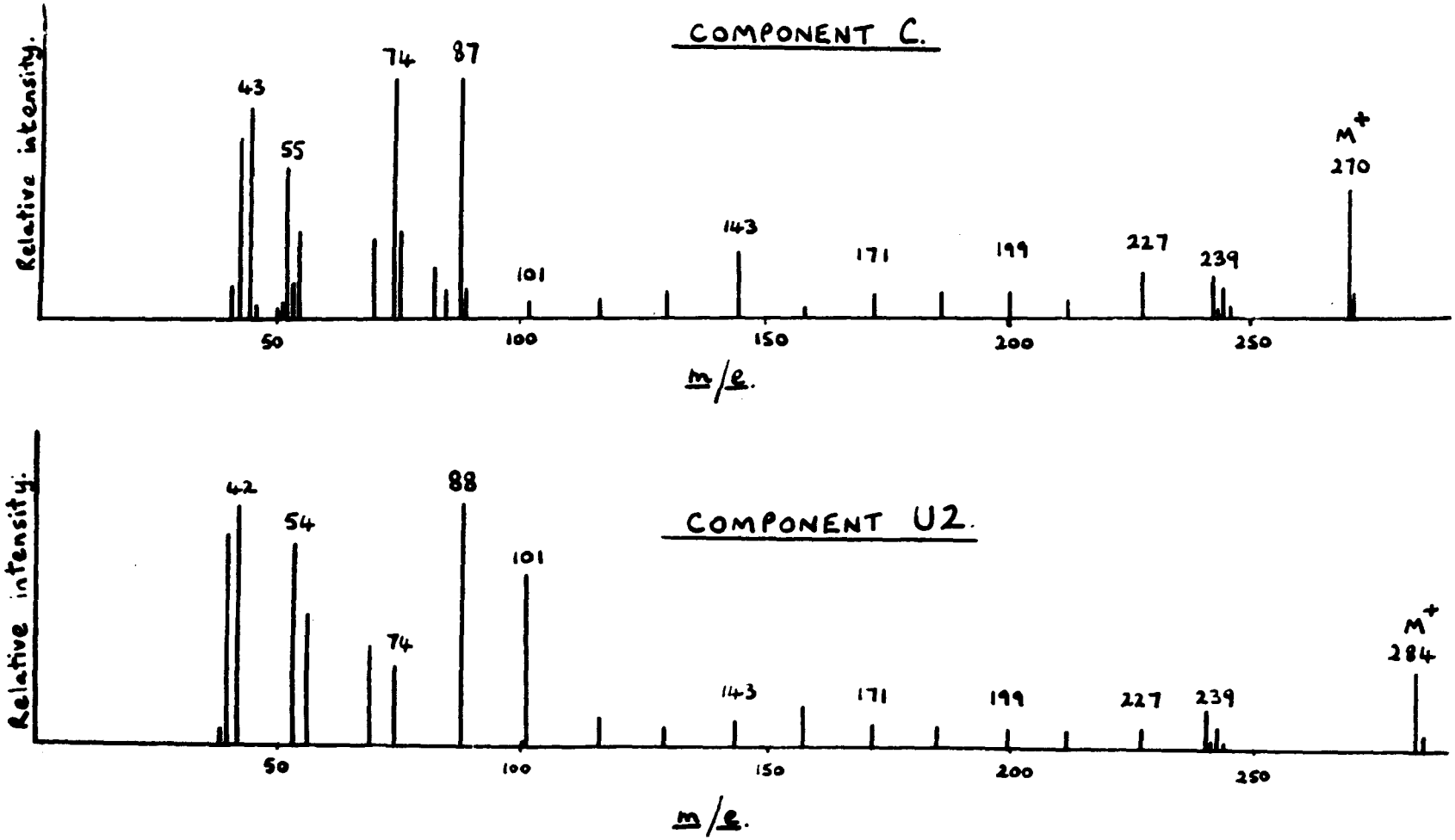
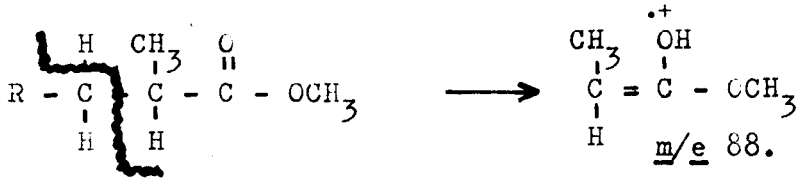
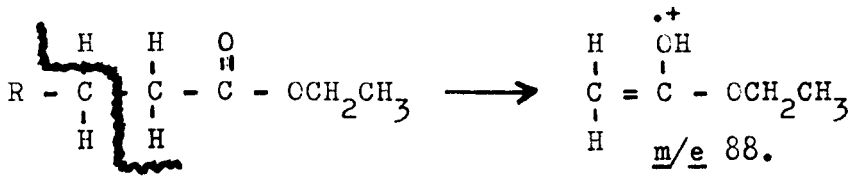


Fig.11. Mass spectra of representative "known" and "unknown" esters of fatty acids from mucoraceous fungi: Component C (methyl palmitate) and Component U2 (ethyl palmitate). (M^+ represents the molecular ion).

I. 

II. 

In Component C the loss of the methoxyl group resulted in a peak at m/e 239 (molecular ion - methoxyl group, i.e. 270 - 31 = 239). If Component U2 were an ∝-methyl branched chain methyl ester a peak at m/e 253 would be expected due to loss of a methoxyl from the molecular ion. However, in the mass spectrum of Component U2 no peak occurs at m/e 253, though there is a peak at m/e 239 consistent with the loss of an ethoxyl group from the molecular ion (284 - 45 = 239).

Examination of the mass spectra of all the "unknown" fatty acid esters (U1 - U6) showed the absence of a peak equivalent to the loss of a methoxyl group from the molecular ion (molecular ion - 31 mass units), and the presence of a peak due to the loss of an ethoxyl group from the molecular ion

(molecular ion - 45). Therefore all the unknown components could be shown to be, not α-methyl branched methyl esters, but normal ethyl esters. Further evidence that Components U1 - U6 were ethyl esters was the presence due to β-cleavage of strong peaks at m/e 88 in each case and the absence of strong absorption at m/e 74 characteristic of straight chain methyl esters.

The ethyl esters U1 - U6 were present as artefacts in the mixture of fatty acids isolated from fungi, and their formation was the result of the carry-over of very small amounts of ethanol from the lipid hydrolysis stage to the subsequent esterification. In early experiments the hydrolysis was carried out by refluxing the crude lipid with ethanolic KOH. When, in all later experiments the lipid was hydrolysed with methanolic KOH the components U1 - U6 were not formed.

6.13 The Growth, Lipid Content and Fatty Acid Composition of Temperature-Adapted Mucoraceous Fungi.

Data for the effect of incubation temperature on the growth, lipid yield and fatty acid composition are presented, for psychrophiles (Table 30), mesophiles (Table 31), thermotolerants (Table 32) and thermophiles (Table 33).

TABLE 30 . The Effect of Incubation Temperature on the Fatty Acid Composition of the Lipids of Psychrophilic Fungi.

Organism	M. strictus (CBS.100.66)								M. strictus (CBS.576.66)								M. strictus (CBS. 575.66)							
Incubation Temperature.	10°				20°				10°				20°				10°				20°			
Incubation Time (days)	10	14	21	28	10	14	21	28	10	14	21	28	10	14	21	28	10	14	21	28	10	14	21	28
Growth (mg.dry wt./ml.)	0.61	0.71	1.14	1.51	0.31	0.69	1.56	2.27	0.42	0.47	0.80	1.33	0.29	0.47	1.24	2.37	0.42	0.57	0.85	1.69	0.33	0.70	1.42	2.35
Lipid Yield (% of dry weight).	13.2	19.1	20.5	16.9	15.8	16.5	20.4	21.5	7.8	16.9	20.8	19.8	15.0	21.4	18.5	24.4	11.1	17.2	19.3	16.2	15.2	17.6	18.4	21.4
Fatty Acid																								
14:0	6.5	6.9	6.4	8.3	4.5	4.3	3.3	5.0	9.9	7.4	5.2	5.6	3.6	5.7	3.4	9.6	3.6	4.7	7.0	7.9	4.3	5.2	5.2	4.8
16:0	10.5	14.0	15.2	15.0	25.0	18.5	18.0	21.5	15.2	20.0	20.5	22.4	19.3	18.0	18.4	15.7	19.7	17.0	14.0	16.2	17.5	20.9	24.4	23.1
16:1	1.8	2.1	2.5	2.4	2.4	2.5	2.8	2.5	2.8	1.8	2.2	1.9	2.6	2.5	2.4	2.0	3.0	2.3	1.7	2.4	2.4	2.4	2.6	2.3
18:0	7.5	5.5	7.7	9.3	8.1	2.7	4.0	4.2	7.8	8.2	8.9	9.2	12.0	8.3	7.1	6.8	10.3	9.1	9.7	8.0	9.8	4.4	5.1	5.7
18:1	35.6	37.2	34.7	32.7	35.9	35.0	45.0	41.2	27.6	36.0	36.1	34.8	40.0	42.5	45.0	41.0	36.7	33.4	34.7	34.6	42.5	41.3	46.3	42.4
18:2	14.2	16.6	13.1	12.0	12.6	19.3	13.9	13.2	16.0	13.6	11.8	11.6	12.7	11.5	14.8	13.2	14.5	17.5	17.7	12.7	12.8	13.5	9.8	11.4
18:3	20.6	17.7	20.3	20.0	11.4	16.0	12.9	12.3	18.2	17.4	15.1	14.2	9.8	11.5	8.7	11.8	10.2	16.0	15.2	18.1	11.6	11.8	6.7	10.4
Degree of Unsaturation	1.26	1.26	1.24	1.13	0.98	1.24	1.14	1.07	1.14	1.18	1.08	1.03	0.98	1.03	1.04	0.94	0.99	1.19	1.19	1.17	1.06	1.06	0.89	0.99

TABLE 30 (continued). The effect of Incubation Temperature on the Fatty Acid Composition of the Lipids of Psychrophilic Fungi.

Organism	M. oblongisporus (CBS. 173.27)						M. oblongisporus (CBS.220.29).						
Incubative Temperature	10°				25°		10°				25°		
Incubation Time (days)	10	14	21	28	10	28	10	14	21	28	10	14	28
Growth (mg. dry wt./ml.)	0.44	0.47	0.69	0.94	0.07	0.16	0.48	0.55	0.66	0.76	0.40	0.70	1.22
Lipid yield (% dry weight).	10.0	19.1	13.2	20.0	2.5	16.0	7.6	14.2	17.2	10.8	4.5	9.8	10.3
Fatty Acids													
14:0	1.0	5.8	9.5	7.2	5.1	10.0	5.3	5.0	4.2	8.0	5.3	4.2	4.0
16:0	23.0	20.6	20.2	14.5	30.0	22.0	16.6	21.8	18.8	16.0	18.8	14.0	12.1
16:1	2.8	2.4	2.9	2.4	28.	2.4	2.8	2.6	2.2	1.9	2.2	2.1	1.9
18:0	13.1	7.8	16.0	14.3	20 .4	10.1	9.1	12.0	9.2	10.8	10.8	11.7	10.2
18:1	37.3	38.2	24.6	28.1	30.6	21.8	34.5	32.3	29.4	26.6	28.3	27.5	29.6
18:2	14.0	15.9	14.5	19.6	12.7	18.9	16.1	14.1	18.1	20.0	16.6	19.2	20.6
18:3	9.1	11.2	11.4	14.0	8.4	14.9	12.0	12.3	17.9	19.7	17.7	21.3	21.3
Degree of Unsaturation	0.95	1.03	1.01	1.12	0.84	1.07	1.05	1.00	1.22	1.29	1.17	1.30	1.37

TABLE 31 . Fatty Acid Composition of the lipids of Mesophilic Fungi during growth at 25°.

Organism.	M. ramannianus			M. racemosus			M. mucedo			M. hiemalis +			M. hiemalis -		
Time of Harvest(days).	4	7	14	4	7	14	4	7	14	4	7	14	4.	7	14
Growth (mg.dry wt./ml.)	1.10	1.25	3.52	0.58	0.86	1.54	0.69	1.25	4.44	0.75	1.75	2.69	0.82	1.77	2.75
Lipid yield (% of dry weight)	19.8	16.6	15.2	8.2	19.0	9.8	12.8	15.2	12.0	18.8	18.0	18.1	15.1	19.3	17.2
Fatty Acid															
14:0	1.4	1.6	1.6	4.1	3.1	2.7	4.1	0.8	1.1	2.3	1.8	1.8	4.3	2.8	2.4
16:0	16.2	17.6	18.5	18.7	17.8	16.9	15.5	12.0	16.8	16.2	15.5	15.2	22.2	15.6	14.8
16:1	2.7	3.1	2.6	3.4	3.3	2.9	3.0	3.1	1.4	2.8	2.0	3.2	3.4	3.3	3.1
18:0	7.2	5.9	4.4	5.5	5.9	4.6	6.1	9.3	11.3	20.0	17.2	9.6	16.2	12.8	9.7
18:1	35.4	28.7	28.0	31.3	38.2	36.5	22.1	26.1	30.5	31.3	38.6	36.8	27.5	26.5	32.6
18:2	15.8	13.1	13.5	14.1	12.7	16.6	27.5	25.3	32.9	13.2	18.5	19.4	14.6	19.8	18.8
18:3	21.1	29.1	30.9	20.9	18.8	19.4	22.3	20.2	6.4	11.6	18.2	14.8	12.4	18.9	19.2
Degree of Unsaturation	1.33	1.45	1.50	1.25	1.23	1.28	1.47	1.40	1.16	0.96	1.19	1.21	0.96	1.26	1.29

TABLE 32 . The effect of Incubation Temperature on the Fatty Acid Composition of the Lipids of Thermotolerant Fungi.

Organism	(a) *Rhizopus* sp.1						(b) *Rhizopus* sp.II.					
Incubation Temperature	28°			48°			28°			48°		
Incubation Time (days)	4	7	14	4	7	14	4	7	14	4	7	14
Growth (mg.dry wt./ml.).	0.69	1.17	1.49	1.25	1.63	1.54	1.05	0.95	1.38	0.69	1.25	1.19
Lipid Yield (% of dry weight)	43.2	45.3	25.7	21.5	23.0	11.6	20.8	32.8	24.6	22.9	25.8	29.8
Fatty Acid												
14:0	trace	1.2	1.4	1.5	3.0	trace	2.5	1.0	1.7	3.4	0.8	trace
16:0	24.6	18.4	20.6	29.4	20.2	23.3	24.3	19.1	24.6	22.7	25.4	24.3
16:1	2.8	2.3	2.3	3.0	2.5	3.0	3.0	2.8	2.5	2.8	3.0	3.0
18:0	8.2	10.5	5.2	10.6	11.3	18.6	10.0	10.0	3.9	13.0	8.2	7.9
18:1	35.9	32.5	30.0	35.6	36.1	34.9	30.7	26.4	29.4	42.3	37.2	33.8
18:2	17.8	23.8	28.6	14.1	20.6	21.8	19.4	28.0	27.5	19.7	19.8	24.4
18:3	9.3	12.4	11.9	6.4	5.8	5.1	8.9	13.6	12.3	15.0	5.5	6.3
Degree of Unsaturation	1.02	1.14	1.22	0.80	0.95	0.94	0.99	1.24	1.20	1.29	0.94	1.04

TABLE 33 . The effect of Incubation Temperature on the Fatty Acid Composition of the Lipids of Thermophilic Fungi.

Organism.	(d) Mucor sp. I.						(e) Mucor sp.II.						(f) Mucor sp.III (Mucor miehei)				(g) Rhizopus sp.III.			
Incubation Temperature	28°			48°			28°			48°			28°			48°	36°		48°	
Incubation Time (days)	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14	14	7	14	7	14
Growth (mg.dry wt./ml.)	1.00	2.95	4.34	2.90	4.34	4.75	1.81	2.80	4.00	3.01	3.46	3.61	0.67	1.66	1.74	4.38	0.32	1.12	0.85	1.87
Lipid Yield (% of dry weight).	21.8	24.8	22.6	19.4	13.2	35.9	36.4	25.2	19.4	25.4	26.0	8.4	25.1	24.6	7.8	11.9	21.0	8.8	32.5	13.1
Fatty Acid																				
14:0	1.7	1.2	1.0	1.7	0.9	0.8	trace	trace	trace	trace	trace	trace	1.3	1.4	1.6	1.8	trace	1.3	1.9	1.6
16:0	16.5	18.8	22.2	20.9	19.7	22.5	21.2	21.2	18.8	20.9	32.0	29.1	20.3	22.4	22.8	27.7	24.0	34.0	44.8	30.0
16:1	3.0	2.8	3.2	2.9	3.0	3.2	3.3	3.0	3.8	3.3	2.9	2.9	3.5	3.4	3.0	3.0	2.8	3.0	3.1	3.2
18:0	9.2	4.8	2.3	2.7	1.7	2.3	4.1	3.8	1.7	2.4	3.3	3.8	5.7	5.8	4.7	6.3	5.3	6.2	3.3	7.0
18:1	47.1	53.4	49.1	49.7	54.9	55.1	49.2	52.4	55.1	50.1	45.4	57.8	47.9	43.7	47.8	48.0	53.5	39.5	33.4	43.5
18:2	14.1	12.5	13.6	17.6	15.3	12.6	15.0	13.5	12.6	19.4	13.7	10.1	16.4	14.5	15.8	10.3	11.9	14.3	8.5	15.3
18:3	7.8	5.0	8.4	6.0	4.2	3.1	4.6	4.0	4.0	2.9	1.8	3.2	5.2	5.8	4.1	2.0	2.0	2.2	3.0	3.3
Degree of Unsaturation	1.01	0.96	1.04	1.03	1.04	0.91	0.96	0.96	1.06	1.00	0.79	0.91	0.98	0.93	0.95	0.76	0.84	0.76	0.60	0.86

The thermotolerant and thermophilic fungi all grew well at 28° and 48° except Rhizopus sp. III which barely grew at 28° and was therefore grown at 36° and 48°. The mesophilic fungi grew well at 25°. All the psychrophilic species grew well at 10°, but at 25° only M. oblongisporus (CBS.220.29) was capable of good growth; M. oblongisporus (CBS.173.27) growing poorly and M. strictus not at all. M. strictus was grown at an upper temperature of 20°.

In mesophiles and psychrophiles the percentage of lipid in the mycelium remained fairly constant throughout the incubation period, but in thermotolerants and thermophiles the amount of mycelial lipid varied with the ageof the mycelium, usually reaching a peak in the early stages of culture. In the high temperature species, concentration of mycelial lipid invariably reached 30 - 40% of the dry weight of the fungus. In mesophiles and psychrophiles however, the fungal lipid rarely exceeded 20% of the dry weight. Possible explanations of the higher concentration of mycelial lipid in fungi grown at high temperatures are discussed in Chapter 8.

Qualitatively all the fungi tested were very similar in that they invariably contained the same seven fatty acids, namely myristic, palmitic, palmitoleic, stearic, oleic, linoleic,

and linolenic acid; lauric, pentadecanoic, pentadecenoic and heptadecanoic acid were present in trace quantities.

In the present study the absence of α-linolenic acid was confirmed; the triply unsaturated C18 acid was identified as γ-linolenic acid, which is in agreement with the findings of previous workers that only the γ-isomer is present in phycomycete fungi (Bernhard and Albrecht 1948, Shaw 1965, 1966, 1966a, White and Powell 1966, and Tyrrell 1967).

There were marked differences in the relative proportions of fatty acid esters of psychrophilic, mesophilic and thermophilic fungi (Fig. 12). The lipids of thermophilic fungi characteristically contained large quantities of oleic acid (around 45% of the total fatty acids) and much smaller quantities of linoleic acid (approximately 15%) and linolenic acid (3 - 5%). Thermotolerant, mesophilic and psychrophilic fungi all contained lipids in which oleic acid was present in smaller proportions (30 - 38%) and linoleic acid (15 - 24%) and linolenic acid (8 - 19%) in much larger proportions.

Differences in the relative proportions of monounsaturated and polyunsaturated acids led to differences in the overall saturation/unsaturation ratio of the lipid. Such differences are conveniently expressed by calculating the degree

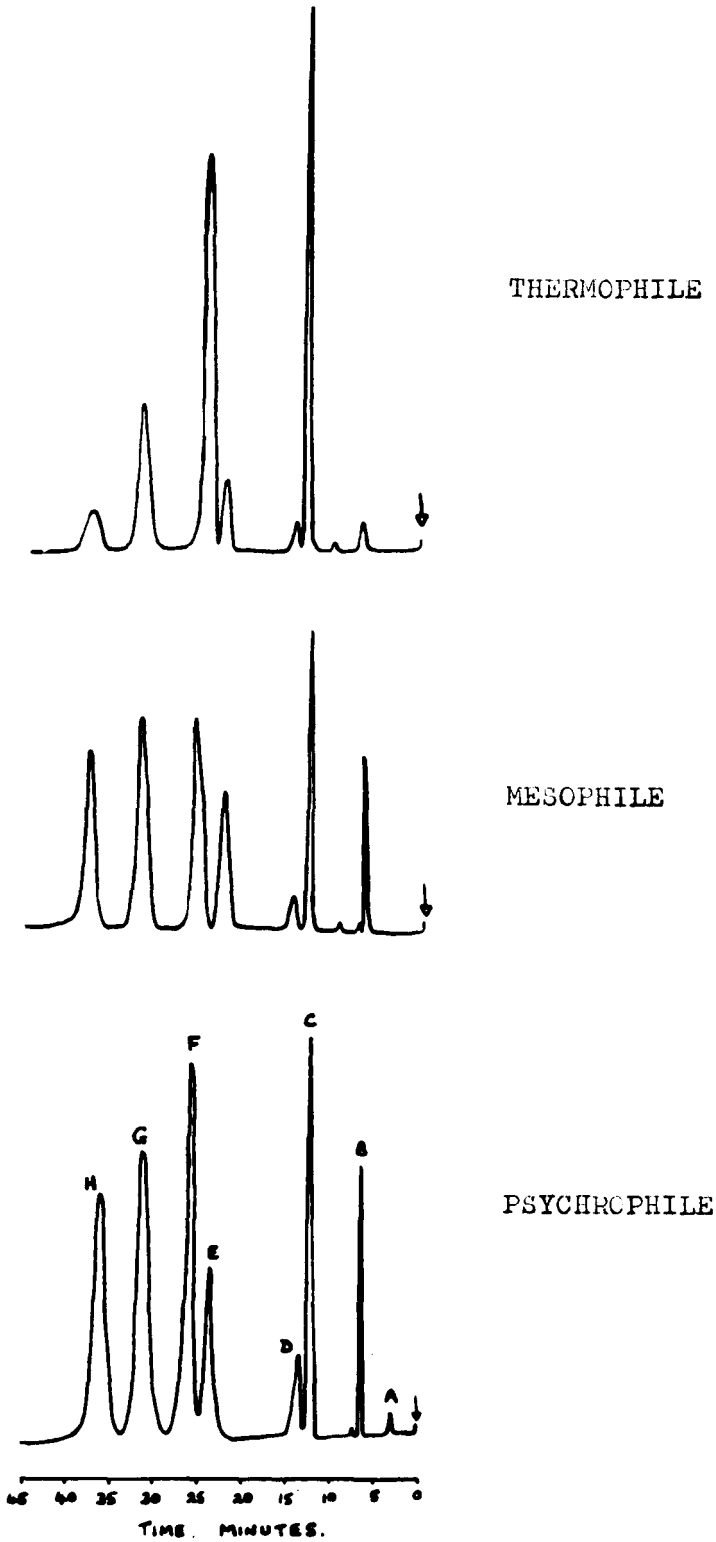


Fig.12. Gas chromatographs of methyl esters of fatty acids from psychrophilic, mesophilic and thermophilic fungi in the Mucorales. (A = laurate, B = myristate, C = palmitate, D = palmitoleate, E = stearate, F = oleate, G = linoleate, H = linolenate).

of unsaturation of the lipid (Kates and Baxter 1962) which is expressed as the number of double bonds/mole, and is calculated by adding the percentage of individual fatty acids multiplied by their number of double bonds and dividing by 100.

The lipids of psychrophilic and mesophilic fungi had similar degrees of unsaturation and were more unsaturated than the lipids of thermophilic fungi. The effect of incubation temperature on the lipids of thermotolerant fungi was especially marked. When thermotolerant fungi were grown at 28° their lipids had degrees of unsaturation similar to those of psychrophiles and mesophiles, but when grown at 48° the lipids of thermotolerants showed a degree of unsaturation similar to that of thermophiles.

Among the high-temperature fungi, the most thermophilic species were Rhizopus sp.III and M. miehei with maximum growth temperatures of 60° and 57° respectively. Their lipids were the most saturated encountered in this study, the low degrees of unsaturation being due to unusually small proportions of the polyunsaturated fatty acids (linoleic and linolenic acid).

The influence of environmental temperature on the degree of unsaturation of the lipids was identical in all the fungi examined. As a broad generalisation the mycelial lipids

were more highly unsaturated when the organism was grown at a lower, rather than a higher incubation temperature. There were exceptions however. In M. oblongisporus (CBS. 220.29) the lipids of cultures grown at 25° were more unsaturated than those grown at 10°. In addition, the lipids of young cultures were more highly unsaturated when grown at the upper temperature; as the fungus aged the lipids became more unsaturated in cultures grown at the lower incubation temperature. The same effect was shown in young cultures of the mesophilic phycomycete Pythium ultimum grown at 20° and 30° (Bowman and Mumma 1968) and the psychrophilic yeast Candida lipolytica grown at 10° and 25° (Kates and Baxter 1962).

These results indicate quite clearly that growth at different temperatures does result in changes in the fatty acid composition of the lipids. In mature cultures the lipids are more unsaturated when the organism is incubated at a lower temperature. However, the fact that in young mycelium the lipids are more unsaturated in cultures grown at the upper incubation temperature, suggests that other factors influence the degree of unsaturation of fungal lipids. Nutritional factors, such as the relative amounts of nitrogen and carbon source accumulated by the young rapidly growing cultures, or the

pH changes in the medium consequent upon the uptake of these nutrients, may affect the fatty acid composition.

In the following chapter the effects of nutritional factors, such as type and concentration of carbon and nitrogen source, and physical factors, such as pH, incubation temperature and oxygen concentration, on the fatty acid composition of fungal lipids are described.

6.2. The Fatty Acid Composition of the Lipids of Spores of Temperature-Adapted Filamentous Fungi.

There have been several investigations of the fatty acid composition of the lipids of fungal spores. In some studies unusual fatty acids have been found to form a significant proportion of the spore oil. In the order Uredinales (the rusts) cis -9,10 -epoxyoctadecanoic acid formed up to 40% of the spore oil (Tulloch, Craig andLedingham 1959, Tulloch and Ledingham 1962). In the order Erysiphales an analysis of the fatty acids of spores of two species of mildew showed that Sphaerotheca humili contained 42% behenic acid, which is rarely found in fungal lipids and then only in trace amounts, while in Erysiphe graminis the spore oil contained 45% of an unidentified fatty acid thought to have a branched chain, or cyclic system (Tulloch and Ledingham 1960). Sclerotia of Claviceps purpurea contained 34% ricinoleic acid

(12-hydroxystearic acid), though the presence of this fatty acid was not demonstrated in any other member of the order Hypocreales so far examined (Shaw 1965). It may be, therefore, that the presence of ricinoleic acid is specific to the genus Claviceps.

Linoleic acid has been found as a major component of the lipids of spores in widely different groups of fungi, amounting to 60% of the total fatty acids of sclerotia of Sclerotium rolfsii (Howell and Fergus 1964), and 65% of the spore fatty acids of Penicillium atrovenetum (Van Etten and Gottlieb 1965). In the fruiting bodies of some Basidiomycetes, linoleic acid accounted for over 70% of the total fatty acids (Hughes 1962, Bentley et al. 1964, Shaw 1966), whilst spore fatty acids of the smut, Tilletia foetens contained 63% linoleic acid (Tulloch and Ledingham 1960).

Of the groups so far mentioned the class Phycomycetes is conspicuously absent, and to remedy this position the fatty acid composition of spores of phycomycete fungi was examined. The experiments described in this section were performed with three objects in view. Firstly to determine whether the fatty acid composition of sporangiospores was essentially similar to that of related mycelium. The second aim was to see whether the spore lipids were influenced by the incubation

temperature in the same way as mycelial lipids. The third was to investigate whether spore lipids were affected by the composition of the culture medium.

6.21 The Effect of Incubation Temperature on the Lipids of Sporangiospores of Temperature-Adapted Fungi.

6.211 Experimental Procedure. The species used in this experiment were as follows:-

(i) mesophilic. M. mucedo. M. racemosus. M. ramannianus and M. hiemalis (+ strain).

(ii) thermotolerant. Rhizopus sp. I.

(iii) thermophilic. M. miehei. and M. pusillus.

The culture media as described in Section 6.11 containing agar (20 g./l.) were poured aseptically into flat medicine bottles and allowed to solidify into agar slopes. Mesophiles were incubated at 25°, thermotolerants and thermophiles at 25° and 48° for a period of 14 days.

By scraping lightly with a spatula, spores were removed from the surface of mature cultures and taken up into ice-cold distilled water. Spore suspensions from a number of replicate cultures (usually 20-25 medicine flat bottles) were pooled and the spores separated by centrifugation. The spore pellet was dried, powdered in a small grinder and the lipids

extracted in a Soxhlet apparatus. Spore lipids were hydrolysed, the fatty acids methylated and analysed by GLC.

In early experiments the lipids were extracted from spores harvested from only six replicate cultures. In these experiments, GLC analyses revealed, in addition to the usual fatty acids, the presence of a group of high molecular weight fatty acid esters which constituted a major proportion of the total. These esters, which were identified by combined gas chromatography-mass spectrometry as isomeric dinonylphthalates, were present as contaminants in the benzene used for lipid extraction in parts per hundred thousand, probably arising from contact with plastic material, in which dinonylphthalate is commonly used as a plasticiser. All solvents were therefore redistilled before use, and to prevent possible extraction of dinonylphthalate from the pipette teat, dropper pipettes were used in which a bulb had been blown in the stem to prevent contact between solvent and pipette teat. Using these precautions dinonylphthalate was no longer detected.

6.212 Results. Data for the lipid yield and fatty acid composition of the spore lipids of temperature-adapted mucoraceous fungi are presented in Table 34.

The lipid yield and fatty acid composition of the spores and mycelium of any fungus were found to be essentially similar. The lipid content of spores of mesophilic fungi was much lower than

TABLE 34 . The effect of incubation temperature on the fatty acid composition of spores of mesophilic, thermotolerant and thermophilic fungi.

Temperature Status.	MESOPHILIC				THERMOTOLERANT.		THERMOPHILIC.			
Species	M.mucedo.	M.ramanniamus.	M.racemosus	M.hiemalis	Rhizopus Sp.I.		M. miehei.		M. pusillus.	
Temperature	25°	25°	25°	25°	25°	48°	25°	48°	25°	48°
Lipid Yield (% of dry wt.)	3.7	7.6	4.1	8.4	16.1	10.4	29.4	11.3	19.3	16.1
Fatty Acid										
14:0	2.5	4.1	4.9	2.0	6.0	5.8	5.6	4.0	1.0	1.3
16:0	21.3	18.7	21.8	15.0	27.3	22.6	24.4	26.3	25.4	28.6
16:1	3.5	3.4	3.8	2.1	2.9	2.8	3.1	4.8	3.0	2.9
18:0	12.6	5.5	9.1	17.1	12.1	13.8	11.3	9.9	4.8	7.1
18:1	27.2	31.2	31.4	28.0	28.8	40.1	32.6	44.0	42.2	39.4
18:2	21.0	14.1	14.9	17.9	10.6	11.0	13.1	5.9	19.0	17.2
18:3	12.0	20.9	14.1	18.0	12.1	4.0	9.3	4.4	4.5	3.4
Degree of Unsaturation.	1.08	1.25	1.07	1.20	0.89	0.77	0.88	0.72	0.97	0.87

that of spores of thermotolerant and thermophilic fungi; an exact parallel of the situation in mycelia of temperature-adapted fungi.

In each species examined, the fatty acid composition of spore and mycelial lipids were extremely similar, though generally the lipids of spores were more saturated than the lipids of the parent mycelium. In these respects the spore lipids of the mucoraceous fungi examined here were similar to Pithomyces chartarum (Hartman et al. 1960, 1962) in which the spore lipids closely resembled the mycelial lipids; the spore lipids of P. chartarum also were more saturated than the mycelial lipids.

Spore lipids were influenced by incubation temperature in the same way as mycelial lipids being more unsaturated when grown at the lower incubation temperature. The patterns of fatty acids in spore lipids of mesophiles, thermotolerants and thermophiles were identical with those in mycelial lipids of these fungi; the lipids of mesophiles containing greater proportions of the polyunsaturated fatty acids (linoleic and linolenic acid) and lower proportions of oleic acid, compared with lipids of thermotolerants and thermophiles.

6.22 The Effect of the Composition of the Culture Medium on the Fatty Acid Composition of the Lipids of Sporangiospores of M. pusillus.

6.221 Experimental Procedure. The media used in this experiment were as follows:

(i) Glucose-Ammonia Agar, in which glucose (20 g./l.), ammonium sulphate (250 mg. N/l.), and agar (20 g./l.) were added to the basic medium.

(ii) Glucose-Ammonia-Yeast Extract Agar was similar to glucose-ammonia agar except for the addition of yeast extract (5 g./l.).

(iii) Glucose-Nitrate Agar in which glucose (20 g./l.), sodium nitrate (250 mg. N/l.), agar (20 g./l.) were added to the basic medium.

(iv) Potato-Dextrose Agar, "Oxoid" proprietory brand.

Sterile medium were poured into sterile medicine flat bottles and allowed to cool into agar slopes. Twenty-five medicine bottles of each medium were dispensed. The bottles were inoculated with spores of M. pusillus, incubated at 48° for fourteen days, and the spores removed by scraping lightly into ice-cold deionised water. The spores were separated by centrifugation, dried, powdered in a small grinder and the lipids

extracted in a Soxhlet apparatus. The lipids were hydrolysed, the fatty acids methylated and analysed by GLC.

6.222 Results. Data for the effect of different nutrient culture conditions on the lipid yield and fatty acid composition of the lipids of spores from M. pusillus are presented in Table 35. The composition of the culture medium had an effect on the lipid content which varied from 7.5-14.3% of the total dry weight of the spores. The fatty acid composition however appeared unaffected by differences in nutrient culture conditions.

These experiments indicate that the lipids of spores are affected by some of the environmental factors which affect the synthesis and composition of the mycelial lipids. A number of factors affecting the synthesis and degree of unsaturation of the mycelial lipidsof M. pusillus are investigated in Chapter Seven.

TABLE 35 . The effect of different nutrient culture conditions on the fatty acid composition of the spores of *M. pusillus*.

Culture Medium.	Glucose Ammonia Agar.	Glucose Nitrate Agar.	Glucose Ammonia Yeast Extract Agar.	Potato Dextrose Agar.
Lipid yield (% of dry wt.)	14.3	7.5	10.5	12.2
Fatty Acids				
14:0	1.3	1.5	1.2	1.5
16:0	29.0	32.8	26.9	30.7
16:1	2.6	3.1	3.4	3.0
18:0	7.9	4.1	8.6	4.7
18:1	38.8	37.3	39.7	39.4
18:2	17.3	18.0	17.2	17.3
18:3	3.0	3.3	3.0	3.1
Degree of Unsaturation.	0.85	0.86	0.86	0.82

CHAPTER 7. ENVIRONMENTAL FACTORS AFFECTING THE DEGREE OF UNSATURATION OF THE LIPIDS OF MUCOR PUSILLUS.

In the previous chapter it was shown that, in general, the lipids of fungi grown at a lower temperature were more unsaturated than those grown at a higher temperature. However there was an exception: in the early stages of growth, cultures synthesised more unsaturated lipids when grown at the higher, rather than the lower, temperature.

It was considered therefore, that factors other than temperature influence lipid synthesis, and the effect of a number of environment variables on the fatty acid composition of *Mucor pusillus* were studied.

7.1 The Fatty Acid Composition of the Neutral and Compound Lipid Fractions of *M. pusillus*.

Separation of the lipids of *Euglena* (Erwin and Bloch, 1964), blue-green algae (Levin *et al.* 1964), ciliate protozoa (Erwin and Bloch, 1963) and basidiomycetes (Leegwater *et al.* 1962, Talbot and Vining 1963, and Bentley *et al.* 1964) into neutral and compound lipids revealed differences in the specific fatty acid composition of the two fractions. In all these organisms the polyunsaturated fatty acids were found mainly in the compound lipids,

while the neutral lipids were rich in saturated and monoenoic fatty acids.

In the literature there are conflicting reports about the distribution of specific fatty acids between the lipid fractions of phycomycete fungi. Shaw (1966) separated the mycelial lipids of Cunninghamella blakesleeana and found the fatty acid compositions of the neutral and the compound fractions to be almost identical. However, in Pythium ultimum separation of the lipids revealed marked qualitative and quantitative differences in the fatty acid compositions of the phosopholipid, monoglyceride, free fatty acid and triglyceride fractions (Bowman and Mumma 1968).

In view of this apparent contradiction, coupled with the fact that the distribution of fatty acids between various lipid fractions may have phylogenetic significance (Shaw 1966a) it was decided to study the fatty acid composition of the lipids of Mucor pusillus after separation into neutral and compound fractions on Davison's silica.

7.11 Experimental Procedure. To the basic medium was added glucose (20 g./l.), ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.). Medium was dispensed in 200 ml. quantities into 700 ml. Erlenmeyer flasks and autoclaved at 15 lbs./in.^{2} for 15 mins. Each flask was inoculated with 5 ml.

standard spore suspension of M. pusillus and incubated at 48°. Cultures were harvested after 3, 6, 10 and 14 days incubation, the mycelial mats removed and dried prior to extraction of the lipid.

After Soxhlet extraction for 8 hr. in benzene: ethanol (594:257) most of the solvent was removed from the lipid by evaporation in vacuo; the remainder being taken to dryness under a stream of nitrogen. The crude lipid was quickly weighed, then taken up in benzene and fractionated into neutral and compound lipids on a column of Davison's silica prepared in benzene. Neutral lipids were eluted with benzene, and compound lipids with ethanol. The composition of each lipid fraction was checked by thin-layer chromatography. The solvent was removed from each fraction under nitrogen and the lipids quickly weighed before hydrolysis with o.6 M methanolic KOH. The resulting fatty acids were methylated and analysed by GLC.

7.12 Results. Data for the growth and lipid synthesis of M. pusillus and its fatty acid composition of the total, neutral and compound lipids are presented in Table 36.

The highest lipid content of the mycelium was found after three days incubation and then fell steadily in the later stages of culture. The fall in lipid content was due

TABLE 36 . The fatty acid composition of the total, neutral and compound lipids at different times in the growth cycle of M. pusillus.

Incubation Period(days).	3			6			10			14		
Growth (mg.dry wt./ml.)	2.85			3.19			3.71			3.75		
Lipid yield (% of dry wt.)	24.0			16.0			13.0			3.7		
Lipid fraction.	Total Lipids	Neutral Lipids	Compound Lipids	Total Lipids	Neutral Lipids	Compound Lipids	Total Lipids	Neutral Lipids	Compound Lipids	Total Lipids	Neutral Lipids	Compound Lipids
Weight of fraction (mg)	298	183	94	271	69	188	293	56	220	280	79	193
Fatty acid												
14:0	1.2	1.0	1.1	1.5	1.6	1.3	1.1	1.4	1.5	1.6	1.1	1.9
16:0	29.9	27.6	28.2	31.3	29.7	29.8	31.3	29.0	29.0	28.6	28.5	30.0
16:1	2.8	1.7	1.4	2.4	1.6	1.2	3.1	1.9	2.1	3.6	1.3	1.9
18:0	4.8	5.5	5.3	3.5	2.2	3.5	4.0	5.0	3.4	3.9	5.3	4.9
18:1	36.6	41.8	42.2	40.0	43.6	39.6	38.3	40.2	40.5	39.7	44.5	39.6
18:2	20.2	18.8	18.5	17.7	19.0	21.8	17.4	20.0	20.5	18.3	19.0	18.5
18:3	4.5	3.5	3.2	3.7	2.4	2.9	4.9	2.6	3.3	4.2	1.3	3.2
Degree of Unsaturation	0.93	0.91	0.90	0.89	0.90	0.93	0.91	0.90	0.93	0.92	0.88	0.88

to a reduction in the neutral fraction, which was composed mainly of triglyceride, together with a small proportion of sterol ester. The Compound fraction was composed mainly of phospholipid, together with free fatty acids and sterols.

There was a marked similarity between the fatty acid composition of the total (unfractionated), the neutral and the compound lipids, at all stages during the growth cycle of M. pusillus. This result would seem to confirm the findings of Shaw (1966) with C. blakesleeana that there is no differential distribution of fatty acids in the lipid fractions of phycomycetes. However in view of fatty acid distribution in the lipids of P. ultimum (Bowman and Mumma 1968) it is clear that not all phycomycete fungi show this proportionality. It may be that in this respect oomycete fungi (e.g. P. ultimum) differ from zygomycete fungi (e.g. C. blakesleeana and M. pusillus).

7.2 The Effect of Different Carbon Sources.

In common with other members of the Mucorales M. pusillus cannot use cellulose or hemicellulose as a carbon source (Yung Chang 1967). This fungus was also unable to grow with inulin, ethanol, ethyl acetate, mesoerythritol, lactic

acid, sorbose or citric acid as carbon source (Scholer and Muller 1966). However, starch, glycogen, disaccharides and monosaccharides all formed a suitable source of energy (Scholer and Muller 1966, Yung Chang 1967), as did glycerol and succinic acid (Scholer and Muller 1966) and paraffin (Fergus 1966).

Although the synthesis of lipid by mucoraceous fungi has been studied (Galloway 1949, Woodbine, Gregory and Walker 1951, Chesters and Peberdy 1965 and Shaw 1966) no investigation has been performed on the effect of different carbon sources on the fatty acid composition of the lipids of these fungi. In a study of the effect of different culture conditions on the fatty acid composition of the lipids of the Ascomycetes, Penicillium sophi and Aspergillus niger (Salmonowicz and Niewiadomski 1965) it was found that altering the source of carbon had an extremely strong influence on the degree of unsaturation of the lipids.

In view of the absence of comparable data for any group other than the Ascomycetes, the effect of carbohydrate and non-carbohydrate carbon sources on the fatty acid composition of the lipids was investigated in M. pusillus.

7.21 Experimental Procedure. The following carbon sources were incorporated into the basic medium plus ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.):-

(i) glucose (20 g./l.)

(ii) maltose (20 g./l.)

(iii) starch (20 g./l.)

(iv) paraffin wax (9g./l.)

(v) sodium stearate (10 g./l.)

(vi) sodium succinate (27 g./l.)

(vii) sodium acetate (27 g./l.)

The carbon concentration of all media was the same - equivalent to glucose (20 g./l.).

Media were dispensed in 200 ml. portions into 700 ml. Erlenmeyer flasks, medium containing paraffin wax being dispensed after warming to melt the paraffin (m.pt.45° - 50°). In order to dissolve the sodium stearate in the culture medium a few drops of "SPAN 20"(Sorbitan monolaurate. BDH, Reagent) were added.

After autoclaving at 15 lb/in^2 for 15 mins. media were inoculated with 5 ml. standard spore suspension of M. pusillus and incubated at 50°. Cultures were harvested in triplicate after 3,6, 9 and 12 days incubation, when the mycelium was removed and dried prior to the extraction of the lipid. After extraction and hydrolysis of the lipid the fatty acids were methylated and analysed by GLC.

7.22 Results. Data for the growth, lipid yield and fatty acid composition of the lipids of M. pusillus grown on different carbon sources are presented in Table 37.

The fungus grew well on carbohydrate carbon sources, badly on paraffin wax or succinate, and not at all on stearate or acetate. Because only slight growth occurred on paraffin wax and succinate only one harvest was possible for these sources.

On the carbohydrate sources, both growth and lipid synthesis increased throughout the incubation period. The lipid content of mycelium grown on a succinate medium was rather less than that of mycelia grown on media containing carbohydrate carbon sources. It was not possible to measure accurately the growth or lipid content of mycelium grown on paraffin wax medium because harvested mycelium contained globules of wax adhering to the hyphae.

The fatty acid composition and degree of unsaturation of the lipids were remarkably constant at all times of harvest and on all the media tested except for young (3-day) cultures, in which the degree of unsaturation was higher than at other stages of growth.

These results are apparently in direct contrast with the situation in the ascomycete fungi. Penicillium sophi and

TABLE 37 . The effect of different carbon sources on the growth, lipid yield and fatty acid composition of M. pusillus.

Carbon Source	Glucose						Maltose		Starch				Paraffin wax	Sodium Succinate
Incubation period (days)	3	6	9	12	3	6	9	12	3	6	9	12	12	12
Growth (mg.dry wt./mL)	1.98	3.06	3.51	3.77	2.06	3.55	3.96	4.08	1.92	3.13	3.39	3.84	0.39	0.10
Lipid yield (% of dry wt.)	17.7	15.0	24.1	34.5	14.0	18.2	11.2	32.4	16.9	13.0	14.8	30.0	-	12.5
Fatty Acid														
14:0	1.4	1.3	1.6	1.6	1.3	1.9	1.4	1.6	1.5	1.4	1.6	1.8	1.5	1.4
16:0	29.2	31.3	31.2	31.6	27.0	29.7	31.9	29.8	29.4	29.7	31.7	28.9	29.8	29.7
16:1	2.0	2.9	2.2	4.2	2.1	1.9	2.8	3.7	1.4	2.2	2.4	5.0	3.6	3.9
18:0	4.1	5.6	5.0	5.4	3.5	6.5	5.4	5.0	4.5	6.9	5.3	8.3	6.0	4.9
18:1	36.8	35.1	35.7	34.0	35.7	36.0	35.3	38.7	37.3	36.2	35.6	34.3	34.4	35.6
18:2	22.7	20.2	20.9	19.4	25.4	20.5	20.0	17.6	21.7	20.3	20.3	18.1	21.1	18.8
18:3	3.8	3.6	3.4	3.7	4.9	3.5	3.1	3.6	4.0	3.3	3.2	3.6	3.2	3.9
Degree of Unsaturation	0.95	0.89	0.90	0.88	1.03	0.89	0.87	0.88	0.94	0.88	0.88	0.86	0.90	0.89

Aspergillus niger, in which the effect of different carbon sources on the fatty acid composition and degree of unsaturation of the lipids was extremely strong (Salmonowicz and Niewiadomski 1965). For example, when Pen. sophi was grown on media containing similar carbon sources such as glucose, maltose or starch, the degrees of lipid unsaturation were, respectively, 1.40, 0.88 and 1.26; on a sucrose medium the lipids were extremely saturated (degree of unsaturation 0.38). Similar results were obtained for Asp.niger. It should be mentioned however that in the investigation by Salmonowicz and Niewiadomski the fatty acids were analysed on only one occasion during the growth cycle of these fungi. In addition, the length of the incubation period was not stated, neither was it clear whether all treatments were incubated for the same length of time.

In an experiment to investigate the effect of harvesting cultures of different age, it was found that in Pen. sophi the fatty acid composition of the lipid was affected by the age of the culture, the lipids becoming more saturated as the fungus aged (Salmonowicz and Niewiadomski, 1965). After 5 days growth on a glucose medium the degree of lipid unsaturation was 1.50 and this value fell progressively to 1.26 after 10 days, and 0.54 after 16 days incubation.

It seems probable therefore that the changes in fatty acid composition of the lipids of Pen. sophi and Asp. niger, resulting from altering the source of carbon in the medium, are attributable to differences in the rate at which the cultures mature on each particular energy source. Time-course experiments with analysis of the fatty acid composition at several stages of growth of Pen. sophi and Asp. niger would provide information on this point.

7.3 The Effect of Different Nitrogen Sources.

7.31 Experimental Procedure. The following nitrogen sources were used:-

(i) ammonium sulphate.
(ii) ammonium sulphate + sodium succinate (5 g./l.).
(iii) ammonium nitrate.
(iv) sodium nitrate.
(v) sodium nitrite.
(vi) casein hydrolysate.
(vii) leucine.

The nitrogen sources were autoclaved separately and then added at a level of 250 mg. N/l. to sterile basic medium plus glucose (20 g./l.) The complete media were dispensed aseptically in 200 ml. portions into sterile 700 ml. sterile Erlenmeyer flasks and inoculated with 5 ml. standard spore suspension of M. pusillus. The flasks were incubated at 48° and harvests of three replicate flasks were made

4, 7 and 14 days after inoculation. Mycelial mats were dried and weighed prior to extraction of the lipid. After hydrolysis of the lipid, the fatty acids were methylated and analysed by GLC.

7.32 Results. Yields of mycelium and lipid after 4, 7 and 14 days incubation at 48° are shown in Table 38, and Fig.13.

When casein hydrolysate, buffered ammonium sulphate, ammonium nitrate and sodium nitrate were nitrogen sources the fungus produced large quantities of mycelium of high lipid content. Growth was also good on leucine, though the mycelium accumulated a relatively small proportion of lipid. In contrast, sodium nitrite and unbuffered ammonium sulphate supported poor growth, though the mycelium had a high lipid content.

GLC analysis of the fatty acids of M. pusillus indicated that the source of nitrogen in the culture medium, and the time of harvest of the mycelium significantly affect the fatty acid composition of the lipids. When ammonium sulphate was a nitrogen source in unbuffered conditions the pH of the medium became very acid and no increase in dry weight occurred after the fourth day of incubation. On this source the lipids became more saturated in the later stages of culture. However on all other nitrogen-sources tested the lipids became more unsaturated as the fungus aged (Fig.14) and older cultures generally contained higher proportions of linoleic and linolenic acids. On these sources the pH drift was less acid than on unbuffered ammonium sulphate, and growth continued until the fourteenth day of culture.

TABLE 38 . The effect of different nitrogen sources on the growth, lipid yield and fatty acid composition of M.pusillus.

Nitrogen source.	Ammonium sulphate.			Ammonium sulphate and sodium succinate.			Ammonium nitrate.			Sodium nitrate.			Sodium nitrite.			Hydrolysed casein.			Leucine.		
Incubation period (days)	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14
Growth (mg. dry wt./ml.)	2.20	2.45	2.42	2.65	3.35	5.42	2.78	3.90	5.15	1.41	2.20	4.85	1.56	2.08	2.25	3.00	4.42	5.87	1.00	1.65	3.70
Lipid yield (% of dry weight).	13.8	20.1	17.8	7.8	23.1	13.8	11.6	21.8	28.5	8.5	21.3	18.0	8.3	24.1	17.3	16.7	19.9	13.2	8.6	11.8	6.5
Fatty acids																					
14:0	0.9	1.1	1.5	1.0	1.0	1.2	1.9	0.8	1.0	1.1	1.0	1.3	1.4	1.5	1.0	0.9	1.0	1.5	1.2	1.1	1.3
16:0	20.3	23.6	21.3	23.5	19.6	21.5	25.2	17.7	25.1	25.8	27.5	21.0	39.1	24.0	23.0	29.0	20.4	23.6	20.3	27.5	21.0
16:1	4.0	4.2	4.5	3.5	4.0	3.7	3.0	3.2	3.3	2.2	4.0	3.6	4.0	3.5	4.1	3.6	3.0	3.4	3.2	3.2	3.1
18:0	5.7	7.7	8.5	6.7	1.9	3.2	9.3	1.0	4.0	8.3	2.6	6.7	8.0	5.9	2.3	4.6	2.8	4.1	5.0	2.8	2.2
18:1	47.8	47.1	51.3	41.5	42.1	43.2	40.5	47.7	43.5	40.5	43.2	46.7	33.0	41.5	44.0	39.2	45.2	41.7	48.2	37.8	48.0
18:2	18.1	14.0	8.7	19.7	29.3	20.1	18.4	23.6	17.3	17.8	19.2	16.6	12.1	20.6	18.4	19.7	24.4	18.9	18.2	23.7	18.4
18:3	3.0	2.0	4.0	4.1	2.0	7.4	2.6	6.0	6.0	4.1	3.0	4.6	2.4	3.0	7.2	3.0	3.1	6.7	3.9	4.4	6.5
Degree of Unsaturation	0.97	0.85	0.85	0.97	1.11	1.09	0.88	1.16	0.99	0.90	0.94	0.97	0.68	0.95	1.06	0.90	1.06	1.03	0.99	1.01	0.97

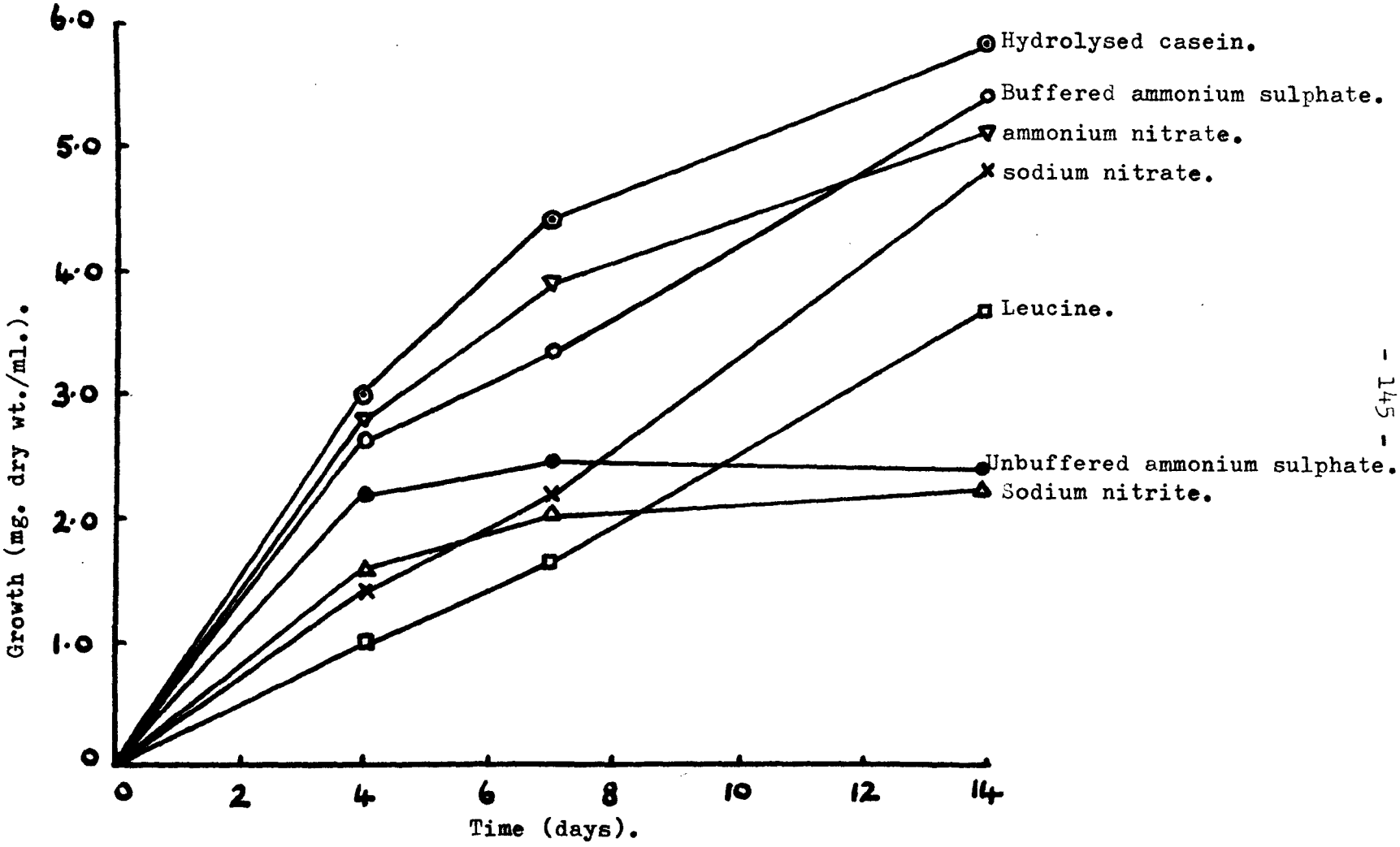


Fig. 13. Effect of different nitrogen sources on the growth of M. pusillus.

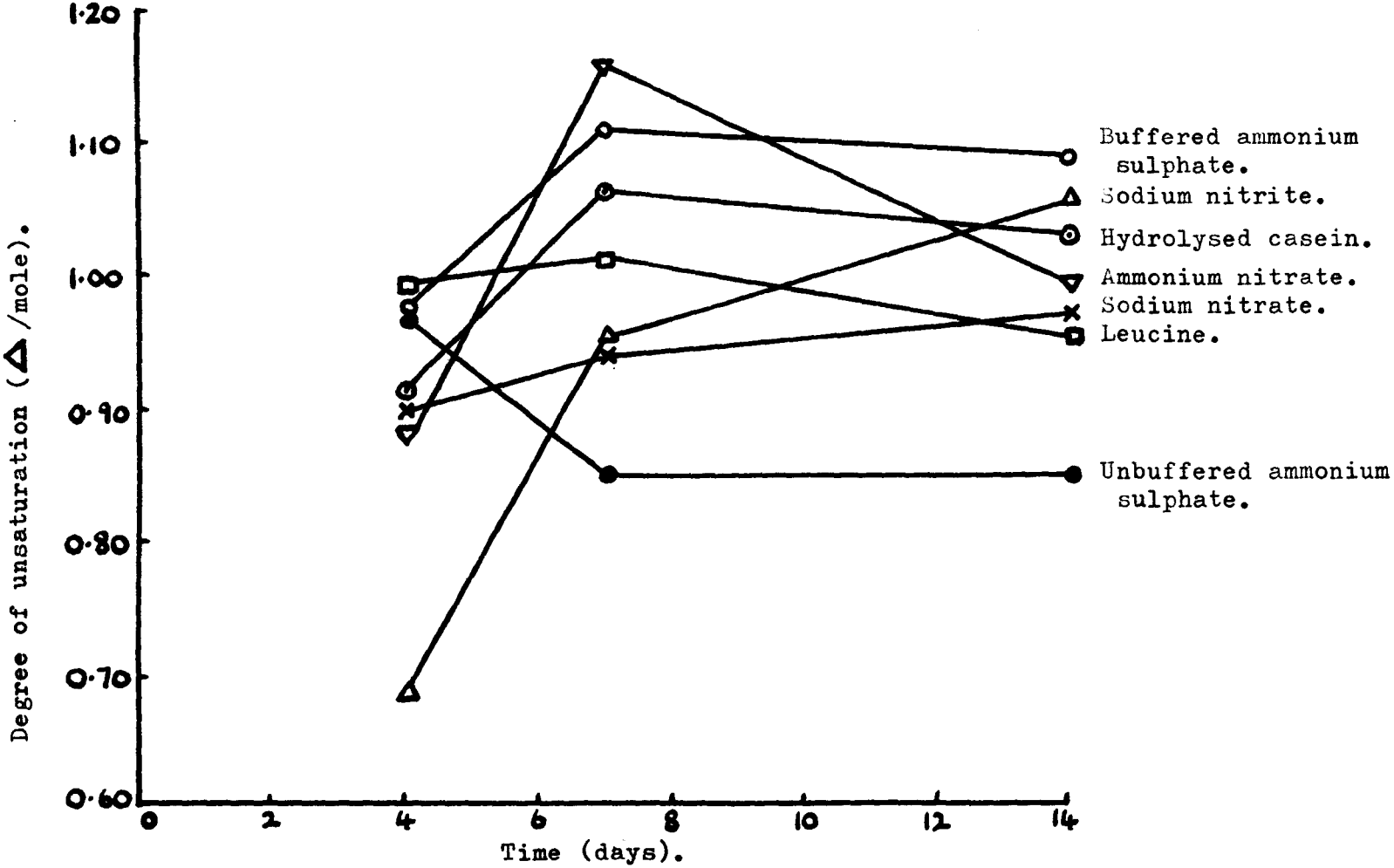


Fig. 14. Effect of different nitrogen sources on the degree of unsaturation of the lipids of M. pusillus.

7.4. Effect of the Initial pH of the Culture Medium.

The effect of pH on the fatty acid composition of the lipids was investigated by incubating pre-grown mycelial mats in media of different hydrogen ion concentration.

7.41 Experimental Procedure. To the basic medium was added glucose (20 g./l.) casein hydrolysate (250 mg. N/l.) and sodium succinate (5 g./l.). The medium was then divided into 5 portions and the pH of each portion adjusted with 1 N NaOH or 1 N HCl to give media of the following pH values: 2.0, 4.0, 6.0, 8.0, 10.0. The culture medium, dispensed in 200 ml. aliquots into 700 ml. Erlenmeyer flasks, was autoclaved and inoculated with mycelial mats from three small flasks of inoculum. The inoculum was grown on a medium of glucose (10 g./l.), casein hydrolysate (125 mg. N/l.) and sodium succinate (2.5 g./l.) dispensed in 20 ml. portions into 100 ml. Erlenmeyer flasks.

Cultures were incubated at 48° and three replicate flasks harvested after 1, 4, 7 and 14 days incubation. The

mycelium was filtered from the medium, dried and weighed, and the pH of the culture filtrate was recorded. The fungal lipid was extracted and hydrolysed, and the fatty acids methylated and analysed by GLC.

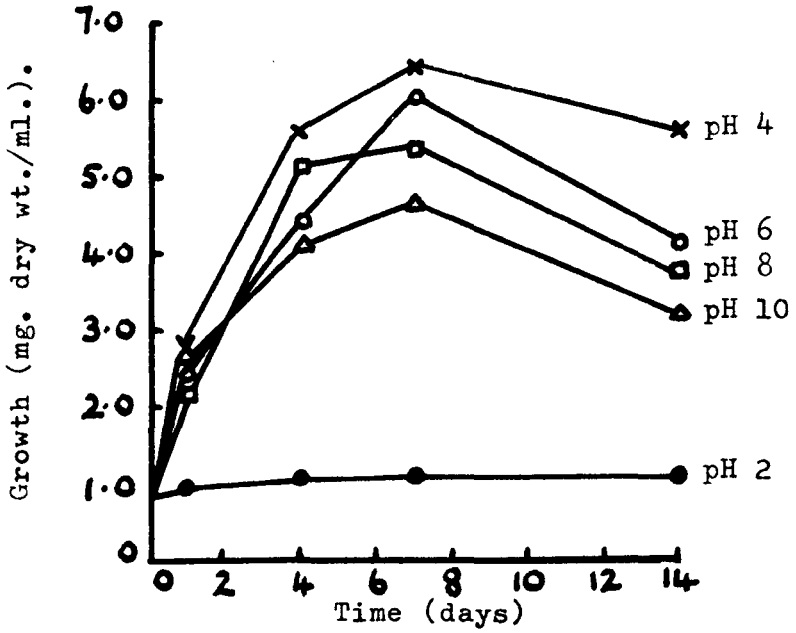
7.42 Results. Of the hydrogen-ion concentrations tested, the optimum for growth was pH 4.0 (Table 39, Fig.15). At this value the pH of the medium remained only slightly acid throughout incubation and little mycelial autolysis occurred. No growth occurred at pH 2. Where the initial pH of the medium was adjusted to 6.0, 8.0 or 10.0, the pH drift early in incubation was acid, though in the later stages of culture it became alkaline. Considerable mycelial autolysis occurred under these conditions.

The fatty acid composition of the mycelium varied through the incubation period of the experiment. Except for cultures grown at pH2 it is unlikely that the pH of the culture medium significantly affected the degree of unsaturation of the mycelial lipids (Fig. 16).

TABLE 39 .

The effect of the hydrogen ion concentration of the medium on growth and fatty acid composition of the lipids of M. pusillus.

Initial pH.		pH 2.				pH 4.				pH 6.				pH 8.				pH 10.			
Incubation Period(days)	Zero (Inoc-ulum)	1	4	7	14	1	4	7	14	1	4	7	14	1	4	7	14	1	4	7	14
pH Drift in Medium	6.5	1.8	1.9	2.0	2.0	3.7	4.2	5.0	5.3	5.5	5.7	6.5	8.1	6.0	6.1	6.7	8.2	6.0	6.3	6.9	7.9
Growth (mg. dry wt. /ml.	0.95	0.94	1.01	1.11	1.09	2.72	5.60	6.37	5.63	2.64	4.36	6.04	4.06	2.52	5.08	5.39	3.79	2.25	4.26	4.62	3.27
Fatty Acids																					
14:0	1.0	1.1	1.0	1.0	1.4	1.0	1.1	1.7	1.2	1.2	1.1	0.8	1.0	1.2	1.0	1.8	1.0	1.0	1.1	1.0	1.3
16:0	26.6	23.0	29.4	29.8	29.8	21.4	28.2	23.8	26.1	21.5	25.0	24.7	23.5	22.8	21.6	23.0	25.8	25.7	20.3	22.2	25.9
16:1	3.2	3.3	3.8	3.2	2.9	3.2	3.4	2.9	3.0	3.5	3.3	2.8	3.3	3.1	3.5	3.1	3.3	3.3	3.1	3.5	3.6
18:0	6.1	6.6	6.1	6.1	7.0	6.1	5.1	3.9	5.0	6.1	3.0	6.5	5.7	6.8	5.8	2.9	4.1	4.4	4.7	4.8	4.2
18:1	35.2	36.5	37.3	42.6	39.3	43.6	40.5	43.2	42.8	39.4	44.9	45.0	45.3	38.0	43.1	41.2	44.1	38.2	42.7	47.3	42.2
18:2	24.3	28.3	19.5	14.7	17.4	20.8	18.3	20.8	19.4	23.8	19.0	18.0	19.0	25.0	22.0	23.3	19.0	24.1	24.8	17.8	17.7
18:3	3.4	2.8	2.7	2.8	1.9	3.5	3.1	3.5	2.6	4.0	3.5	2.2	2.2	3.3	3.0	3.4	2.8	3.3	3.2	2.4	5.0
Degree of Unsaturation	0.97	1.02	0.88	0.83	0.83	0.99	0.90	0.98	0.92	1.02	0.96	0.90	0.93	1.01	1.00	1.01	0.94	0.99	1.05	0.94	0.96



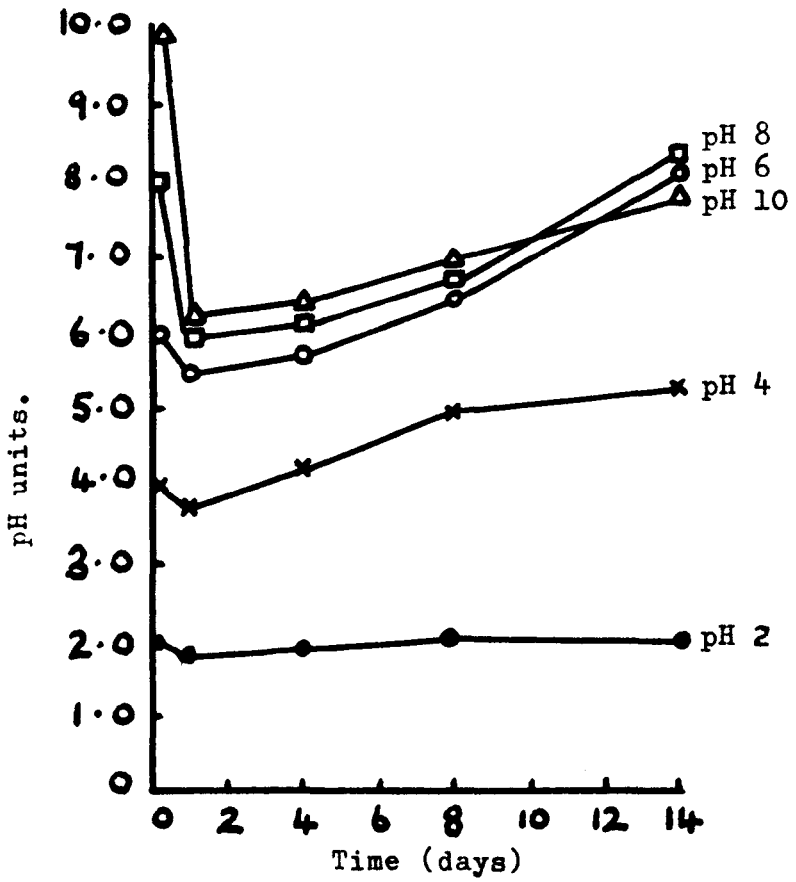


Fig. 15. Effect of initial pH on growth of M. pusillus and the pH drift of a glucose/casein hydrolysate/succinate medium.

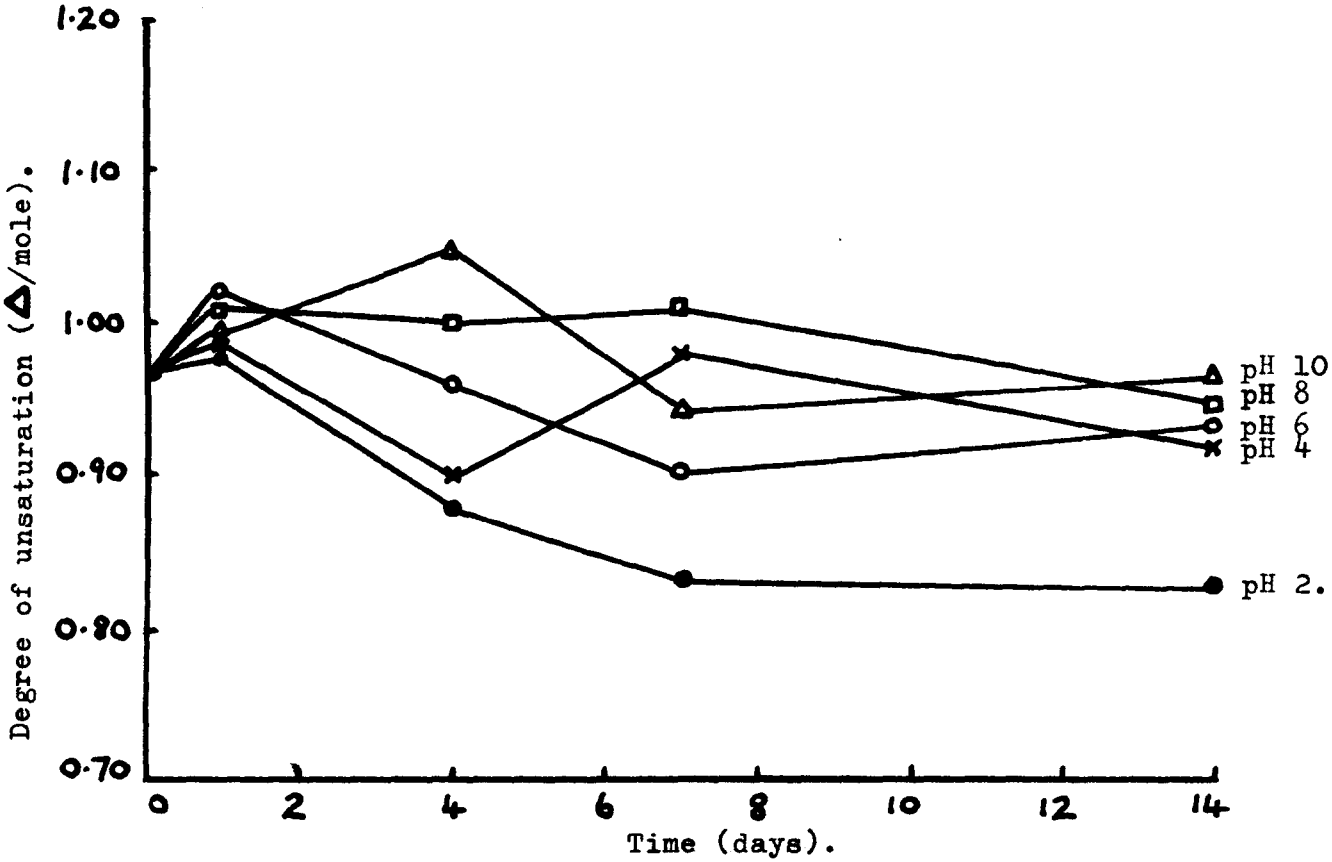


Fig. 16. Effect of initial pH of the culture medium on the degree of unsaturation of the lipids of M. pusillus.

7.5 The Effect of the Carbon : Nitrogen Ratio in the Culture Medium.

7.51 Experimental Procedure. Media containing glucose and ammonium sulphate were prepared in which carbon and nitrogen in ratios of 1:1, 34:1 and 1280:1 were prepared as follows:

Medium (1) glucose (20 g./l.) + ammonium sulphate (76 g./l.) ≡ 1:1 ("high nitrogen" medium).

Medium (2) glucose (20 g./l.) + ammonium sulphate (2.36 g./l.) ≡ 34:1("balanced" medium).

Medium (3) glucose (400 g./l.) + ammonium sulphate (1.18 g./l.) ≡ 1280:1 ("high carbon" medium).

The pH of the culture medium was buffered by the inclusion of sodium succinate (5 g./l.). The carbon content of sodium succinate was not taken into account in the calculation of carbon : nitrogen ratios.

Media were dispensed in 200 ml. aliquots into 700 ml. Erlenmeyer flasks, autoclaved and inoculated with 5 ml. of a standard spore suspension of M. pusillus. Cultures were incubated at 25° and 50°; 3 replicate flasks were harvested after 4, 7 and 14 days incubation. Harvested mycelium mats were filtered from the culture medium, dried and weighed. The mycelial lipid was extracted and hydrolysed and the fatty acids methylated and analysed by GLC.

7.52 Results. The influence of the carbon:nitrogen (C:N) ratio of the culture medium on the growth and lipid synthesis of M. pusillus is shown in Table 40 and Fig. 17. The lipid content of the mycelium varied both with the C:N ratio of the medium and the age of the mycelium. On a "high nitrogen" medium the mycelial lipid content fell considerably during growth due probably to the utilisation of "storage lipids" as an energy source consequent on the depletion of the exogenous energy source.

On a "high-carbon" medium the lipid content of mycelium grown at 50° was extremely high after four days incubation, but fell rapidly in the later stages of growth. On this medium, lipid accumulation ceased after exhaustion of the nitrogen source, and though growth increased after four days it was probably at the expense of "storage lipid" as an energy source.

The lipids were invariably more unsaturated when cultures were grown at the lower temperature. However, irrespective of temperature, the degree of unsaturation of the lipids was also affected by the C:N ratio of the medium; lipids were more unsaturated when the fungus was grown on a "high nitrogen" medium (Fig. 18).

TABLE 40 . The effect of different carbon : nitrogen ratios on the growth, lipid yield and fatty acid composition of *M. pusillus* at 25° and 50°.

Carbon : nitrogen ratio.	1 : 1						34 : 1						1280 : 1					
Incubation temperature	25°			50°			25°			50			25°			50°		
Incubation period	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14	4	7	14
Growth (mg.dry wt./ml.)	1.93	1.14	2.16	1.41	3.94	4.84	1.91	3.70	6.04	4.70	7.76	6.62	1.90	2.40	4.98	2.58	3.67	5.63
Lipid yield (% of dry wt.)	22.5	12.8	6.2	18.2	9.8	6.9	24.0	17.5	19.3	23.5	26.6	21.8	7.2	15.8	17.4	43.6	13.4	8.3
Fatty acid																		
14.0	1.0	1.0	1.2	0.9	1.2	1.0	0.6	1.0	1.1	1.0	1.2	1.8	1.0	1.8	1.8	1.1	1.8	1.5
16.0	24.4	22.7	22.2	21.0	24.8	24.2	27.4	23.9	28.4	22.7	28.2	34.2	25.3	26.1	25.6	23.9	27.6	31.1
16.1	2.9	3.9	3.8	3.4	4.0	3.0	3.1	3.9	3.2	3.0	4.0	3.1	3.3	3.5	3.2	3.1	3.5	3.3
18.0	6.6	5.4	6.2	5.1	5.4	5.5	8.5	3.6	5.1	4.4	4.3	4.5	5.8	8.5	6.4	4.3	6.4	5.2
18.1	42.5	42.5	41.6	45.0	43.6	44.9	38.5	45.3	43.0	49.0	41.7	39.0	41.5	32.5	39.8	44.6	39.3	43.9
18.2	17.2	18.1	17.9	18.5	15.1	16.2	18.0	16.6	14.5	15.0	16.1	16.0	16.4	21.0	19.3	19.4	18.4	13.6
18.3	5.5	6.7	6.6	5.8	5.8	4.1	3.6	5.9	4.7	3.7	4.3	1.8	6.4	6.5	3.7	3.9	2.8	1.5
Degree of Unsaturation	0.96	1.03	1.01	1.02	0.95	0.92	0.88	1.00	0.91	0.93	0.91	0.79	0.97	0.97	0.91	0.99	0.88	0.79

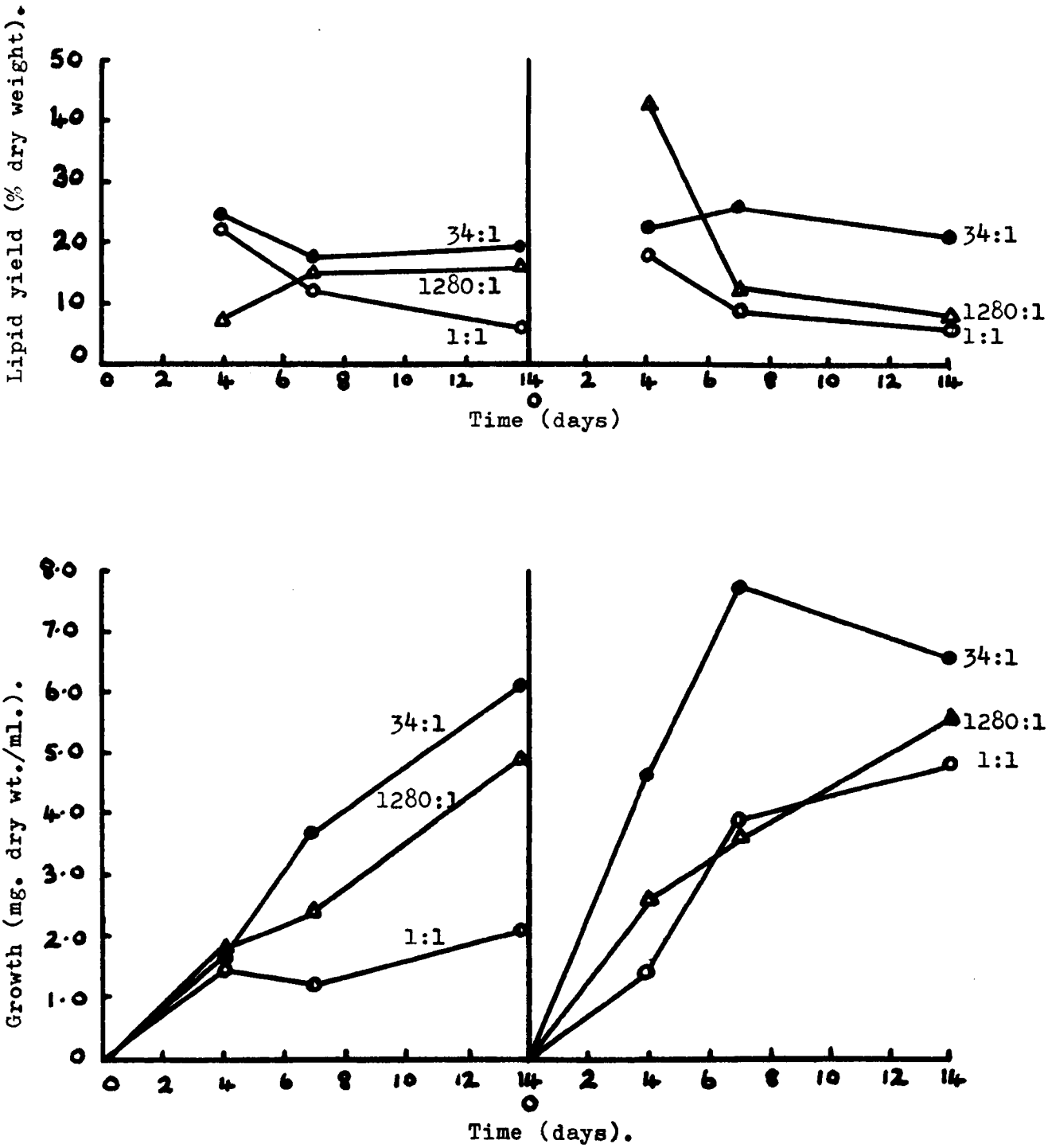


Fig. 17. Effect of different C:N ratio in the culture medium on growth and lipid accumulation of M. pusillus at 25° or 50°.

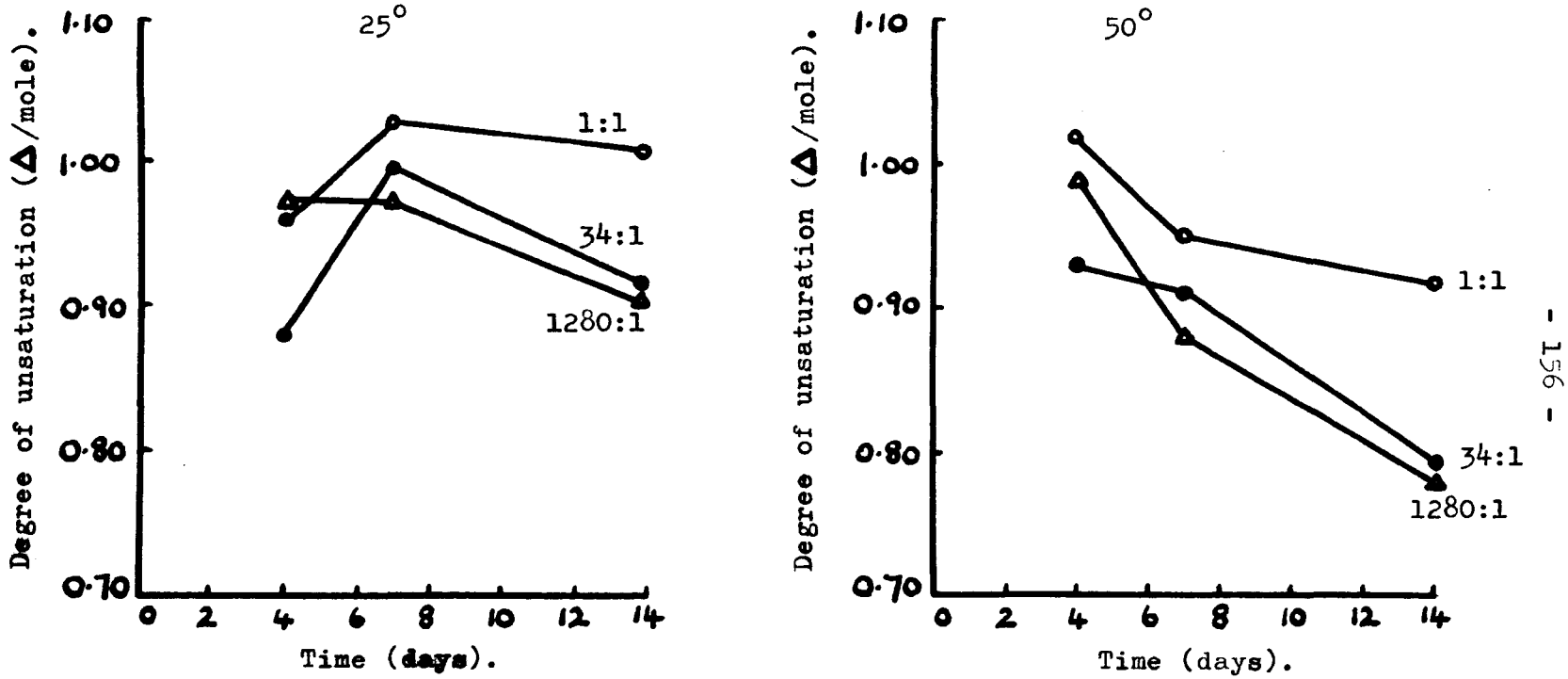


Fig. 18. Effect of different C:N ratios on the degree of unsaturation of the lipids of M. pusillus grown at 25° or 50°.

7.6 The Effect of Different Concentrations of Carbon and Nitrogen at a Constant C:N Ratio.

In the previous experiment M. pusillus was grown on "high nitrogen", "balanced" and "high carbon" media. On the "high carbon" medium, cultures were subjected to conditions of nitrogen limitation, and on the "high nitrogen" medium to carbon limitation. It was noted that the greatest amounts of growth and lipid synthesis occurred on a "balanced" medium C:N ratio 34:1.

The effect of different concentrations of carbon and nitrogen at a fixed C:N ratio on the growth, lipid synthesis and fatty acid composition of M. pusillus was therefore investigated at a C:N ratio of 34:1.

7.61 Experimental Procedure. The following amounts of glucose and ammonium sulphate were added to the basic medium plus sodium succinate (5g./l.).

Medium	1	glucose	(2g./l.),	ammonium sulphate	(0.118g./l.)
"	2	"	(5g./l.),	"	(0.295g./l.)
"	3	"	(20g./l.),	"	(1.18 g./l.)
"	4	"	(100g./l.),	"	(5.90 g./l.)
"	5	"	(200g./l.),	"	(11.8 g./l.)

In all media the ratio of carbon to nitrogen was 34:1. Culture medium was dispensed in 30 ml. amounts into 150 ml. Erlenmeyer flasks and sterilised by autoclaving at 15lbs./in.^{2} for 15 mins. Each flask was inoculated with 1 ml. of a standard spore suspension of M. pusillus and incubated at 48°. Replicate cultures (at least six) were harvested after 2, 5, 8 and 12 days incubation and the partial pressure of oxygen (pO_2) determined as described in Section 1.72.

The mycelial mats were removed from the flasks and dried prior to extraction of lipid. After extraction and hydrolysis of the lipid the fatty acids were methylated and analysed by GLC.

7.62. Results. Data for the growth, lipid yield and fatty acid composition of M. pusillus grown on media of different nutrient concentration are presented in Table 41.

As the nutrient concentration increases it is clear that growth is increased (Fig.19). This is not exactly paralleled by the mycelial lipid content, although there are indications that higher concentrations are more productive of lipid than lower (Fig. 19). On medium 1 the fungus did not form a mycelial mat but grew as discrete tangles of submerged hyphae. On medium 2 a mycelial mat was formed which did not cover the whole surface of the medium, while on media 3, 4 and 5, the fungus grew as a thick mat covering the entire surface.

The nutrient status also had a marked effect on the fatty acid composition of the mycelial lipids. Generally, the degree of unsaturation of the lipids was increased at higher nutrient concentrations. However, when the fungus was grown on the dilute media (media 1 and 2) the lipids were more unsaturated than those of mycelium harvested from medium 3 (Fig. 20).

Determination of the oxygen concentration of culture media after 12 days incubation at 48°(Table 42) showed that while in media 1 and 2 the oxygen concentration had fallen from 146 µM/l. to 110 µM/L. and 69 µM/l. respectively, media 3, 4 and 5 had an oxygen concentration of 28 µM/l.

TABLE 41 . The effect of different concentrations of carbon and nitrogen at a constant C : N ratio, on the growth, lipid yield and fatty acid composition of M. pusillus.

Medium	1				2				3				4				5			
Incubation period (days)	2	5	8	12	2	5	8	12	2	5	8	12	2	5	8	12	2	5	8	12
Growth (mg. dry wt./ml.).	0.20	0.30	0.20	0.17	0.73	0.90	0.83	0.93	4.30	5.70	5.83	5.30	7.23	18.03	21.20	26.10	9.06	22.80	26.10	33.23
Lipid yield (% of dry wt.)	Insufficient lipid obtained for accurate calculation of mycelial lipid content.				14.7	18.1	16.1	12.1	15.9	22.2	18.0	6.8	34.2	39.6	31.6	24.7	39.3	38.7	34.1	12.6
Fatty acid																				
14.0	1.5	2.5	1.0	1.2	1.7	1.2	1.3	1.4	1.4	1.1	1.4	1.4	1.4	1.1	1.1	1.1	1.3	1.5	1.1	1.4
16.0	29.5	32.6	25.1	27.5	31.8	33.2	30.9	28.9	31.0	29.8	32.2	29.0	27.1	30.8	29.2	25.0	28.8	29.2	24.5	23.7
16.1	4.7	3.5	2.1	3.2	2.6	3.6	3.2	1.4	1.7	2.8	2.1	2.2	2.3	2.6	2.1	2.0	1.6	2.3	3.1	2.8
18.0	6.8	5.6	6.1	11.3	5.1	4.6	5.2	5.7	8.1	12.4	11.4	6.1	5.1	5.4	4.2	4.6	4.1	3.9	4.8	4.7
18.1	37.3	38.6	47.0	35.0	40.8	40.5	40.5	40.6	37.1	40.0	40.9	46.0	37.3	36.0	41.4	45.6	37.0	42.0	44.0	42.7
18.2	16.1	14.4	16.3	16.2	15.0	14.3	16.1	16.2	18.3	12.1	10.7	12.7	20.5	19.7	18.3	18.3	19.2	16.6	18.0	18.9
18.3	4.0	2.8	3.6	5.5	2.6	2.6	2.9	4.0	2.3	1.9	1.4	2.8	6.1	4.4	3.7	3.4	8.1	4.6	4.6	5.7
Degree of Unsaturation	0.86	0.79	0.92	0.87	0.81	0.80	0.84	0.86	0.84	0.73	0.69	0.82	0.99	0.91	0.91	0.94	1.02	0.91	0.97	1.02

Table 42. Determination of the partial pressure of oxygen (pO_2) of cultures of M. pusillus grown at 48° for 12 days on media of different concentrations of carbon and nitrogen.

(Data expressed as pO_2 in mm.Hg.).

	Uninoculated medium at 48°.	Medium 1	2	3	4	5
pO_2 determination of replicate cultures. (mm.Hg.).	148	101	63	24	21	36
	136	86	58	30	29	26
	138	109	61	25	33	24
	141	91	51	26	20	20
	140	98	73	26	26	25
Mean pO_2 (mm.Hg.)	140	97	61	26	26	26
Oxygen concentration expressed in µM/l. water.	146	110	69	28	28	28

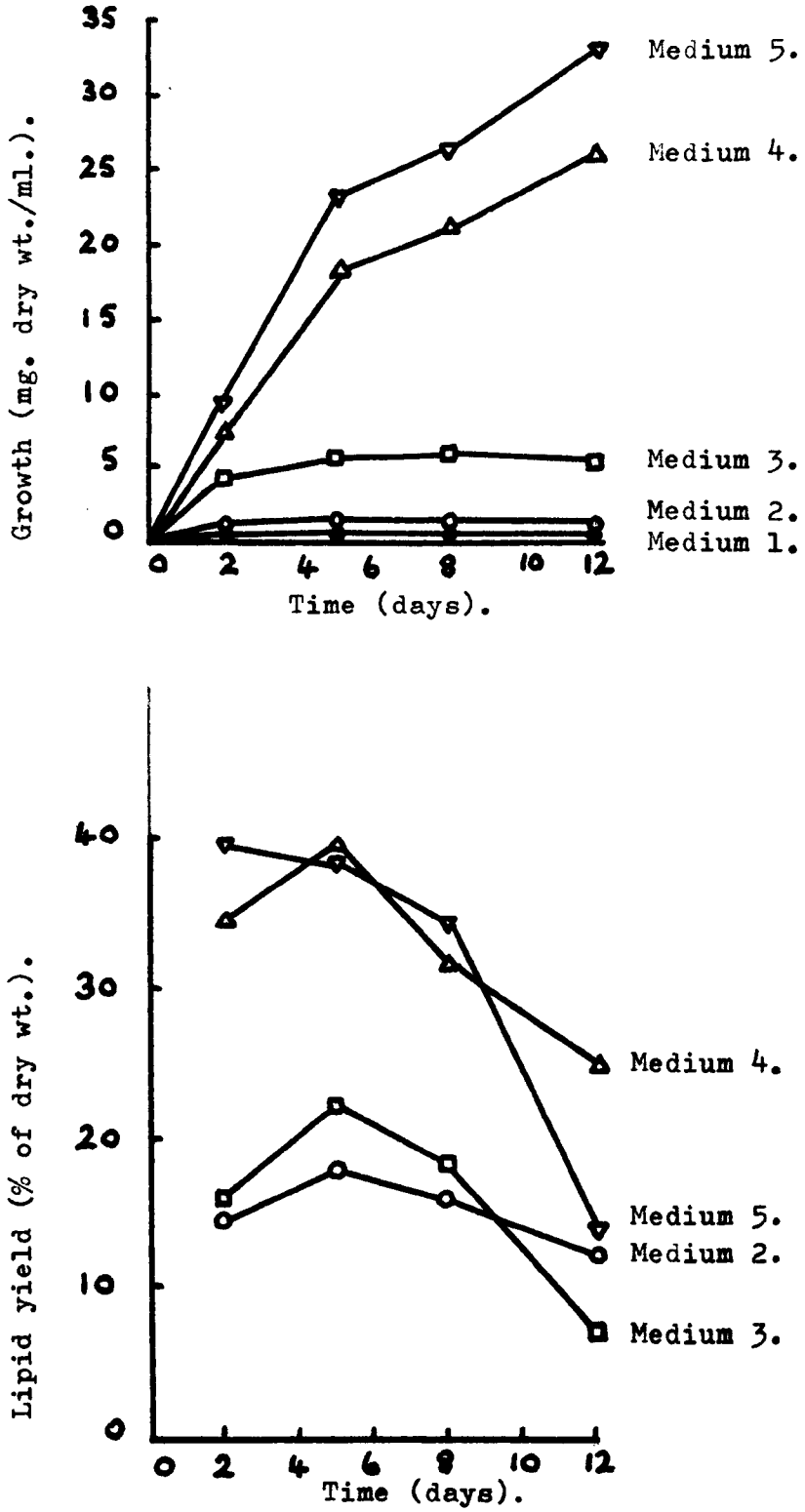
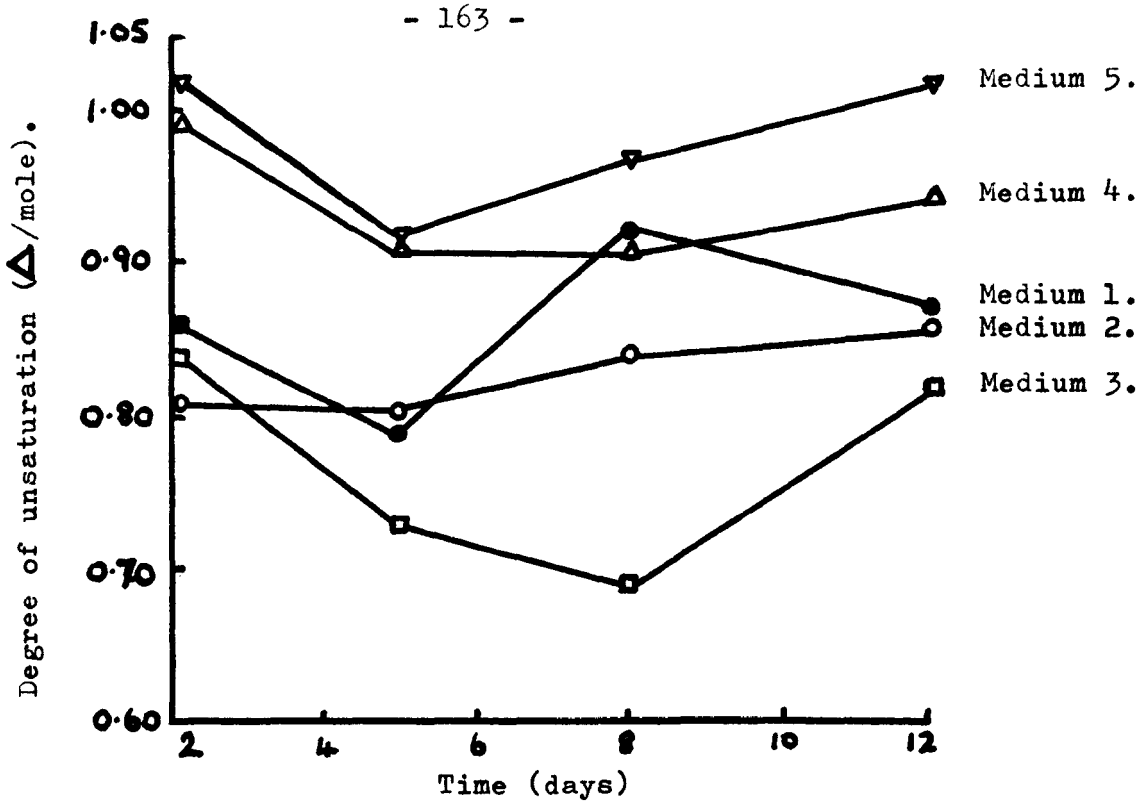


Fig. 19. Growth and lipid yield of M. pusillus grown on medium containing different concentrations of carbon and nitrogen at a constant C:N ratio.



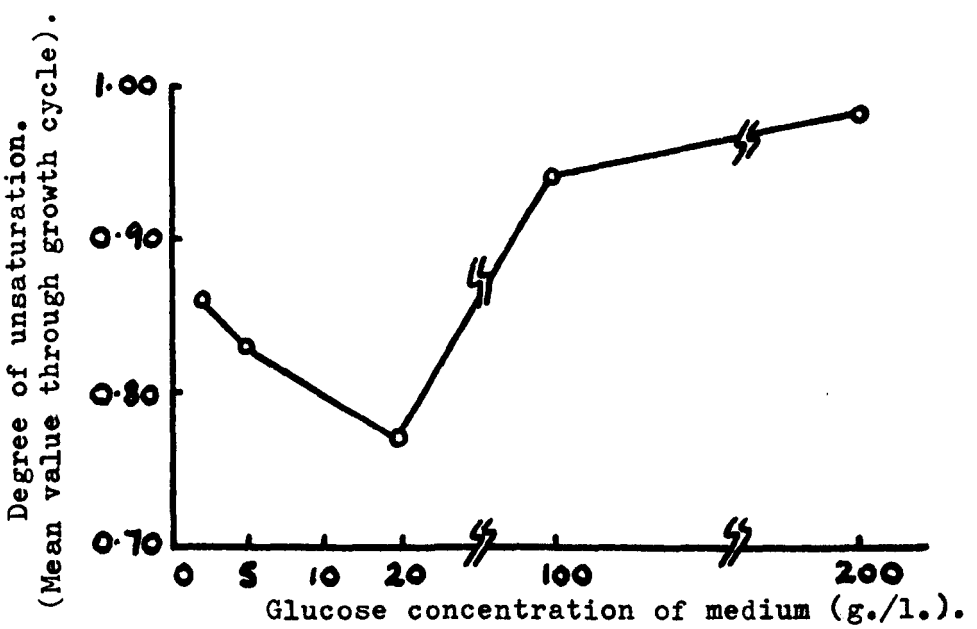


Fig. 20. Effect of different concentrations of carbon and nitrogen at a constant C:N ratio, on the degree of unsaturation of the lipids of M. pusillus.

These results indicate that when the mycelial mat completely covers the surface of the medium, the rate of oxygen diffusion between atmosphere and medium is slower than the rate at which oxygen is utilised from the medium. On medium 1 the fungus did not form a complete mycelial mat and it is concluded that this medium did not become oxygen-deficient. On the other hand, in media 3, 4 and 5 there is a marked degree of oxygen depletion, while medium 2 stands in an almost intermediate position.

In oxygen-limited cultures, therefore, the degree of unsaturation of the lipids is influenced by the concentration of carbon and nitrogen in the medium; on a medium of lower nutrient status (e.g. medium 3) the lipids are more saturated than that from media of higher nutrient concentration (media 4 and 5).

However, when cultures were grown on media of low nutrient status (media 1 and 2), the type of growth of the fungus allowed some diffusion from the atmosphere so that oxygen was less limiting in the culture medium. Under these conditions the lipids had a high degree of unsaturation despite having been grown on nutrient-poor media.

It seems possible therefore that the degree of lipid unsaturation can be affected by concentration of nutrients and by the oxygen concentration of the medium. The effect of oxygen concentration is investigated further in Sections 7.10, 7.11 and 7.12.

7.7 The Effect of the Surface Area : Volume Ratio (SA:V ratio) of the Medium.

7.71 Experimental Procedure. The basic medium plus glucose (20 g./l.) ammonium sulphate (250 mg.N/l.) and sodium succinate (5 g./l.) was dispensed into 700 ml. Erlenmeyer flasks in the following quantities:

(i) 40 ml. medium; surface-area : volume ratio 2.0.
(ii) 90 ml. " " " " " 1.0.
(iii) 195 ml. " " " " " 0.45.
(iv) 395 ml. " " " " " 0.16.

Culture flasks were autoclaved and inoculated with 5 ml. of a standard spore suspension of M. pusillus. Cultures were incubated in still culture at 48° and triplicate flasks harvested after 3, 7 and 10 days growth. For comparative purposes flasks containing 200 ml. medium (SA:V = 0.45) were grown in shake culture at 48° and harvested in triplicate after 3, 7 and 10 days incubation. After the mycelium had been harvested dried and weighed, the lipid was extracted and hydrolysed and the fatty acids methylated and analysed by GLC.

7.72 Results. Growth of M. pusillus in still culture wasmore rapid on media with high surface-area to volume ratios (Table 43. Fig. 21). At a surface-area to volume ratio of 0.45, growth was more rapid in shake culture than in still culture.

The surface area to volume ratio appeared to have little effect on lipid accumulation, except in the highly aerated cultures (SA : V=2.00) and in shake culture, where there was a pronounced drop in the lipid content as the fungus aged (Fig. 22).

Apart from a higher percentage of oleic acid and lower percentage of linoleic and linolenic acid in shaken cultures there appeared little correlation between the surface area to volume ratio of the medium and the degree of unsaturation of the lipids (Fig. 22). However, lipids obtained from mycelia grown in shake culture were more unsaturated than those from mycelia grown in still culture of the same SA : V. ratio which indicates that aeration may affect the degree of lipid unsaturation.

TABLE 43 . The effect of aeration on growth, Lipid content and fatty acid composition of the lipids of M. pusillus.

Culture Conditions.	Stationary			Stationary			Stationary			Shake			Stationary		
Surface Area to Volume Ratio.	2.00			1.00			0.45			0.45			0.16		
Incubation Period (days).	3	7	10	3	7	10	3	7	10	3	7	10	3	7	10
Growth (mg.dry wt./ml.)	4.54	5.80	5.30	2.32	5.00	5.28	0.98	2.16	3.65	5.18	5.50	4.88	0.01	0.66	1.19
Lipid yield (% of dry wt.).	22.8	17.9	9.5	18.8	19.2	15.8	23.7	11.7	14.5	29.3	19.7	14.8	17.4	12.1	13.2
Fatty Acids															
14.0	2.0	1.7	1.1	1.5	1.4	1.7	1.8	1.3	1.3	1.0	0.9	1.3	1.9	1.3	1.0
16.0	28.4	27.1	30.4	31.8	28.9	26.8	25.0	28.1	28.6	21.4	23.2	20.0	26.7	28.2	29.0
16.1	3.4	5.0	3.3	2.7	2.2	3.0	2.6	1.9	3.0	2.8	3.0	2.9	4.0	1.5	3.0
18.0	3.4	3.9	1.8	3.3	5.6	3.2	5.9	4.5	3.0	2.3	3 .2	2.5	6.9	4.1	2.2
18.1	37.4	46.2	44.3	42.2	41.0	46.0	41.0	42.5	44.3	56.6	52.4	56.0	41.0	43.2	41.0
18.2	21.8	13.3	16.6	16.3	18.2	17.8	20.2	18.3	17.6	14.3	15.4	15.7	17.3	18.4	20.1
18.3	3.7	2.6	2.4	2.2	2.7	1.7	3.5	3.1	1.9	1.6	2.0	1.9	3.1	3.0	3.5
Degree of unsaturation	0.96	0.85	0.88	0.84	0.87	0.90	0.94	0.90	0.88	0.93	0.92	0.96	0.89	0.90	0.94

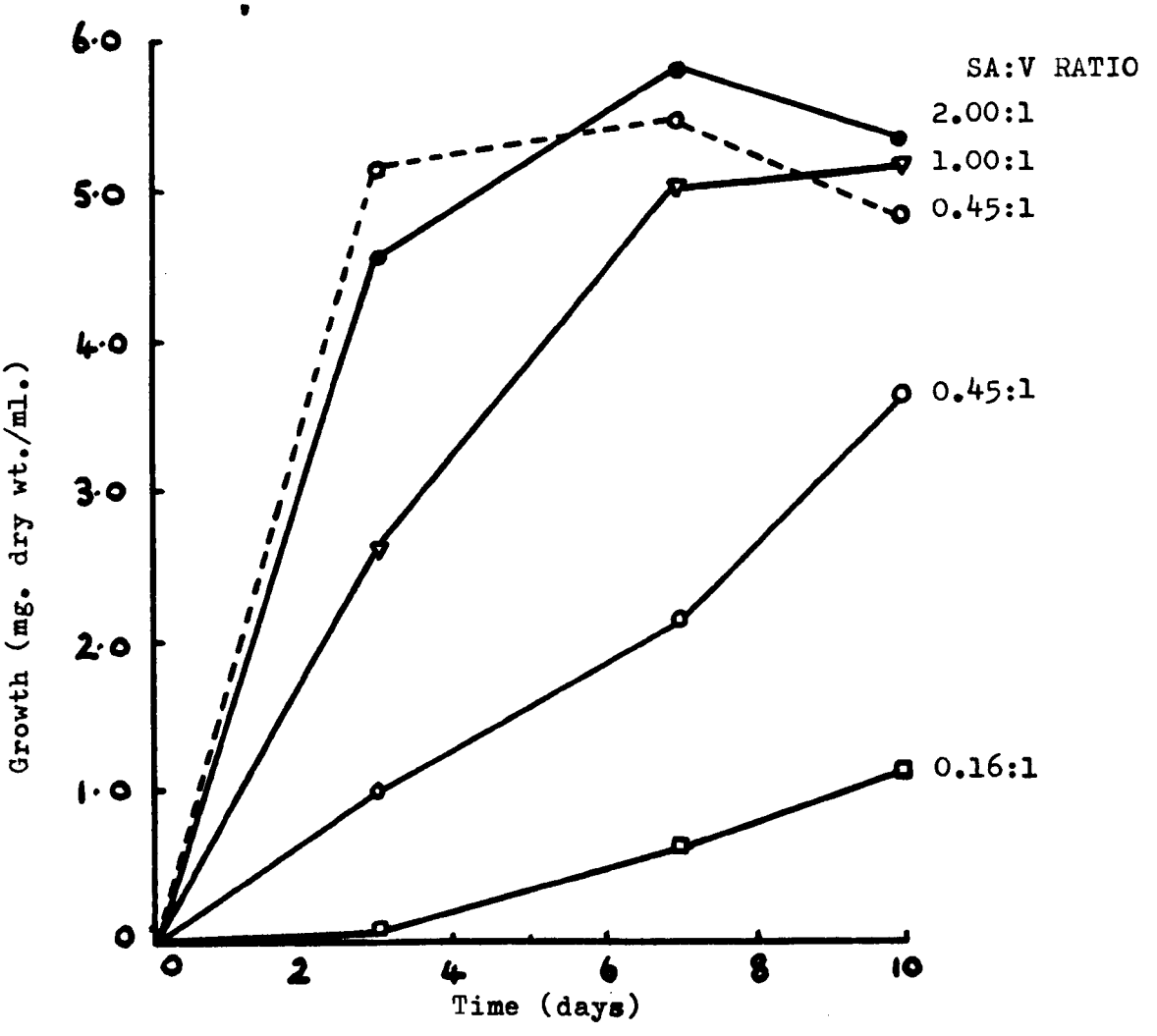


Fig. 21. Effect of aeration on the growth of M. pusillus in still (——) and shake (o---o) culture.

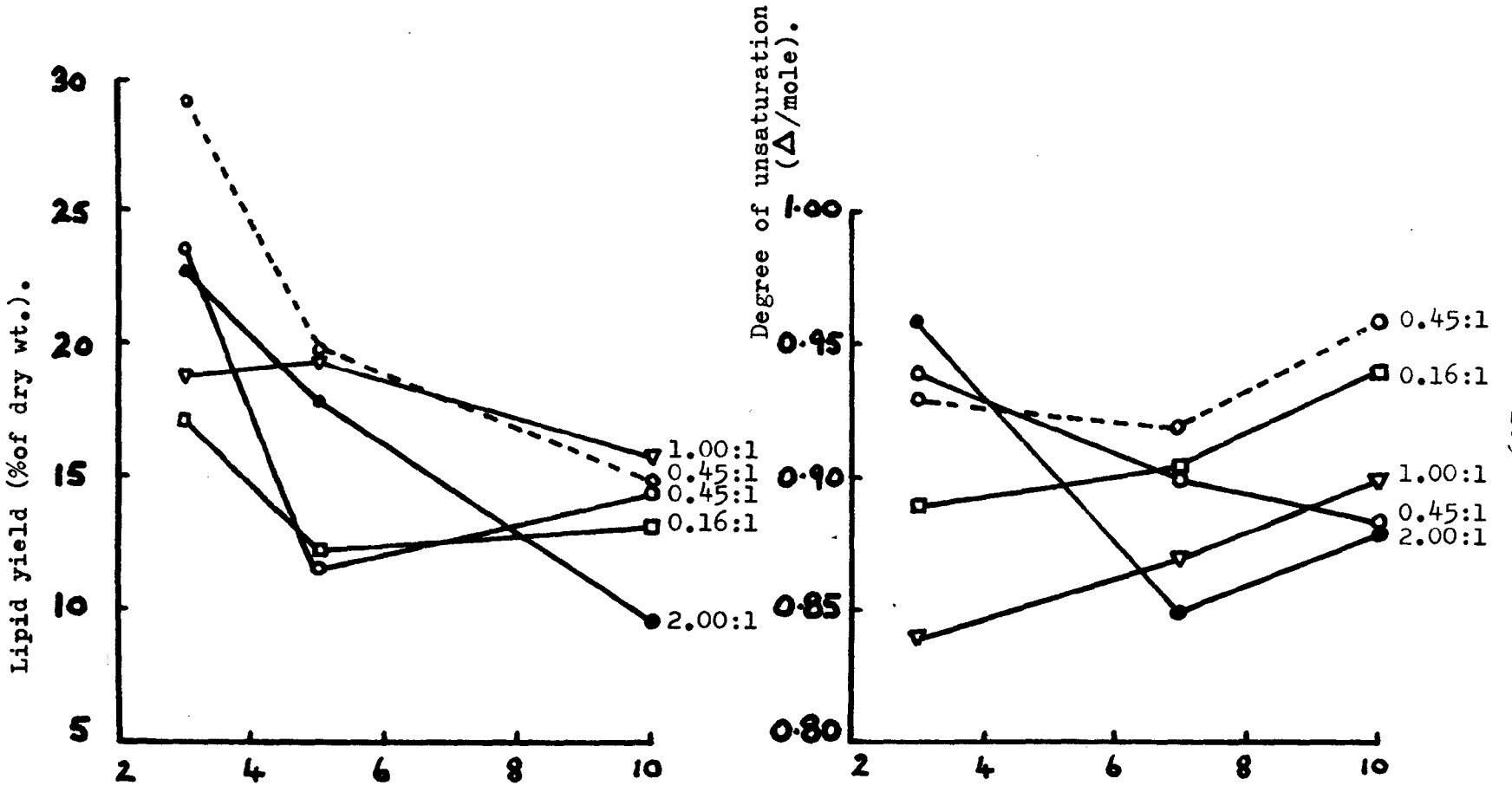


Fig. 22. Effect of aeration on the lipid content and degree of unsaturation. of M. pusillus grown in still (——) and shake (o---o) culture.

7.8 The Effect of Temperature.

7.81 Experimental Procedure. The basic medium plus glucose (20 g./l.), ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.) was dispensed in 25 ml. portions into 100 ml. Erlenmeyer flasks, autoclaved and inoculated with 1 ml. standard spore suspension of M. pusillus. Cultures were incubated at 25°, 37°, 47° and 53° and six replicate flasks were harvested from each temperature at regular intervals. After the mycelium was dried and weighed the lipid was processed in the usual way and the fatty acid methyl esters analysed by GLC.

7.82 Results. The data for the growth and lipid content of M. pusillus grown at 25°, 37° 47° and 53° are presented in Table 44 and Fig. 23. The fungus grew well at all temperatures except 53° at which the cultures grew typically, producing submerged non-sporing mycelium. At the other incubation temperatures cultures grew as aerial, heavily spering mycelial mats.

At 25°, 37° and 47°, mycelial growth and lipid accumulation followed a similar course, both steadily increasing throughout the experiment. Temperature appeared to enhance lipid accumulation, the concentration of mycelial lipid being higher at 47° than at 25° and 37°.

The incubation temperature had a marked effect on the fatty acid composition of the lipids (Fig.24). At higher temperatures the lipids contained a greater proportion of oleic acid and smaller proportions of linoleic acid and linolenic acid. As a consequence the lipids became progressively more saturated as the incubation temperature was raised.

TABLE 44 . The effect of incubation temperature on the growth, lipid content and fatty acid composition of M. pusillus in a glucose, ammonia medium buffered with sodium succinate.

Incubation Temperature	25°						37°						47°						53°					
Incubation Period (days)	2	3	4	6	9	14	1	2	3	4	6	9	14	1	2	3	4	6	9	2	3	4	6	9
Growth (mg.dry wt./ml.)	0.54	0.64	1.90	2.47	3.99	5.88	0.89	2.55	3.69	4.80	4.92	5.80	5.48	1.21	2.74	3.37	3.88	4.64	5.40	0.45	0.85	1.03	0.76	0.94
Lipid yield (% of dry wt.)	10.7	13.2	12.1	18.6	17.5	26.2	12.7	15.5	17.0	22.2	19.7	25.6	19.2	16.3	19.9	18.1	22.2	23.1	30.4	6.7	19.3	9.3	12.5	10.7
Fatty Acid																								
14.0	1.2	0.8	1.0	1.9	1.4	1.2	1.0	1.0	1.7	1.3	1.5	1.9	1.2	2.2	2.1	1.2	1.9	1.3	1.5	1.1	2.3	1.2	1.3	2.0
16.0	25.1	25.8	26.6	24.4	26.7	23.9	28.9	25.0	25.1	23.7	27.1	22.5	24.1	25.4	24.7	23.2	27.0	28.8	26.3	25.7	24.0	25.2	27.5	25.1
16.1	3.0	2.9	2.7	3.0	2.9	3.0	2.8	2.7	3.5	2.8	2.9	2.8	3.0	2.8	2.8	2.4	3.0	2.5	3.0	2.4	2.7	2.5	3.1	3.3
18.0	6.2	7.5	5.6	7.2	5.4	5.6	10.1	9.1	3.1	5.6	9.2	8.3	4.8	5.2	4.6	4.6	3.8	5.0	4.3	5.2	4.6	3.8	6.0	4.7
18.1	41.5	41.9	42.0	43.1	39.0	39.7	39.2	38.4	44.6	45.0	37.5	40.6	44.0	45.0	44.9	46.2	40.0	41.0	42.1	47.3	52.5	51.8	49.6	48.4
18.2	15.3	15.0	16.6	16.3	17.0	20.2	13.7	18.5	17.5	18.1	18.7	20.1	18.5	16.2	17.5	18.9	19.9	17.9	19.2	14.0	11.9	13.0	10.4	14.1
18.3	7.7	5.7	5.6	4.0	7.3	6.3	4.5	5.3	4.5	3.5	3.5	4.1	4.4	3.0	3.3	3.7	4.0	3.0	3.6	4.3	2.0	2.5	2.3	2.6
Degree of unsaturation	0.98	0.92	0.95	0.91	0.98	1.02	0.83	0.94	0.95	0.94	0.88	0.96	0.97	0.89	0.92	0.97	0.95	0.87	0.93	0.90	0.84	0.87	0.80	0.87

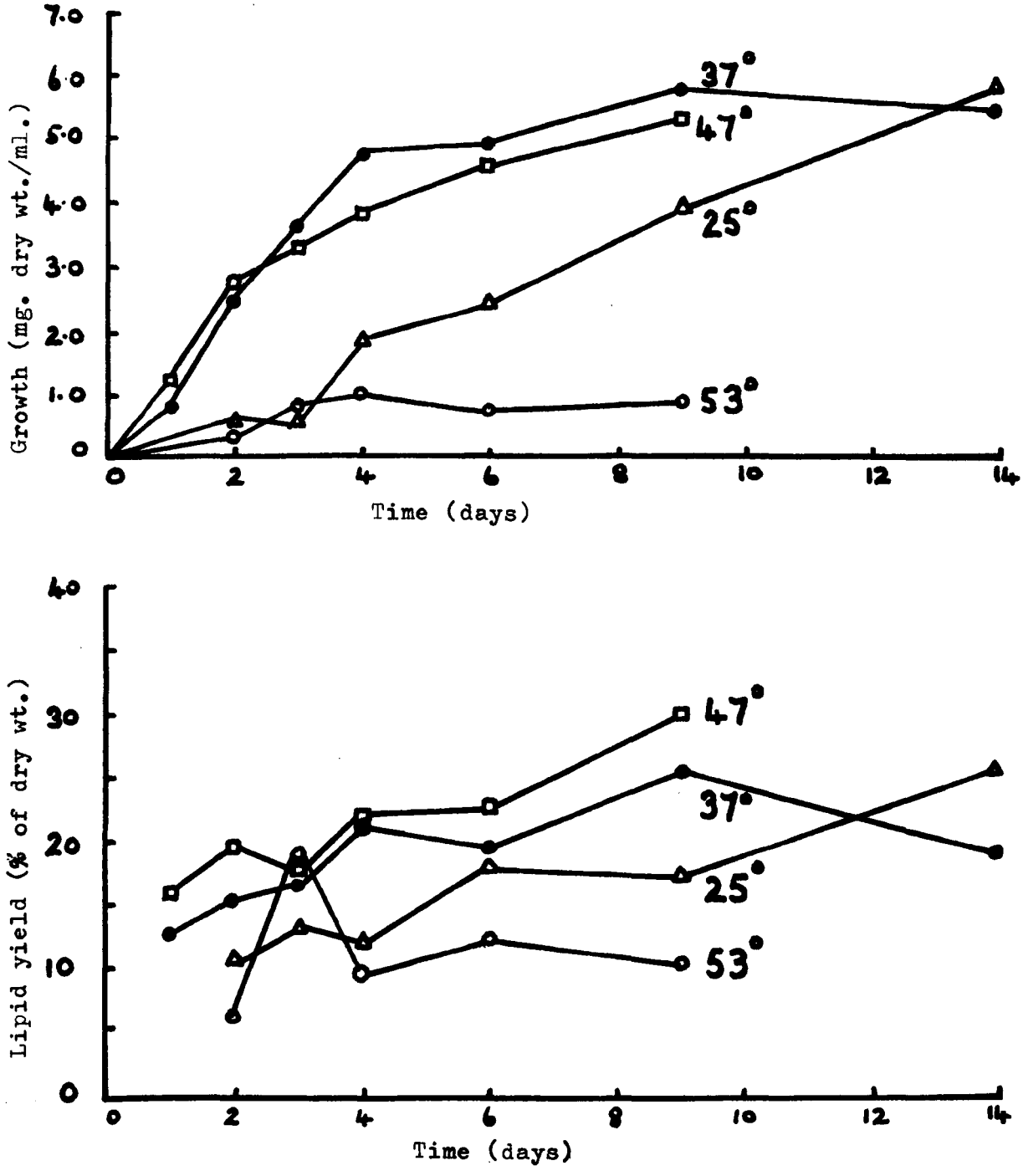


Fig. 23. Effect of incubation temperature on growth and lipid accumulation of M. pusillus.

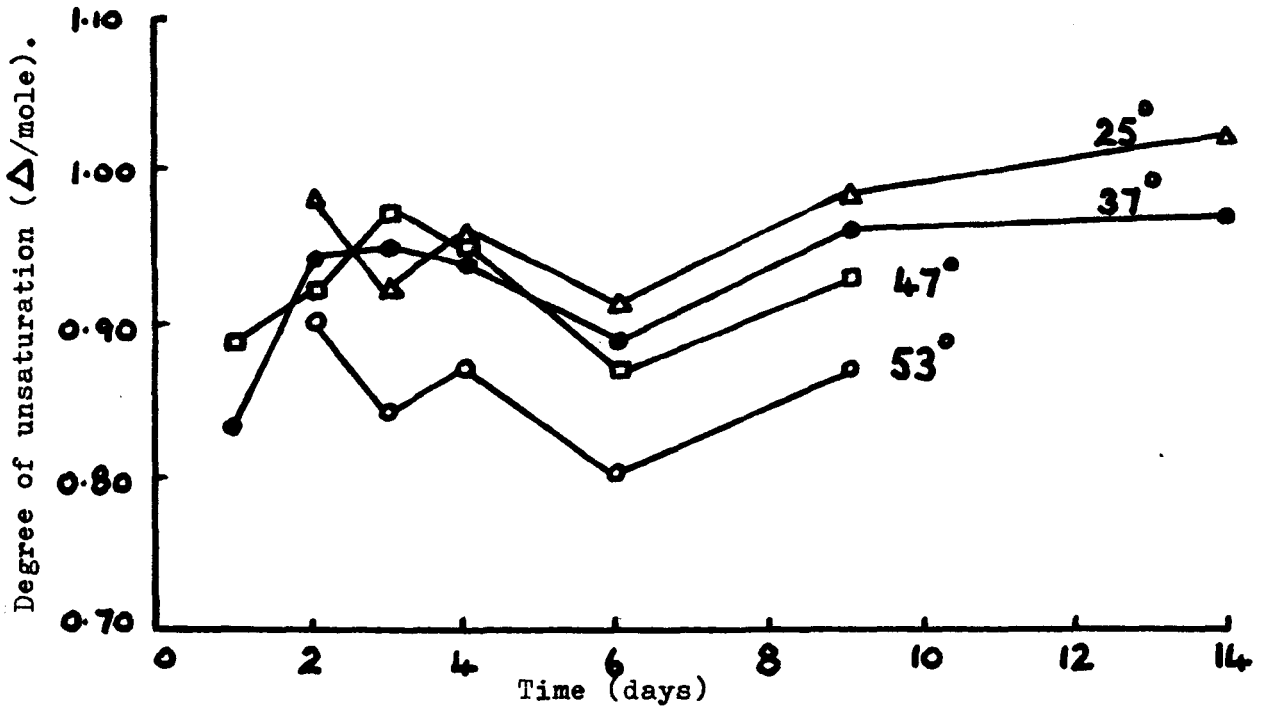


Fig. 24. Effect of incubation temperature on the degree of unsaturation of the lipids of M. pusillus.

7.9 The Effect of Change in Incubation Temperature.

The effect of transferring cultures from 50° to 25°, and vice versa, on growth, lipid synthesis and fatty acid composition was investigated at several stages in the growth cycle of M. pusillus.

7.91 Experimental Procedure. The basic medium plus glucose (20g/l.) ammonium sulphate (250 mg. N/l.) and sodium succinate (5g./l.) was dispensed in 20 ml. portions into 100 ml. Erlenmeyer flasks. The flasks were autoclaved at 15 lb./in.^{2} for 15 mins., inoculated with 1 ml. standard spore suspension of M. pusillus and incubated at either 25° or 50°. Harvests were carried out after 3,6,9,12 and 15 days incubation, 6 replicate flasks being used on each occasion. Because the culture medium had dried out it was not possible to analyse cultures which had been grown at 50° for 15 days.

At each harvest, cultures were transferred in batches from the 50° to the 25° incubator, and vice versa, the following alterations in incubation temperature were made:

(i) cultures incubated 12 days at 50°
(ii) " " 3 " " ", then transferred to 25°.
(iii) " " 6 " " " " " " "
(iv) " " 9 " " " " " " "
(v) " " 15 " " 25°
(vi) " " 3 " " " then transferred to 50°.
(vii) " " 6 " " " " " " "
(viii) " " 9 " " " " " " "
(ix) " " 12 " " " " " " "

After the cultures were harvested, the mycelial mats were removed, dried and weighed, and the lipid extracted. The lipid was hydrolysed and the resulting fatty acids methylated and analysed by GLC.

7.92 Results. Data for the growth, lipid synthesis and fatty acid composition of cultures of M. pusillus transferred from 50° to 25° are presented in Table 45, and for transfer from 25° to 50° in Table 46.

Cultures transferred from 25° to 50° invariably grew better than those remaining at 25°, and the lower lipid content of these cultures indicates that utilisation of endogenous lipids was greater at the higher temperature.

The behaviour of cultures transferred from 50° to 25° appeared more complex. Young (3-day and 6-day) cultures did not grow as well following transfer to a lower temperature, but cultures grown for 9 days at 50° grew better when switched from 50° to 25° (Fig. 25). Cultures transferred from 50° to 25° had a higher mycelial lipid content than cultures grown at 50°, and this is taken to indicate a reduction in metabolic rate following transfer to the lower incubation temperature (Fig. 26).

Altering the incubation temperature had a marked effect on the fatty acid composition of the mycelial lipids (Fig.27). The effect of temperature on the lipids was identical with that

TABLE 45 . The effect of change in incubation temperature on the growth, lipid content and fatty acid composition of M. pusillus.

(a) Incubation temperature changed from 50° to 25°.

Time of Harvest (days)	3	6	9	12	6	9	12	9	12	15	12	15
Incubation Temperature	50°				3 days at 50°, then transferred to 25°.			6 days at 50°, then transferred to 25°.			9 days at 50°, then transferred to 25°.	
Growth (mg.dry wt./ml)	4.01	7.15	8.75	9.96	4.48	5.70	5.31	7.70	10.00	10.50	11.60	14.65
Lipid yield (% of dry wt.)	25.1	28.6	19.2	12.9	21.6	25.0	18.7	26.3	27.0	17.2	26.3	18.7
Fatty Acid												
14.0	1.2	1.4	1.8	1.1	1.4	1.0	1.1	1.5	1.0	1.01	2.0	1.0
16.0	29.1	26.5	30.1	26.3	23.3	27.3	24.3	30.2	26.4	23.8	22.2	24.3
16.1	2.7	3.1	3.1	3.8	3.2	3.1	2.9	2.8	3.8	3.6	3.3	2.5
18.0	6.3	2.7	7.5	6.4	5.2	5.2	5.8	5.0	4.8	3.8	3.9	4.3
18.1	40.1	39.4	45.6	45.1	42.1	37.5	38.6	39.4	36.3	38.7	47.0	40.1
18.2	17.7	22.8	11.7	14.2	19.3	20.4	17.7	19.3	21.4	19.8	18.4	21.5
18.3	3.0	4.1	4.2	3.1	5.4	5.6	9.5	2.8	6.3	7.5	3.2	6.3
Degree of unsaturation	0.87	0.97	0.84	0.86	1.00	0.98	1.02	0.89	1.01	1.05	1.05	1.04

TABLE 46 (continued).

(b) Incubation temperature changed from 25° to 50°.

Time of Harvest (days)	3	6	9	12	15	6	9	12	9	12	15	12	15	15
Incubation Temperature	25°					3 days 25°, then transferred to 50°.			6 days 25° then transferred to 50°.			9 days 25° then transferred to 50°.		12 days 25°, then 50°.
Growth (mg. dry wt./ml.)	1.64	4.52	5.29	6.00	6.24	5.45	6.98	9.35	6.38	8.35	7.87	7.50	9.65	6.82
Lipid yield (% of dry wt.)	20.8	32.2	30.7	25.9	18.8	18.2	17.6	21.7	18.4	18.3	14.3	17.9	13.5	17.2
Fatty Acids														
14.0	1.2	1.3	1.0	1.4	1.0	1.5	0.9	1.8	1.3	1.5	1.2	1.5	1.4	1.5
16.0	28.4	21.0	25.4	21.5	23.2	25.5	27.9	25.5	21.0	23.7	23.9	21.9	26.3	23.8
16.1	3.0	2.9	3.0	3.0	2.9	3.5	3.0	2.6	3.0	2.9	3.0	3.3	3.1	3.1
18.0	14.3	11.9	5.8	7.0	4.1	4.2	2.5	3.6	5.8	4.6	3.4	4.2	3.5	4.5
18.1	42.8	39.5	34.7	38.1	36.4	40.5	40.8	41.0	43.9	41.9	35.2	39.5	36.3	42.7
18.2	13.8	17.2	21.5	20.5	22.2	21.2	21.4	22.3	19.6	22.3	25.4	24.0	23.1	19.5
18.3	6.1	6.5	8.5	8.4	10.6	3.5	3.4	3.0	5.4	4.1	6.9	5.4	6.1	4.8
Degree of unsaturation	0.91	0.96	1.06	1.07	1.15	0.97	0.96	0.97	1.02	1.02	1.09	1.07	1.04	0.99

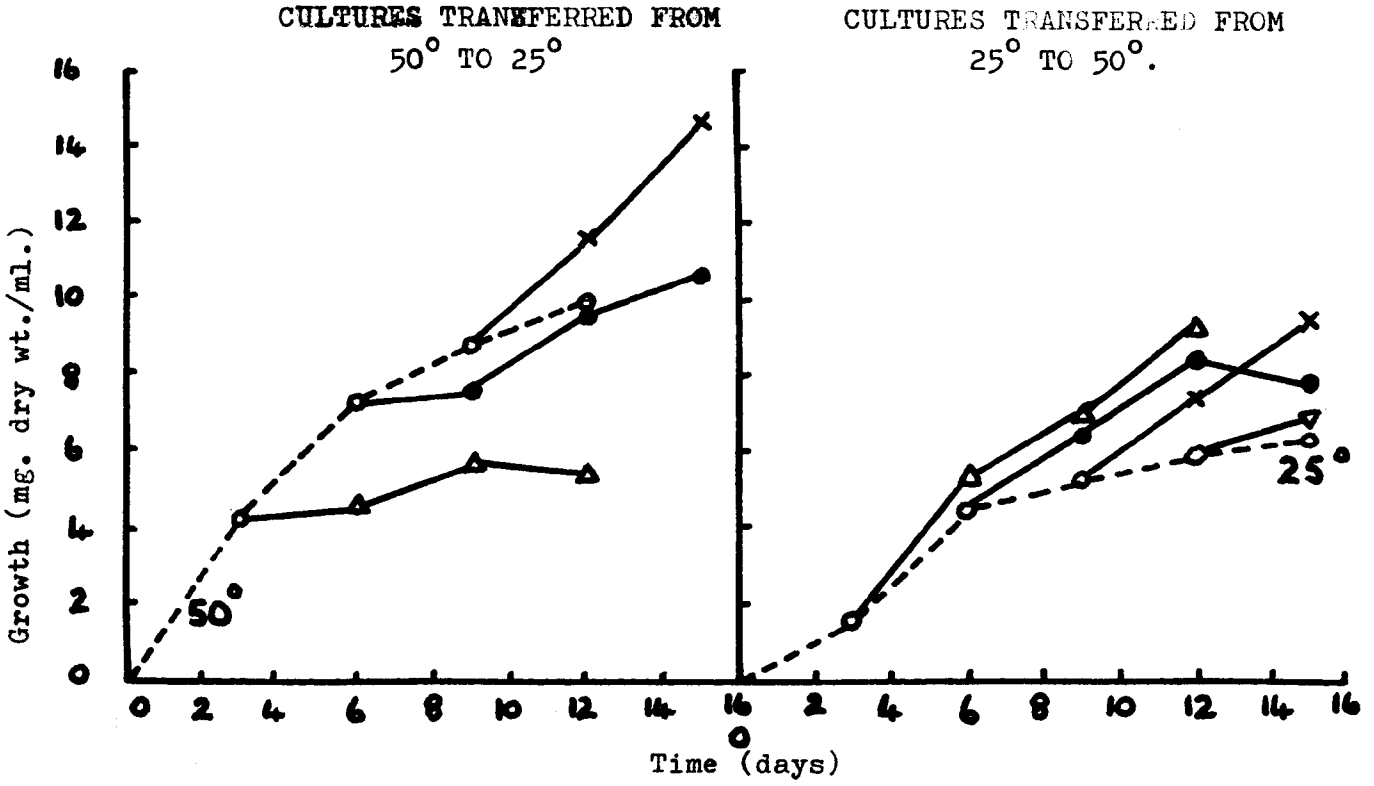


Fig.25. Effect of a change in incubation temperature on the growth of M. pusillus. Cultures grown at 25° or 50° and transferred after 3 days (——), 6 days (o——o), 9 days (x——x) or 12 days (——).

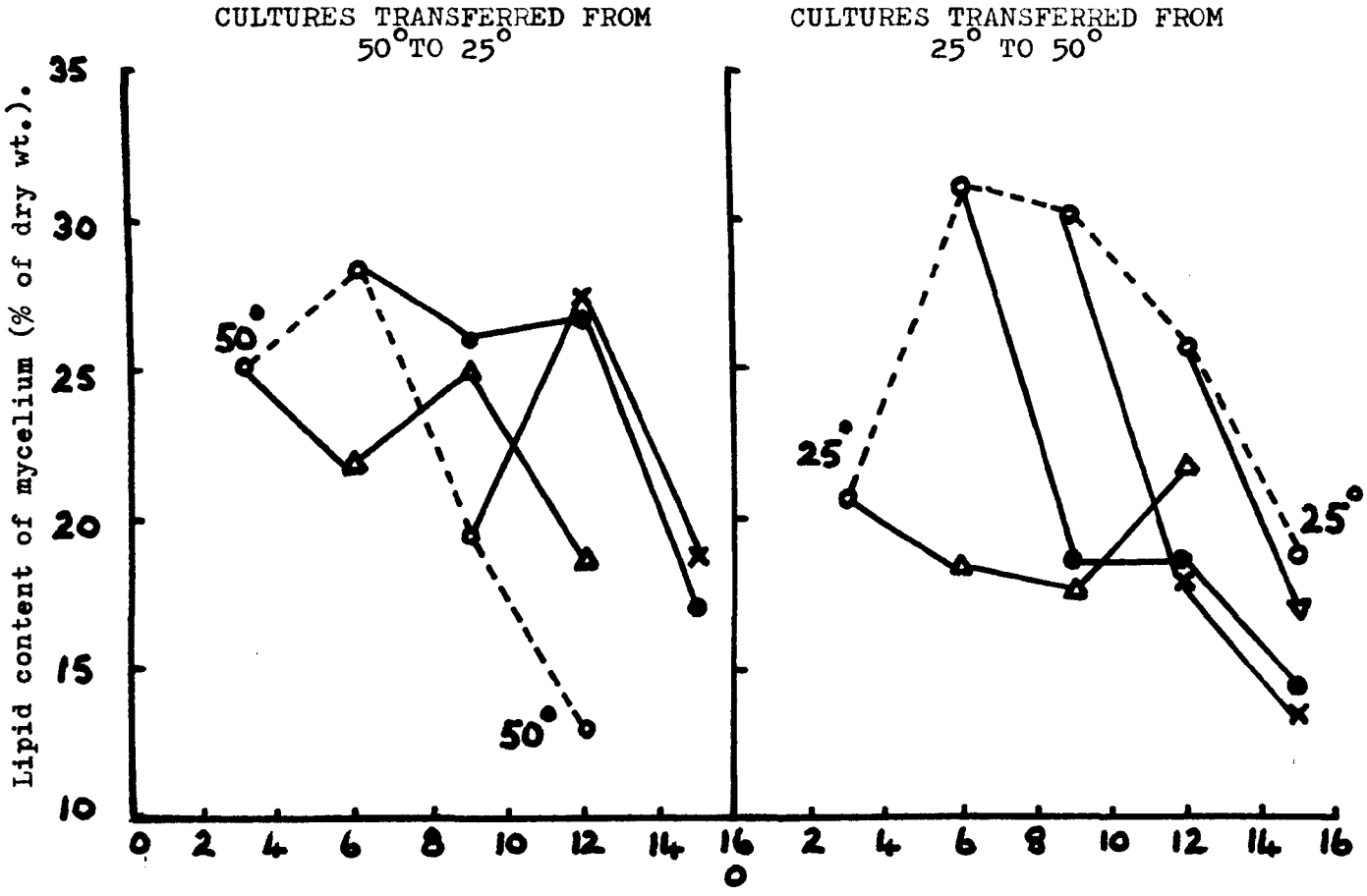


Fig. 26. Effect of a change in incubation temperature on the lipid content of M. pusillus. Cultures grown at 50° or 25° and transferred after 3 days (——), 6 days (o——o), 9 days (x——x), and 12 days (——).

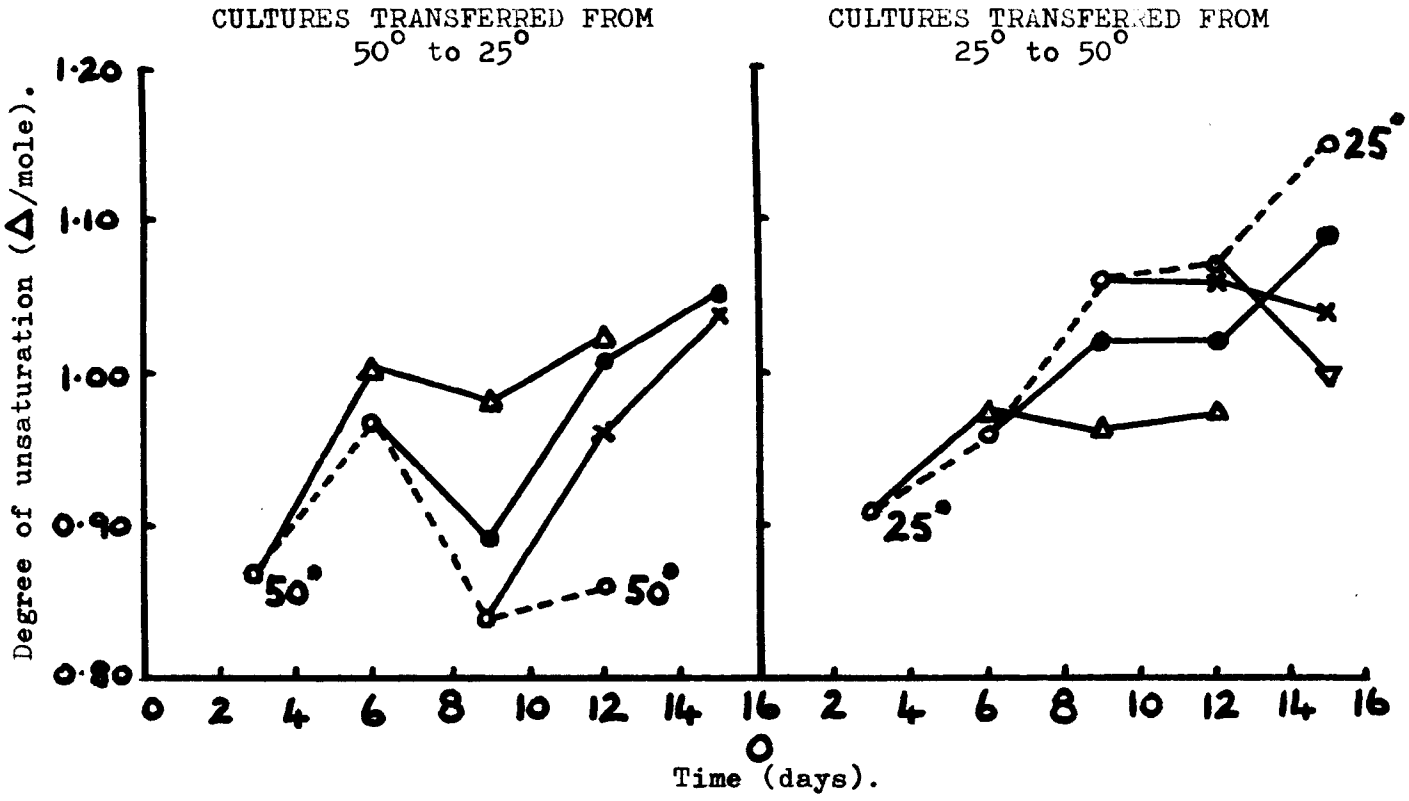


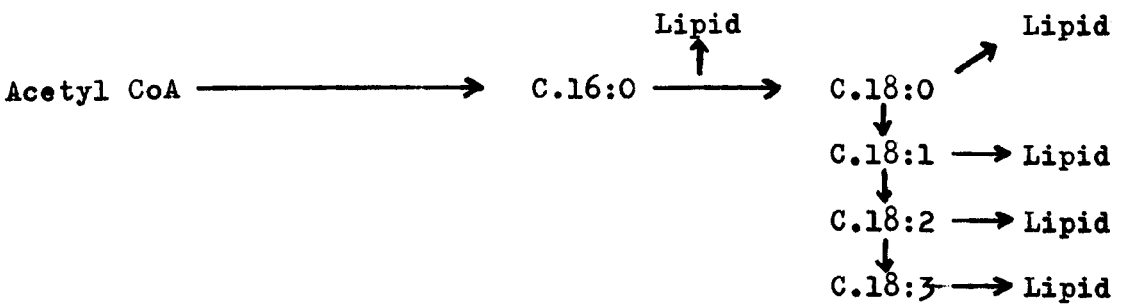
Fig. 27. Effect of a change in incubation temperature on the degree of unsaturation of the lipids of M. pusillus. Cultures grown at 50° or 25° and transferred after 3 days (——), 6 days (o——o), 9 days (x——x), 12 days (——).

described in the previous experiment and also in Chapter 6; when the incubation temperature was raised the mycelial lipids became more saturated, while transfer to a lower temperature resulted in higher degrees of lipid unsaturation.

The effect of a change in temperature on lipid unsaturation was especially marked in older (9, 12 and 15 day) cultures. In older cultures, increases in dry weight were associated with a fall in the lipid content of the mycelium, which could imply the utilisation of endogenous lipid reserves as energy source. After the switching of cultures to a different incubation temperature "new"mycelium containing "new" structural storage lipids was synthesised at the expense of "old"lipids (lipids synthesised at the original temperature). Since in older (9, 12 and 15 day) cultures the lipid content was being steadily reduced, "new" lipids would form an increasingly greater proportion of the total ("new" plus "old") lipids, which may account for the marked changes in degree of unsaturation of older cultures in response to a change in incubation temperature.

7.10 The Effect of Temperature on the Oxygen Content of Cultures Incubated at 25° and 50°.

Although it seems clear that organisms, in general, produce a more unsaturated lipid when grown at a lower temperature the reasons for this effect are not fully understood. The biosynthesis of fatty acids from acetyl coenzyme A may be summarised as follows:-



Kates and Baxter (1962) have stated that in lipid metabolism both the synthesis and the respiratory degradation of lipid would be expected to be temperature-dependent, and therefore to proceed more slowly at a lower temperature. They accounted for the greater proportion of linoleic acid in a micro-organism grown at a lower temperature by suggesting that the reactions by which lipid is synthesised are less retarded at the low temperature than the reactions by which lipid is oxidised. The rate at which linoleic acid is oxidised is therefore slower than the rate at which it is synthesised with the result that this polyunsaturated fatty acid would accumulate in the lipid.

Enzymes exist both for the synthesis of fatty acids (synthetases), and also for the conversion of saturated into unsaturated fatty acids (desaturases). The co-factor requirements for synthetases and desaturases differ in that the former require carbon dioxide and the latter require oxygen; however, both reactions require acetyl CoA, an acyl carrier protein (ACP) and reduced nicotine adenine dinucleotide ($NADH_2$) or reduced nicotine adenine trinucleotide ($NADPH_2$) (Harris and James, 1968. in press).

It appears, therefore, that both the quantity of lipid which an organism synthesises, as well as the degree of unsaturation of the lipid are controlled by the relative concentrations in the environment of oxygen and carbon dioxide. Under aerobic conditions the desaturation reactions will proceed normally. However, if the oxygen concentration falls to a level which becomes rate-limiting for the desaturation process, concentration would tend to favour the activity of lipid synthetase enzymes while depressing the enzyme-catalysed desaturation reactions.

It seemed possible therefore, that one reason why fungal lipids become more saturated at higher incubation

temperatures is that at elevated temperatures the oxygen concentration is rate-limiting for the fatty acid desaturation reaction, with the result that the synthesised lipids contain a low proportion of polyunsaturated acids.

An experiment was performed using an oxygen electrode to measure the partial pressure of oxygen (pO_2) in the medium during the growth cycle of M. pusillus at 25° and 50°; the growth, lipid synthesis and fatty acid composition of the fungus were also investigated at these temperatures.

7.10.1 Experimental Procedure. To the basic medium was added glucose (20 g./l.), ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.). Medium was dispensed in 30 ml. portions into 150 ml. Erlenmeyer flasks and sterilised by autoclaving at 15 lb./in.^{2} for 15 mins. Flasks were each inoculated with 1 ml. of a standard spore suspension of M. pusillus and incubated at 25° or 50°. After 2, 5, 8 and 12 days incubation six replicate flasks were harvested at each temperature; it was not possible to harvest a 2-day culture at 25° as growth had not yet begun.

Determination of pO_2 of cultures was carried out immediately after harvest, as described in Section 1.72. The mycelial mats were removed and dried, prior to extraction of the lipid. The lipid was hydrolysed and the fatty acids methylated and analysed by GLC.

7.10.2 Results. Data for the concentration of oxygen dissolved in the culture medium during growth of M. pusillus at 25° or 50° are presented in Table 47, and data for the growth, lipid synthesis and fatty acid composition in Table 48.

Growth and lipid synthesis proceeded essentially as described in Sections 7.5, 7.8 and 7.9. The fungus initially grew and accumulated mycelial lipid more rapidly at 50° than at 25°, though in the later stages of culture both growth and mycelial lipid content were greater at the lower temperature. The effect of incubation temperature on the fatty acid composition and degree of unsaturation of the lipid also followed the trend as described earlier; the lipid was invariably more unsaturated when the fungus was grown at the lower temperature (Fig. 28).

Determination of pO_2 of the medium and calculation of the oxygen concentration showed that at 50° cultures grew under conditions of low oxygen concentration, the concentration of oxygen dissolved in the medium falling from 148 μM O_2/l., in uninoculated medium incubated at 50°, to around 25 μM O_2/l. The oxygen concentration of cultures incubated at 25° fell by a similar amount, though because of the increased solubility of oxygen at 25° the concentration was reduced from 238 μM O_2/l. in uninoculated medium, to around 100–120 μM O_2/l. in growing cultures (Fig. 28).

TABLE 47 . The effect of incubation temperature on the oxygen concentration of the culture medium during growth of M. pusillus in still culture.

Incubation temperature.	25°				50°				
Incubation period (days)	Uninoculated medium at 25°.	5	8	12	Uninoculated medium at 50°	2	5	8	12
p O_2 of replicate cultures (mm.Hg.).	148	60	79	78	148	35	30	23	18
	149	56	62	80	143	32	26	21	25
	147	60	65	75	138	36	26	15	23
	148	52	86	73	140	34	25	19	19
	145	65	48	78	140	30	24	21	17
	144	73	78	77	136	20	25	21	19
Mean p O_2(mm. Hg.).	147	61	69	77	141	31	26	22	20
Oxygen concentration (μ M O_2/l. water).	238	98	112	124	148	34	28	24	22

Table 48 . The effect of incubation temperature on the growth, lipid synthesis and fatty acid composition of M. pusillus.

Incubation Temperature.	25°			50°			
Incubation Period (days).	5	8	12	2	5	8	12
Growth (mg. dry wt./ml.).	4.02	5.76	9.10	4.30	5.70	5.83	5.30
Lipid Yield (% of dry wt.).	15.8	18.2	19.5	15.9	22.2	18.0	6.8
Fatty acid							
14.0	1.2	1.0	1.2	1.4	1.1	1.4	1.4
16.0	28.4	22.4	27.5	31.0	29.8	32.2	29.0
16.1	2.3	3.5	3.1	1.7	2.8	2.1	2.2
18.0	11.6	11.1	7.9	8.1	12.4	11.4	6.1
18.1	37.4	38.4	34.8	37.1	40.0	40.9	46.0
18.2	14.9	18.8	19.8	18.3	12.1	10.7	12.7
18.3	4.2	5.2	5.5	2.3	1.9	1.4	2.8
Degree of Unsaturation.	0.82	0.95	0.94	0.84	0.73	0.69	0.82

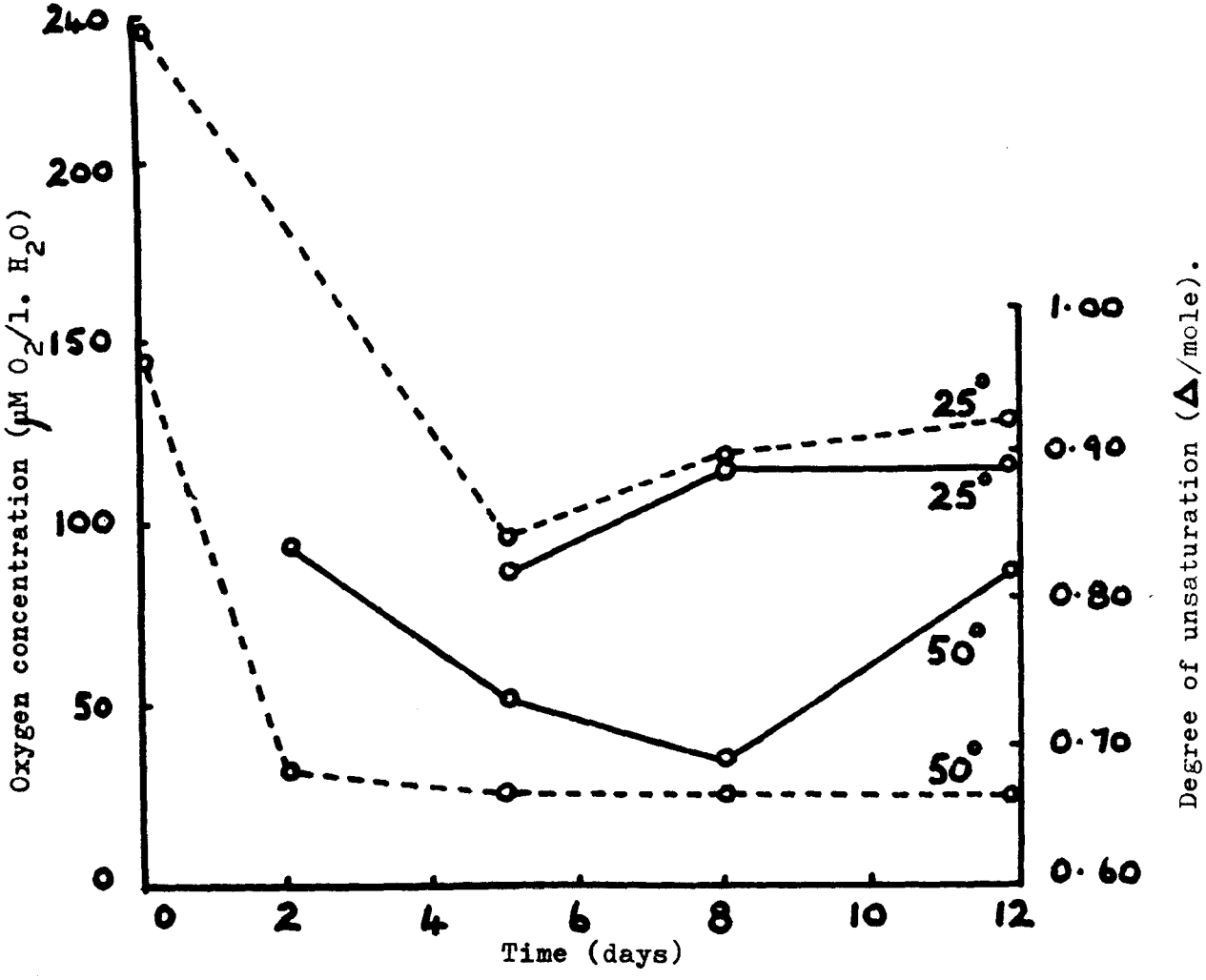


Fig. 28. Effect of incubation temperature on the oxygen concentration of the culture medium (o---o) and the degree of unsaturation of the lipids of M. pusillus (o—o).

It is clear, therefore, that the temperature of incubation has a profound effect on the amount of dissolved oxygen available to the fungus. In cultures grown at 50°, 80/90% of the available oxygen is used up within the first 48 hours incubation and even though gaseous diffusion increases as the temperature is raised, it is not sufficient to balance the rate at which oxygen is depleted from the medium. At 25° there is a fall in the amount of oxygen dissolved in the medium during the early growth of the fungus, when oxygen uptake obviously exceeds oxygen diffusion. However, since the concentration of oxygen in the medium gradually increases throughout the later stages of growth it is probable that after the early, rapid phase of growth, oxygen diffuses into the culture medium more rapidly than it is utilised.

Though at both incubation temperatures the amount of oxygen dissolved in the medium falls, cultures grown at 25° are probably not deficient in oxygen. However, at 50° the fungus almost certainly grows under oxygen-deficient conditions, possibly at a concentration which is rate-limiting for the fatty acid desaturation process. The lower oxygen concentration at 50° may be responsible for the lower proportion of polyunsaturated fatty acids in the mycelial lipid, compared with the proportion of these acids in lipid synthesised at 25°.

7.11 The Effect of Increasing the Oxygen Concentration in the External Environment.

In the previous experiment it was shown that the amount of oxygen dissolved in the medium was much smaller in cultures growing at 50° than in cultures growing at 25°. It was considered that at 50° the oxygen concentration might be rate-limiting for fatty acid desaturation, which could account for the lower degree of unsaturation in cultures grown at the higher temperature .

The effect of increasing the oxygen concentration on the degree of unsaturation of the lipids of two thermophilic fungi, M. pusillus and Rhizopus sp. III was investigated by growing cultures in air-tight containers with different mixtures of oxygen and nitrogen.

7.11.1 Experimental Procedure. The medium used in this experiment was the basic medium plus glucose (20 g./l.), ammonium sulphate (250 mg. N/ml.) and sodium succinate (5 g./l.). Medium dispensed in 15 ml. portions into 50 ml. Erlenmeyer flasks plugged with non-absorbent cotton wool was sterilised by autoclaving at 15 lb./in.^{2} for 15 minutes. Each flask was inoculated with 1 ml. standard spore suspension of M. pusillus or Rhizopus sp.III. Inoculated flasks were placed in polythene

lunch boxes (Ekcoware, 25cm. diameter, 12 cm. deep) modified to allow entry and exit of gas (Fig.29). Each lunch box held a maximum of thirteen 50 ml. Erlenmeyer flasks. Boxes containing cultures of M. pusillus were incubated at 25° and 50°; cultures of Rhizopus sp.III were incubated at 50° only.

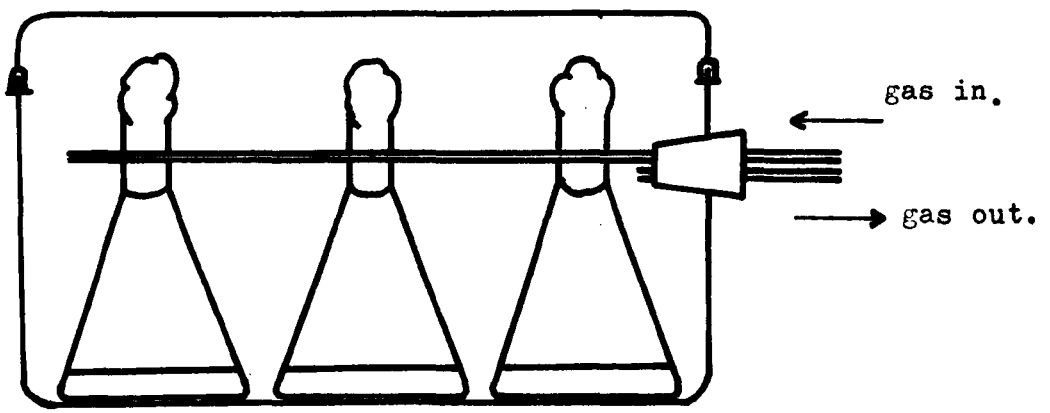


Fig. 29.

Artifical gas mixtures were made from cylinders of oxygen and nitrogen (British Oxygen Company) as follows:
Each cylinder was connected by rubber tubing to a Dreschel bottle (Fig. 30) and the gas flow, which could be regulated using a Hoffman clip (Gallenkamp List No. CP-240) positioned between gas cylinder and Dreschel bottle, was measured by opening or closing the Hoffman clip until the required number of gas bubbles were passing through the Dreschel bottle. Precautions were

taken to ensure that all the Dreschel bottles were of the same design and that the incoming tube in each bottle was submerged beneath the same level of water. After passage through the Dreschel bottles, nitrogen and oxygen gas flows were merged using a T-piece, and the synthetic gas mixture led into the appropriate lunch boxes.

By combining oxygen and nitrogen in different ratios the following gas mixtures were made:

Gas flow through Dreschel bottle (Number of bubbles/min.).		% composition of Gas Mixture.	
Oxygen	Nitrogen	Oxygen	Nitrogen
60	15	80	20
30	45	40	60
15	60	20	80

Cultures were harvested in triplicate after suitable periods of incubation, the mycelial mats were removed and dried prior to extraction of lipid. The lipid was hydrolysed, and the fatty acid methylated and analysed by GLC.

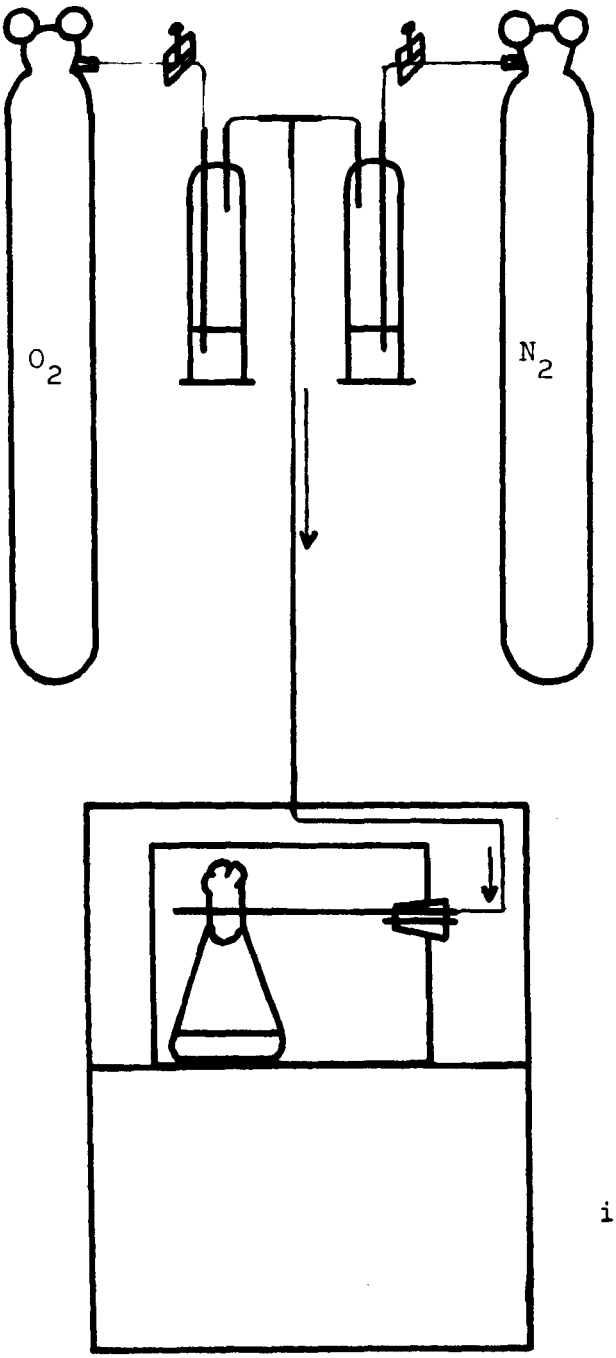


Fig. 30. Production of a synthetic gas mixture and introduction into the gas phase surrounding the culture vessels. (⟶ denotes direction of gas flow).

7.11.2 Results. Data for the growth, lipid synthesis and fatty acid composition of M. pusillus are presented in Table 49, and for Rhizopus sp.III in Table 50.

In Rhizopus sp.III growth was slightly increased in atmospheres enriched with oxygen (Fig.32), while in M. pusillus increasing the oxygen concentration of the environment had no effect on growth.

In M. pusillus the lipids were invariably more unsaturated when grown at 25° than at 50°, irrespective of the oxygen concentration in the external environment (Fig.31). The effect of increasing the external oxygen concentration on the degree of unsaturation was very small, though in M. pusillus at 25° and 50°, and also in Rhizopus sp.III at 50°, there was a suggestion that the lipids of cultures grown in enriched oxygen were more unsaturated than those grown in 20% oxygen (Fig. 32).

It is thought that despite the provision of an atmosphere rich in oxygen outside the culture flask, diffusion of gases through the cotton wool plug was insufficient to raise the oxygen concentration of the cultures significantly. Unfortunately in this experiment the Astrup micro-equipment and oxygen electrode were not available and it was not possible to measure the pO_2 of culture media and calculate their oxygen concentration.

TABLE 49. The effect of different gaseous atmospheres on the growth and fatty acid composition of M. pusillus grown at 25° and 50°.

Atmosphere	OXYGEN : NITROGEN 1 : 4								OXYGEN : NITROGEN 2 : 3							
Temperature	25°				50°				25°				50°			
Incubation Period(days)	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12
Growth (mg.dry wt./ml.)	1.66	5.00	6.13	6.24	3.00	4.80	5.33	7.40	2.06	4.73	5.80	6.00	3.86	4.93	5.73	7.06
Lipid Yield (% of dry wt.)	13.2	33.8	45.3	23.0	6.0	29.0	39.6	19.4	22.4	30.8	44.5	19.3	17.1	25.0	30.5	16.8
Fatty Acids																
14.0	2.0	1.4	1.5	1.6	2.0	1.5	1.6	1.8	1.5	1.1	1.3	1.8	1.4	1.2	1.6	1.8
16.0	25.1	30.0	28.1	25.5	29.2	33.1	30.4	29.8	27.0	22.0	24.4	26.6	28.2	30.2	30.6	28.1
16.1	3.7	2.4	3.1	4.2	4.0	3.7	3.6	4.5	2.8	4.0	3.8	4.1	4.9	5.8	2.5	3.0
18.0	6.8	10.1	9.0	7.8	4.2	6.2	5.1	5.0	10.3	12.4	11.3	9.0	4.5	6.6	5.6	5.5
18.1	43.4	38.6	38.7	39.0	44.0	41.2	44.1	42.8	39.9	41.0	38.2	36.4	41.3	41.3	42.2	43.9
18.2	14.8	15.2	15.3	17.0	14.0	12.3	13.2	13.7	13.4	16.5	16.8	17.7	15.2	12.7	14.6	14.8
18.3	4.1	3.2	4.5	4.7	2.6	2.0	2.0	2.2	5.1	4.0	4.2	4.4	2.9	1.9	2.5	2.5
Degree of Unsaturation	0.82	0.81	0.86	0.92	0.84	0.75	0.80	0.81	0.85	0.90	0.88	0.89	0.85	0.78	0.82	0.84

Atmosphere	OXYGEN : NITROGEN 4 : 1							
Temperature	25°				50°			
Incubation Period(days)	3	6	9	12	3	6	9	12
Growth (mg.dry wt./ml.)	1.53	4.65	6.06	6.53	3.80	4.80	6.00	7.40
Lipid Yield (% of dry wt.)	7.8	27.4	38.4	22.8	16.4	17.9	40.0	14.2
Fatty Acids								
14.0	1.1	1.1	1.0	1.4	1.1	1.0	1.5	1.8
16.0	27.7	23.6	26.4	27.6	32.2	25.1	30.5	29.4
16.1	4.8	3.5	3.0	4.4	5.4	3.5	3.7	5.9
18.0	6.7	10.4	6.4	8.8	4.9	7.3	6.0	5.4
18.1	40.3	42.3	43.4	37.8	39.7	44.1	43.2	40.8
18.2	15.6	16.3	16.6	15.4	14.0	16.0	13.8	13.5
18.3	3.7	3.3	3.6	4.4	2.5	2.9	1.5	3.2
Degree of Unsaturation	0.87	0.88	0.90	0.86	0.80	0.88	0.79	0.83

TABLE 50 . The effect of different gaseous atmospheres on the growth and the fatty acid composition of the lipids of a thermophilic Rhizopus sp.

Atmosphere	Oxygen : Nitrogen 1 : 4			Oxygen : Nitrogen 2 : 3			Oxygen : Nitrogen 4 : 1		
Incubation Period (days).	3	6	9	3	6	9	3	6	9
Growth (mg. dry wt./ml.).	2.53	4.46	5.00	2.66	4.92	5.00	2.86	5.32	5.25
Lipid Yield (% of dry weight).	25.2	30.8	41.6	19.6	29.8	33.5	21.9	33.0	39.0
Fatty Acids									
14.0	2.3	1.2	1.5	1.4	1.1	1.5	1.4	1.8	1.0
16.0	31.5	35.6	30.0	31.6	31.8	30.5	30.3	32.6	29.5
16.1	3.0	3.7	3.8	3.7	4.4	3.4	4.3	2.3	4.5
18.0	8.7	7.7	9.1	6.4	5.7	7.5	6.9	7.9	4.7
18.1	36.9	38.0	43.1	37.2	40.0	41.3	40.8	39.0	40.5
18.2	14.8	12.5	12.2	16.9	14.8	14.2	14.5	14.0	16.5
18.3	2.7	1.5	1.3	2.9	2.5	1.7	2.0	1.8	2.9
Degree of Unsaturation	0.77	0.71	0.75	0.83	0.81	0.78	0.80	0.75	0.86

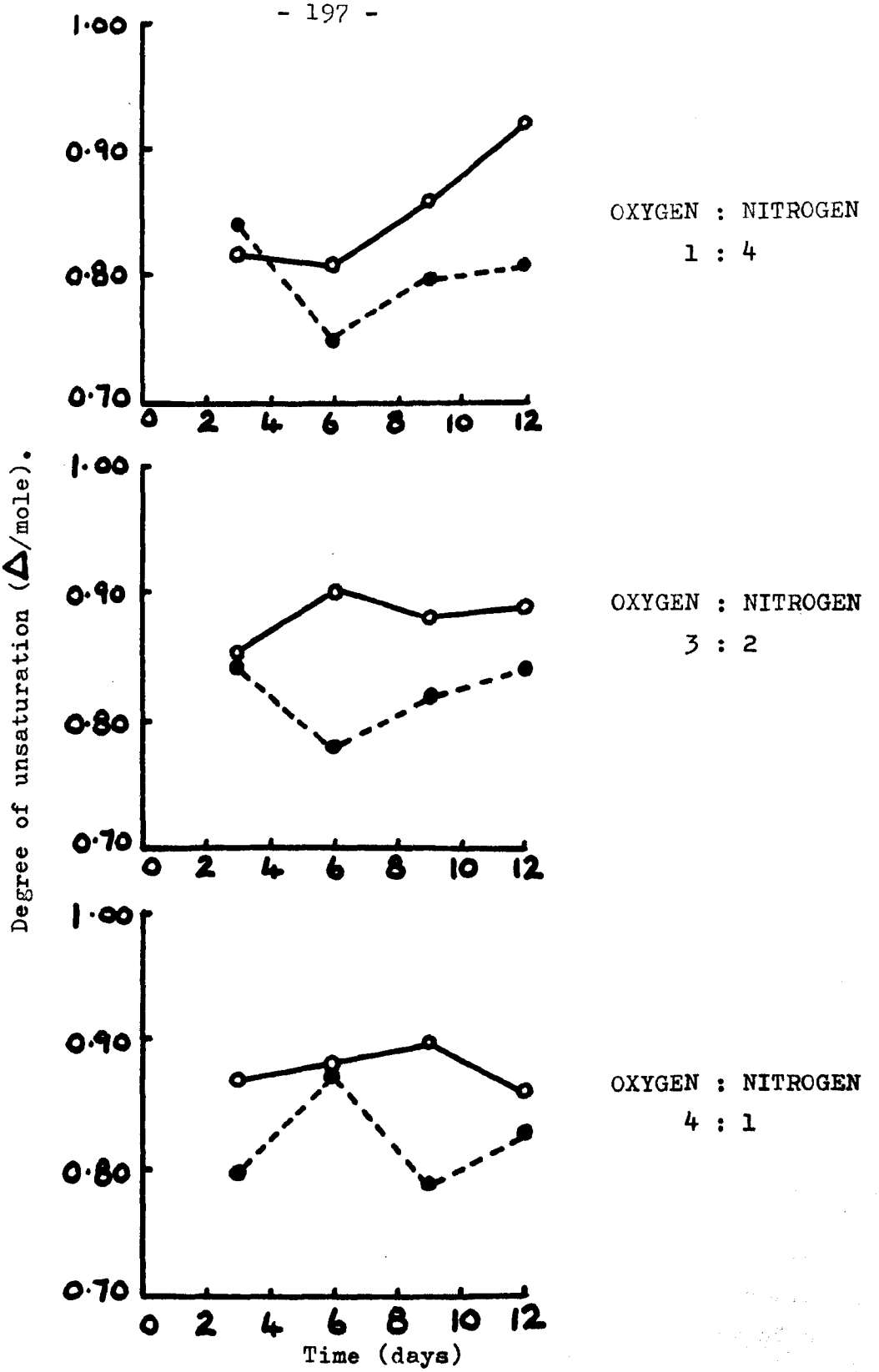


Fig. 31. Effect of different gaseous atmospheres on the degree of unsaturation of the lipids of M. pusillus grown at 25° (o---o) and 50° (o——o).

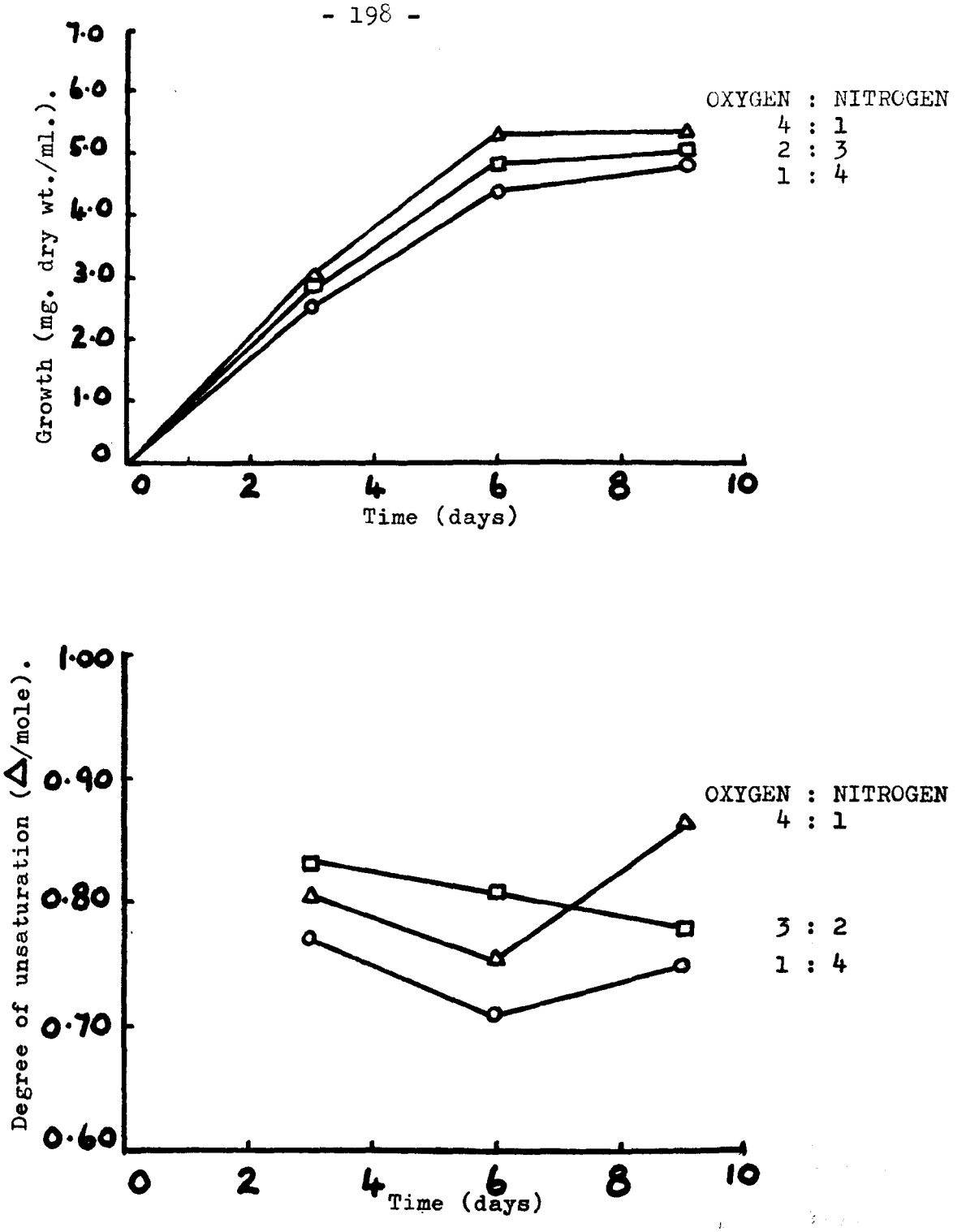


Fig. 32. Effect of different gaseous atmospheres on growth and the degree of unsaturation of the lipids of a thermophilic Rhizopus sp.

7.12 The Effect of Increased Oxygen Concentration within the Culture Flask.

In the previous experiment the effect of increasing the oxygen concentration of the external environment resulted in very small increases in lipid unsaturation. In this experiment the effect of increasing the oxygen concentration within the culture flask on the degree of unsaturation of the lipids was investigated.

7.12.1 Experimental Procedure. The basic medium plus glucose (20 g./l.), ammonium sulphate (250 mg. N/l.) and sodium succinate (5 g./l.) was dispensed in 400 ml. quantities into 1000 ml. Erlenmeyer flasks (Quickfit FE.IL/3) with B.24 ground glass necks. The flasks were plugged with cotton wool and sterilised by autoclaving at 15 lb./in.^{2} for 15 mins. Each flask was inoculated with 10 ml. of a standard spore suspension of M. pusillus.

In order to aerate cultures, a Dreschel bottle head (Quickfit MF.48) was inserted into the neck of each culture flask (Fig. 33). Culture vessels which were to be aerated with the same gas mixture were connected in series by rubber tubing. The gas flow into each flask was sterilised by the use of sterile

cotton-wool filters. The Dreschel bottle heads, cotton wool filters and rubber gas lines were assembled and sterilised by wrapping in aluminium foil and autoclaving at 15 lb./in.^{2} for 15 mins. before inserting the Dreschel bottle heads into the inoculated culture flasks.

Synthetic gas mixtures were made as described in Section 7.11.1. The following treatments were used:

(1) Culture vessels plugged with cotton wool, incubated at 25°.
(2) " " " " " " " " 50°.
(3) " " aerated by 20% O_2, 80% N_2, " " 50°.
(4) " " " " 40% O_2, 60% N_2, " " 50°.
(5) " " " " 70% O_2, 30% N_2, " " 50°.

The rates of aeration in treatments 3, 4 and 5 were the same. For each treatment 8 replicate flasks were used; in treatments 3, 4 and 5 these were connected in series by rubber tubing (Fig. 34). Cultures were harvested in duplicate after 3, 6, 10 and 14 days incubation at the specified temperature.

Mycelial mats were removed, dried and the lipid extracted. The lipid was hydrolysed, and the resulting fatty acids methylated and analysed by GLC.

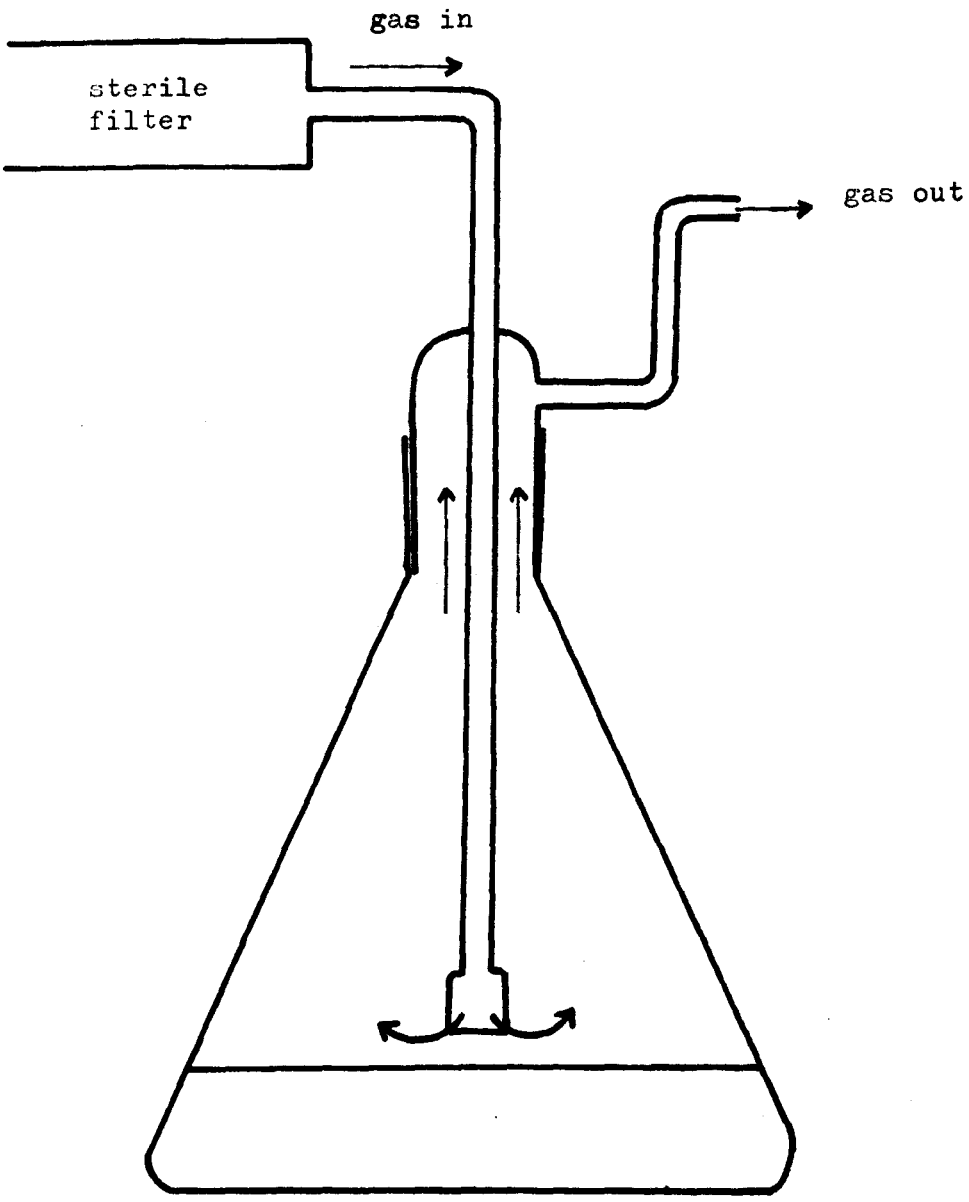


Fig. 33. Passage of gas over surface of growing cultures using a Dreschel bottle head.

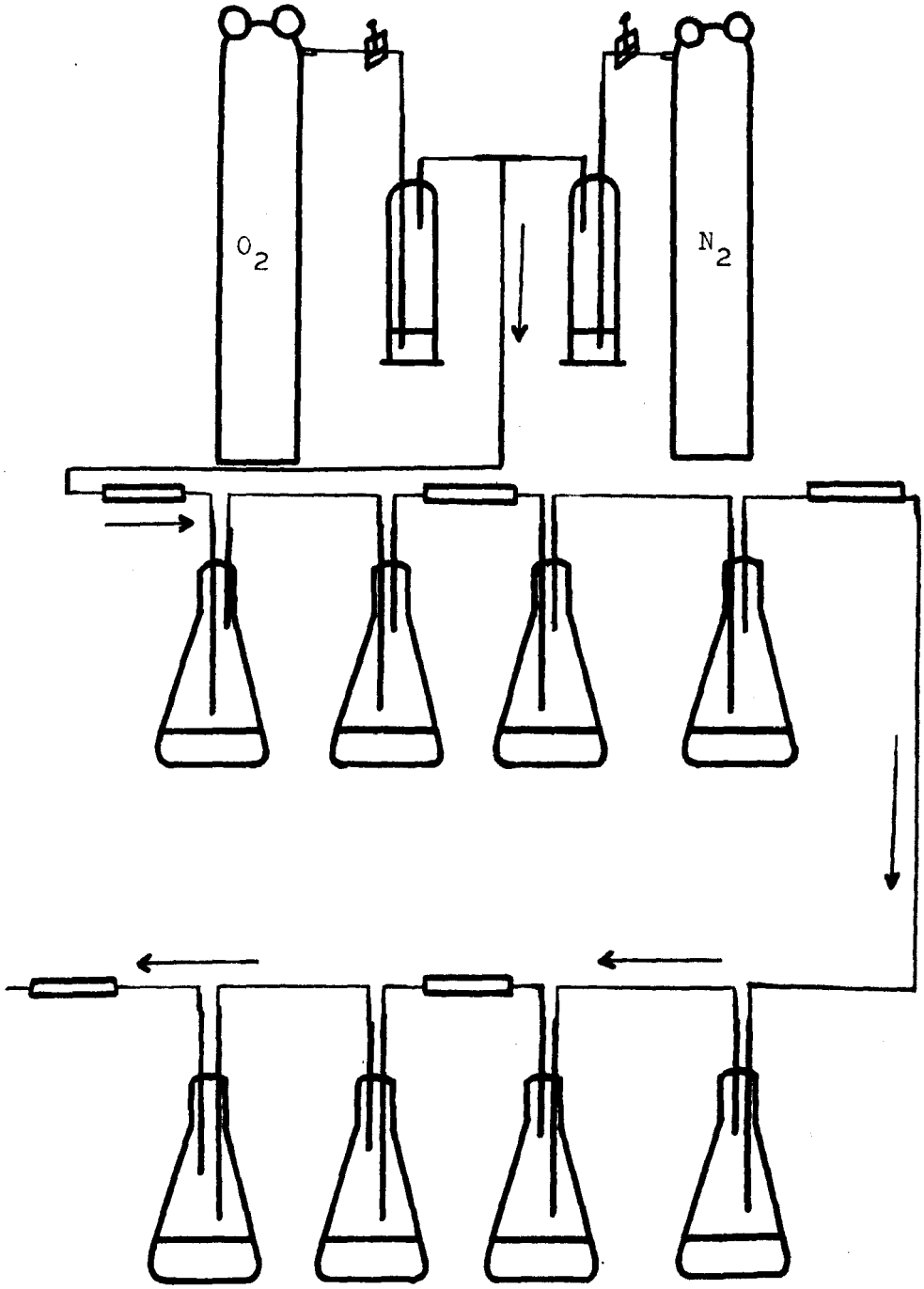


Fig. 34. Production of a synthetic gas mixture and introduction into the gas phase above growing cultures of **M. pusillus**. (⟶ direction of gas flow).

7.12.2 Results. Data for the growth, lipid synthesis and fatty acid composition of M. pusillus in different conditions of aeration are presented in Table 51.

The passage of a stream of gas over the surface of the fungal mat had a small effect on the degree of unsaturation of the mycelial lipids, though this effect persisted throughout the growth cycle. When grown at 50° the lipids of aerated cultures were slightly more unsaturated than those of unaerated cultures grown at the same temperature. Of the three gas mixtures tested, the highest degree of lipid unsaturation was obtained in cultures aerated with a synthetic mixture containing 40% oxygen and 60% nitrogen (Fig. 35).

Determination of the pO_2 and calculation of the oxygen concentration in media of 14 day cultures (Table 52) showed that at 50° the medium of both aerated and unaerated cultures was almost completely devoid of oxygen; the medium of unaerated cultures grown at 25° was also oxygen-deficient though not to the same extent as 50° cultures.

It is clear therefore that the mycelial mat reduces the rate of gaseous diffusion between the atmosphere and culture medium to a level which is insufficient to balance the rate at which oxygen is utilised from the medium and may be rate-limiting

TABLE 51 . The effect of aeration on the growth, lipid yield and fatty acid composition of M. pusillus grown at 25° and 50°.

Culture Conditions.	Non-Aerated				Non-Aerated				Aerated				Aerated				Aerated			
Atmosphere	Air				Air				Oxygen 20% : Nitrogen 80%				Oxygen 40% : Nitrogen 60%				Oxygen 70% : Nitrogen 30 %			
Incubation Temperature	25°				50°				50°				50°				50°			
Incubation Period (days)	3	6	10	14	3	6	10	14	3	6	10	14	3	6	10	14	3	6	10	14
Growth (mg.dry wt./ml.).	0.21	2.22	3.56	4.32	2.85	3.19	3.71	3.75	1.30	2.50	3.00	3.66	1.53	2.71	2.96	4.20	1.73	2.16	3.39	4.15
Lipid Yield (% of dry wt.).	36.8	34.6	24.1	15.8	24.3	16.0	13.0	3.7	22.7	17.2	8.8	1.9	26.3	20.3	9.1	3.2	19.3	12.8	7.4	3.0
Fatty acid																				
14.0	1.5	1.4	1.1	1.8	1.2	1.5	1.1	1.6	1.3	1.4	1.2	1.4	1.2	1.4	1.1	1.5	1.2	1.5	1.5	1.4
16.0	30.2	26.5	27.5	28.6	29.9	31.3	31.3	28.6	29.3	28.5	28.0	29.5	29.4	28.0	26.3	28.8	30.7	30.1	30.7	27.7
16.1	2.8	3.1	3.1	2.8	2.8	2.4	3.1	3.6	4.2	3.5	2.8	2.0	1.6	2.3	2.1	2.0	3.1	2.8	2.1	1.8
18.0	5.0	2.7	5.0	3.4	4.8	3.5	4.0	3.9	4.6	3.8	3.5	3.1	4.8	4.2	2.6	3.4	4.0	3.4	3.4	3.6
18.1	39.4	39.4	37.8	38.0	36.6	40.0	38.3	39.7	36.8	38.6	38.6	37.5	38.0	37.3	40.5	38.8	36.8	41.0	41.0	42.5
18.2	19.3	22.7	20.2	21.8	20.2	17.7	17.4	18.3	17.6	19.1	20.9	22.8	20.9	22.5	21.6	22.0	18.4	17.2	17.5	18.6
18.3	2.8	4.2	5.5	3.7	4.5	3.7	4.9	4.2	6.2	5.2	4.9	3.8	3.6	3.7	5.8	3.6	5.8	4.6	4.1	4.1
Degree of Unsaturation	0.89	0.97	0.98	0.96	0.93	0.89	0.91	0.92	0.95	0.96	0.98	0.95	0.92	0.96	1.03	0.96	0.94	0.92	0.91	0.94

TABLE 52 . Determination of the Partial Pressure ($p O_2$) of Cultures of M. pusillus grown under different conditions of aeration for 14 days at 25° and 50°.

Culture Conditions.	Atmosphere.	Temperature.	Replicate $p O_2$ determination. (mm. Hg).	Mean $p O_2$ (mm. Hg).	O_2 concentration. (μ M O_2/l.H_2O).
Non-aerated	Air	25°	45, 28, 36.	36	58
Non-aerated	Air	50°	10, 18, 13	13	14
Aerated	20% O_2, 80% N_2	50°	10, 12	11	12
Aerated	40% O_2, 60% N_2	50°	14, 14	14	15
Aerated	70% O_2, 30% N_2	50°	13, 13	13	14

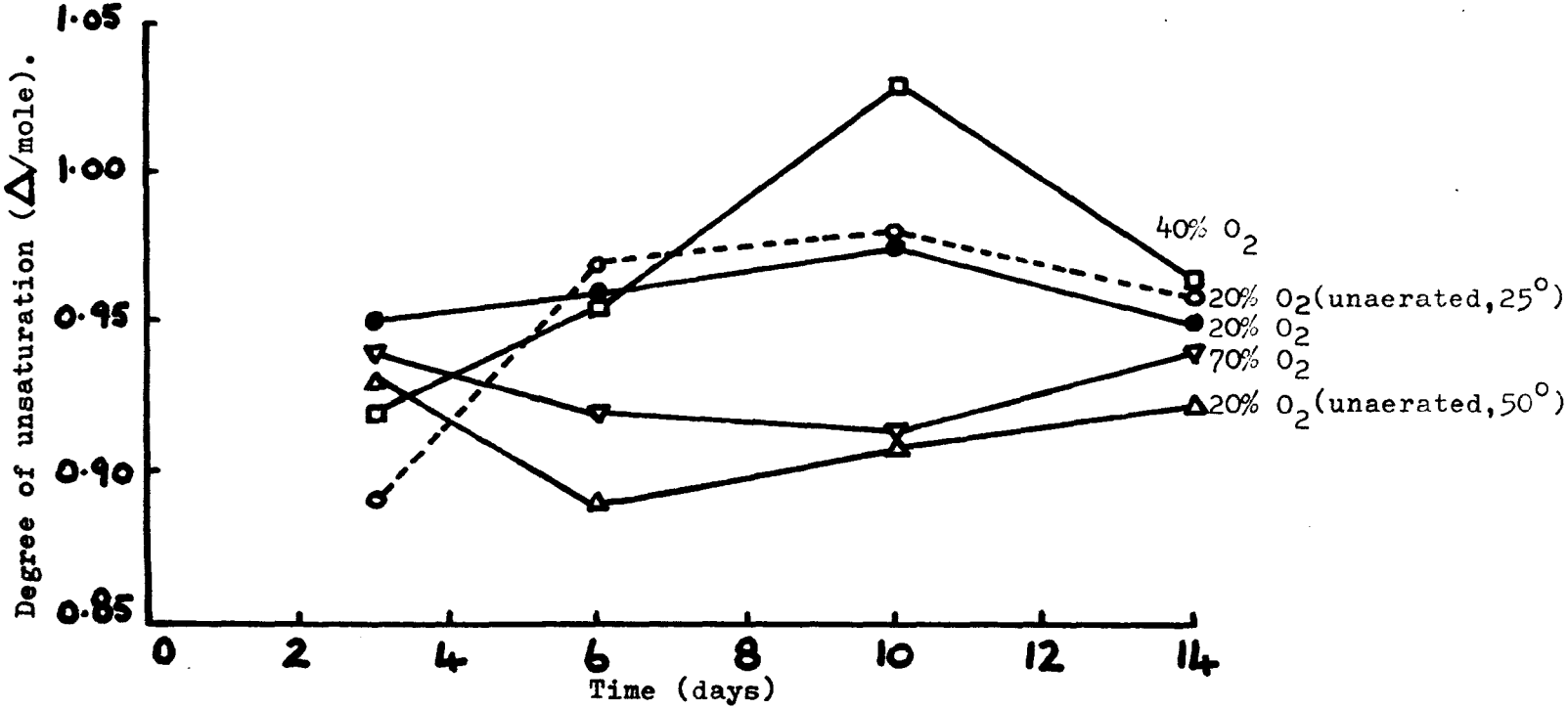


Fig. 35. Effect of different oxygen concentrations on the degree of unsaturation of the lipids of M. pusillus grown at 25° (o---o) or 50° (——).

for the fatty acid desaturation reaction.

With one exception, in all the experiments described in Chapters 6 and 7, the fungi were grown in still culture and developed as a tough mycelial mat. Determination of the pO_2 showed that the oxygen concentration of the medium was reduced in cultures which covered the surface of the medium; reduction of the oxygen concentration was particularly marked in cultures grown at a high temperature.

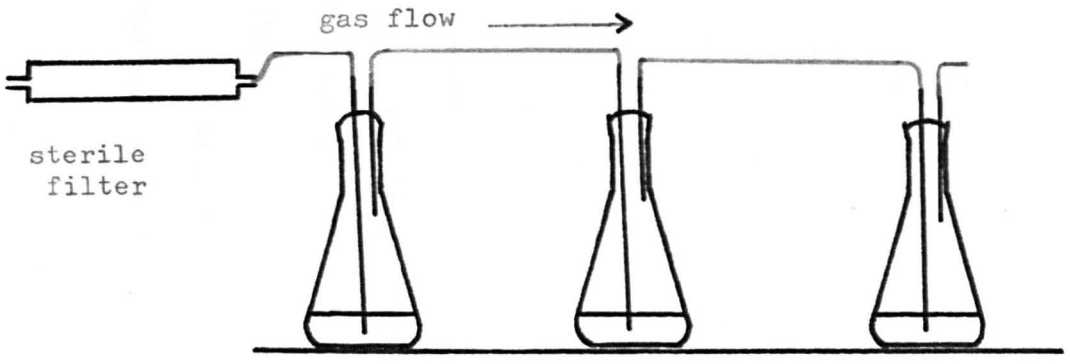
In still culture, therefore, the mycelial mat grows under conditions which are physiologically heterogeneous - the aerial part of the mat growing in aerobic conditions and the submerged part growing in increasingly anaerobic conditions.

Several experiments were set up, the aim in each case being to study the effect of different concentrations of oxygen on the degree of unsaturation of the lipids of M. pusillus grown under physiologically homogeneous conditions. Since none of the methods tried proved satisfactory, the methodology will be only briefly summarised. In those experiments where culture flasks aerated with gas of the same composition were arranged in series (Fig. 36 (i) and (ii)) the fungus grew into the aeration tubes causing a blockage of the gas supply and a consequent blow-out. A glass fermenter was built (Fig. 36 (iii)) from a Quickfit

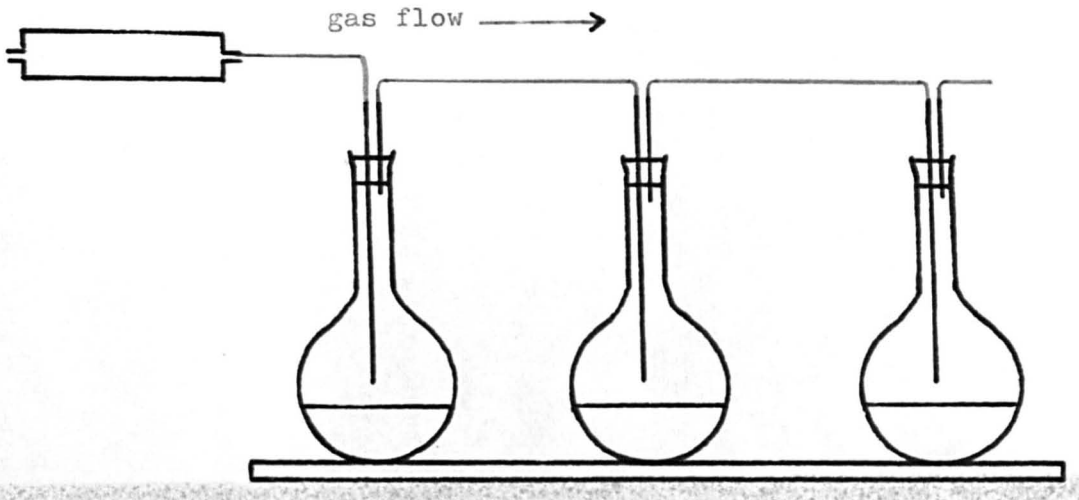
multisocket lid (MAF 2/2) joined by ground glass surfaces to a Quickfit culture vessel (F.V.20 L.). Agitation was provided by an overhead motor driving a Quickfit link stirrer (St.1/1) and air pumped into the fermenter through a sterile cotton wool filter. After sterilisation the fermenter was lowered into a water bath maintained at 50°. In an effort to obtain growth of M. pusillus under fermentation conditions several types of inocula were tried (spore suspension, homogenised mycelial mats, and shake culture mycelium) as were different rates of aeration and agitation. So long as agitation was continued there was little or no growth, but as soon as stirring and aeration were abandoned the fungus grew as a thick surface mat.

All efforts to culture M. pusillus in physiologically homogeneous conditions failed and though it seems possible from still culture experiments that aeration may affect the degree of lipid unsaturation, the physiological heterogeneity of the still culture system probably precludes any wide differences in lipid unsaturation under different oxygen concentrations in the atmosphere above the medium.

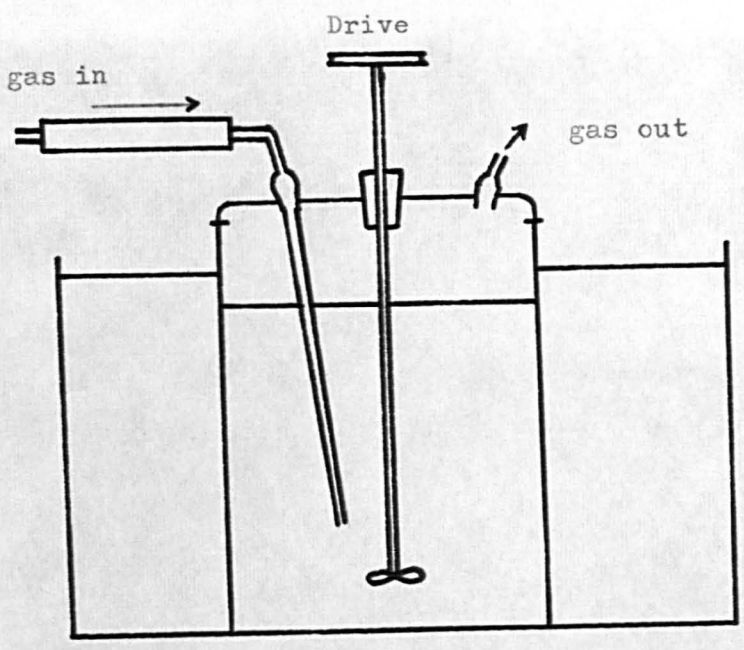
A further study is envisaged on the effect of oxygen concentration on the degree of unsaturation of the lipids of thermophilic fungi grown in fermentation conditions and since mucoraceous fungi are exceptionally difficult to grow in a fermenter it will be necessary to investigate other fungal groups to find a suitable organism.



(i) Aerated,
still culture.



(ii) Aerated,
shake culture.



(iii) Aerated, stirred,
deep culture.

Fig. 36. Methods used for growth of M. pusillus in physiologically homogeneous conditions.

CHAPTER 8. DISCUSSION.

Those organisms which lack extensive tissue differentiation are grouped together in the Protista. The Protista comprises two sub-groups, the lower protists which include bacteria and blue-green algae, and the higher protists - algae, protozoa and fungi (Stanier et al. 1963). Since the advent of gas-liquid chromatography the procedure of fatty acid analysis has become fairly straightforward, with the result that the fatty acids of many protists have been analysed and some correlation with taxonomic classification has become possible (see reviews of Kates 1964, 1966, Lennarz 1966, Shaw 1966a).

In general the lower protists are incapable of synthesising polyunsaturated (polyenoic) fatty acids, the lipids of bacteria and blue-green algae typically containing only saturated and monoenoic fatty acids. One exception is the highly evolved filamentous blue-green alga Anabaena variabilis which synthesises both dienoic and trienoic fatty acids (Levin et al. 1964). The higher protists on the other hand, characteristically synthesise polyenoic fatty acids, which may contain up to six double bonds.

Four main classes of fatty acids have been found in the bacteria: saturated straight-chain, branched-chain and cyclopropane

acids, and straight-chain unsaturated acids. Gram-positive bacteria contain branched-chain acids, often as a major proportion of the total fatty acid; branched-chain acids are found only in trace quantities in Gram-negative bacteria which typically contain saturated, monoenoic and cyclopropane fatty acids. In contradistinction, the higher protists generally contain only two main classes of fatty acids: straight-chain saturated, and unsaturated acids, though branched-chain acids have been reported in trace quantities in ciliate protozoa (Erwin and Bloch 1963). No report could be found in the literature of the occurrence of cyclopropane acids in higher protists.

In the higher protists the ability to synthesise polyunsaturated fatty acids is important taxonomically. Algae and protozoa, which synthesise fatty acids containing up to six double bonds, differ from the fungi which do not synthesise acids containing more than four double bonds. Phylogenetically, evolution of the more advanced protists away from the algal common stock is associated with an increasingly limited ability to synthesise polyunsaturated fatty acids (Fig. 37).

The polyenoic fatty acids of protists have been separated into two groups, an ω 3 group and an ω6 group (Korn et al. 1965). In the ω3 group, fatty acids have a double bond 3 carbon atoms away from the ω carbon atom, e.g. α-linolenic acid (9, 12,

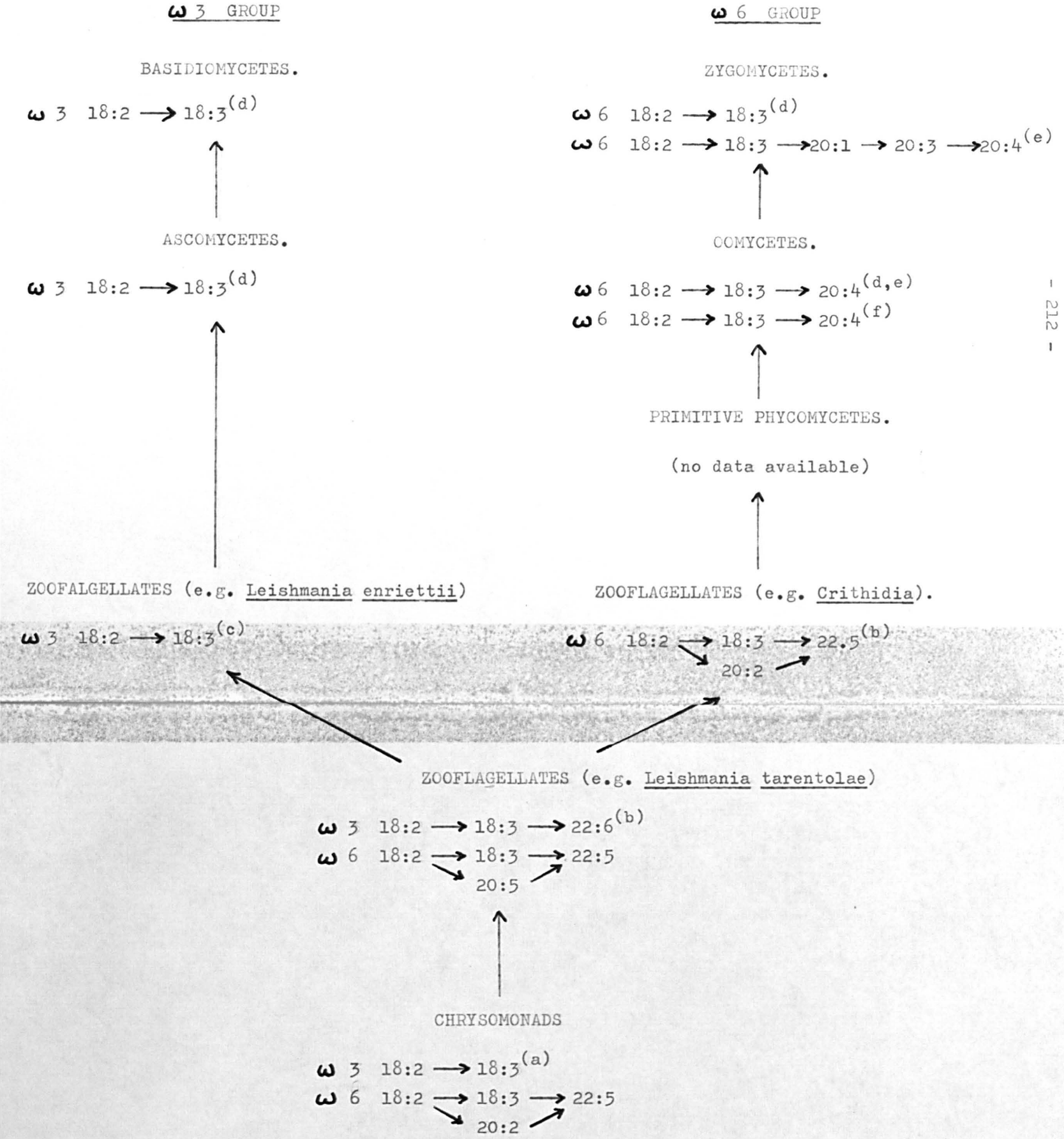


Fig.37. Suggested phylogenetic relationship of some protist groups based on the schemes of Erwin et al. (1964) and Shaw (1966a), and incorporating the results of (a) Haines et al. (1962), (b) Korn et al. 1965, (c) Korn and Greenblatt (1963), (d) Shaw (1965), (e) Tyrrell (1967), (f) Bowman and Mumma (1968).

15-octa-decatrienoic acid); fatty acids of the ω6 group have the extreme methyl-directed double bond 6 carbon atoms from the ω atom e.g. γ-linolenic acid (6, 9, 12-octadecatrienoic acid). In the more advanced protists (fungi, and amoeboid, ciliate and some zooflagellate protozoa) the polyunsaturated fatty acids are of only one type, i.e. either the ω3 or the ω6 group. The more primitive protists however (chrysomonads and some zooflagellate protozoa) retain the ability to synthesise fatty acids of both ω3 and ω6 groups (Fig. 37).

In the fungi, there appear to have been two separate lines of development from common zooflagellate ancestors. One line extends from zooflagellates resembling Leishmania enriettii to ascomycete and basidiomycete fungi. The other line extends from zooflagellates resembling Crithidia, through unicellular Phycomycetes and mycelial oomycete Phycomycetes, to mycelial zygomycete Phycomycetes. All the phycomycete fungi so far examined have been found to contain γ-linolenic acid as opposed to α-linolenic acid, and it seems probable that in these fungi all polyunsaturated acids are of the ω6 type, though no data could be found in the literature regarding the positions of the double bonds of the C20 and C22 polyenoic fatty acids.

Evolution of phycomycete fungi from zooflagellate ancestors has involved a reduction in the ability to synthesise certain long-chain polyenoic fatty acids (Fig. 38). In the more advanced Phycomycetes, the Zygomycetes, fatty acid biosynthesis terminates with linolenic acid (18:3) in the Mucorales and with arachidonic acid (20:4) in the Entomophthorales; oomycete fungi however, are able to synthesise C22 polyenoic acids in addition to linolenic and arachidonic acids. It should be pointed out, however, that so far only the mycelial Phycomycetes have been examined; an analysis of the fatty acid composition of some primitive unicellular Phycomycetes would clearly be of interest from a phylogenetic point of view.

In the last decade, during which fatty acid analysis has come to play an increasingly important role in the taxonomy of micro-organisms, studies on fatty acid biosynthesis have also progressed rapidly, and the subject has been extensively reviewed (Asselineau and Lederer 1960, Wakil 1962, Mead 1963 and Reeves et al. 1967).

It is now known that in fatty acid metabolism the synthetic pathway is distinct from the degradative (β-oxidation) pathway; in the anabolic process NADPH and the D(-)-β-hydroxy

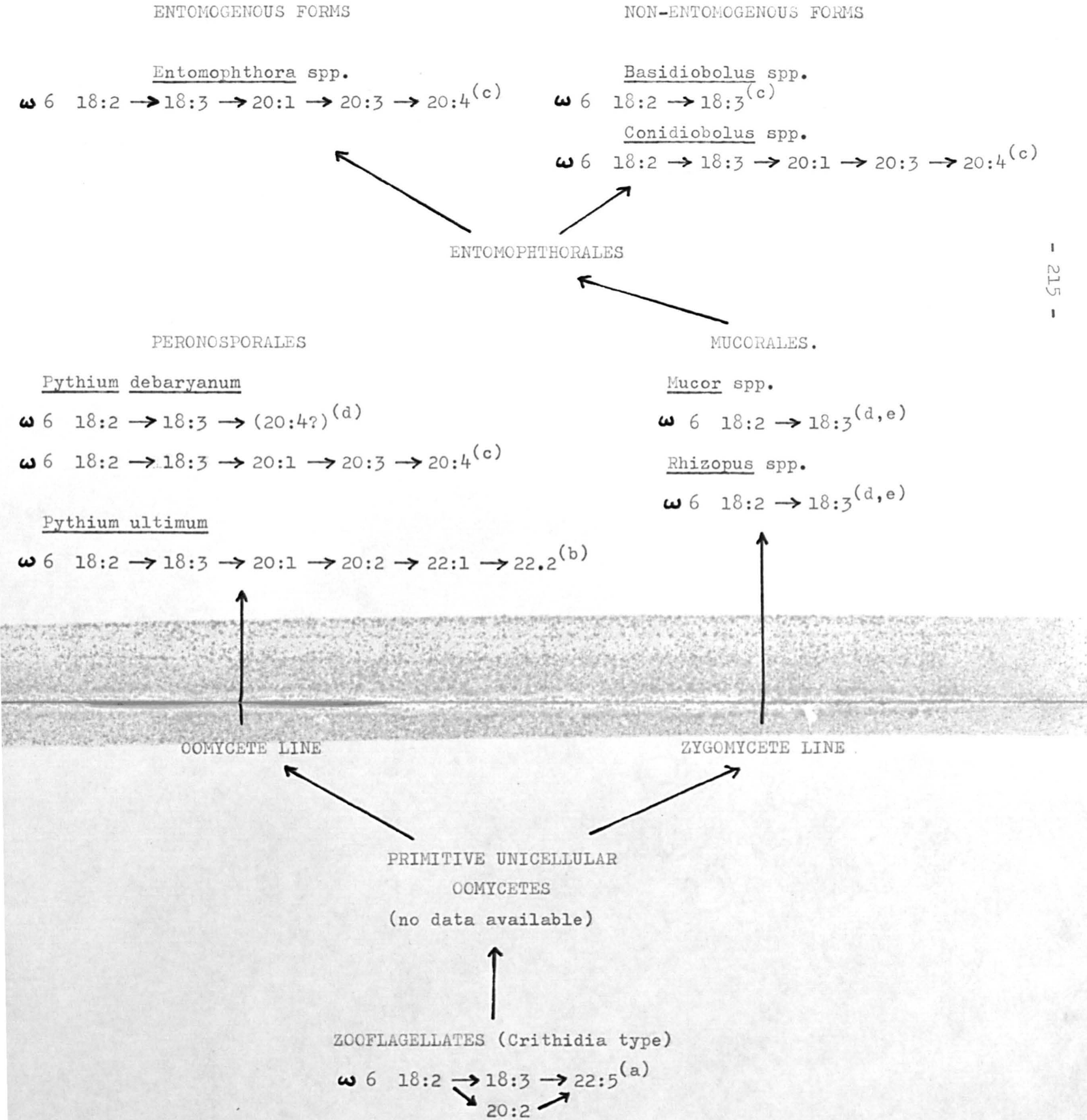


Fig. 2. Suggested lines of evolution in the Phycomycetes, based upon Scheme proposed by Bessey (1950) and incorporating the results of (a) Korn et al.(1965), (b) Bowman and Mumma (1968), (c) Tyrrell (1967), (d) Shaw 1965, (e) Sumner, Morgan and Evans (in press).

acids are involved, while in the catabolic reactions NAD and the L(+)-β-hydroxy acids are involved (Wakil et al. 1964).

The biosynthesis of even-numbered, saturated fatty acids occurs by the malonyl coenzyme A pathway, the overall reaction for the synthesis of palmitic acid being:

$$CH_3CO\text{-}SCoA + 7\ HOOCCH_2CO\text{-}SCoA + 14\ NADPH + 14\ H^+ \longrightarrow \quad 1.$$

$$CH_3(CH_2)_{14}COOH + 7\ CO_2 + 8\ CoA\text{-}SH + 14\ NADP^+ + 6H_2O.$$

In outline, fatty acid biosynthesis involves the initial condensation of one acetyl and one malonyl unit, followed by the stepwise addition of the appropriate number of malonyl units to give the required long-chain acid. The synthesis of malonyl CoA from acetyl CoA is catalysed by the biotin-dependent enzyme, acetyl CoA carboxylase:

$$HCO_3^- + ATP + CH_3CO\text{-}SCoA \xrightarrow{Mn^{++}} {}^-OOCCH_2CO\text{-}SCoA + ADP + P_i \qquad 2.$$

The reactions involved in the addition of each two-carbon unit to the fatty acid chain are catalysed by fatty acid synthetase enzymes. These reactions have been extensively studied in E. coli (Goldman 1964, Alberts et al. 1964, 1965, Majerus et al. 1965, Vagelos et al. 1965) and are shown in Fig.39.

$$\text{acetyl-S-CoA} + \text{ACP-SH} \xrightleftharpoons{\text{acetyl transacylase}} \text{acetyl-S-ACP} + \text{CoA-SH} \qquad 3$$

$$\text{malonyl-S-CoA} + \text{ACP-SH} \xrightleftharpoons{\text{malonyl transacylase}} \text{malonyl-S-ACP} + \text{CoA-SH} \qquad 4$$

$$\text{acetyl-S-ACP} + \text{malonyl-S-ACP} \xrightleftharpoons{\beta\text{-ketoacyl-ACP synthetase}} \text{acetoacetyl-S-ACP} + CO_2 + \text{ACP-SH} \qquad 5$$

$$\text{acetoacetyl-S-ACP} + \text{NADPH} + H^+ \xrightleftharpoons{\beta\text{-ketoacyl-ACP reductase}} \text{D-B-hydroxybutyryl-S-ACP} + NADP^+ \qquad 6$$

$$\text{D-B-hydroxybutyryl-S-ACP} \xrightleftharpoons{\text{enoyl-ACP hydrase}} \text{crotonyl-S-ACP} + H_2O \qquad 7$$

$$\text{crotonyl-S-ACP} + \text{NADPH} + H^+ \xrightleftharpoons{\text{enoyl-ACP reductase}} \text{butyryl-S-ACP} + NADP^+ \qquad 8$$

Fig. 39. Reactions in the biosynthesis of long-chain saturated fatty acids in E. coli.

It will be seen from Fig. 39 that the intermediates remain bound to a protein molecule, called "acyl carrier protein" (ACP). The concept that intermediates of fatty acid metabolism might form protein-bound complexes was originally proposed by Lynen (1961, 1963) and stemmed from his inability to demonstrate free intermediates in fatty acid synthesis in Saccharomyces cerevisiae. Lynen (1961, 1964) provided positive evidence for an intermediate-protein complex by showing that purified yeast fatty acid synthetase catalysed the condensation of acetyl CoA and malonyl CoA to form a protein-acetoacetate complex. The nature of the protein or proteins involved in the binding of intermediates of fatty acid synthesis in yeast have not been characterised. In bacteria however, a "protein derivative" of CoA, acyl carrier protein (ACP), has been found to be the thioester compound to which all the intermediates of fatty acid synthesis are linked (Marjerus et al. 1964). The acyl carrier protein has also been shown to be involved in fatty acid synthesis in plant and animal systems (Overath and Stumpf 1964, Alberts et al. 1964, Wakil et al. 1964, Brooks and Stumpf 1965).

Studies on the fatty acid synthesising systems of E.coli and Clostridium kluyveri have revealed that ACP, which is

readily fractionated from the synthetase enzymes, is stable in 0.1N HCl and boiling water (Lennarz et al. 1962, Alberts et al. 1963), unaffected by DNAase and RNAase, but destroyed by trypsin and chymotrypsin (Alberts et al. 1963). The purified protein has a molecular weight of between 9,000 and 10,000 (Majerus et al. 1964). The prosthetic group has been identified as 4' phosphopantetheine, which is linked to the protein via a phosphodiester linkage to the hydroxyl group of serine (Majerus et al. 1964) (See Fig. 40). Despite the obvious structural similarities between ACP and CoA (c.f. Fig. 41), fatty acid synthetases exhibit a marked specificity for ACP thioesters as opposed to CoA thioesters.

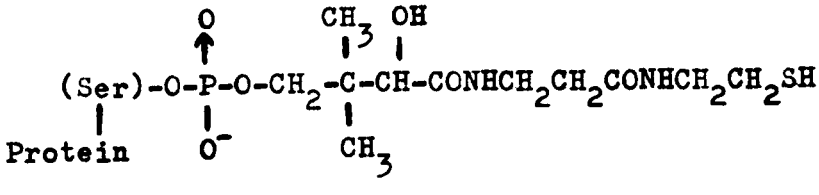


Fig. 40. Structure of the prosthetic group of acyl carrier protein.

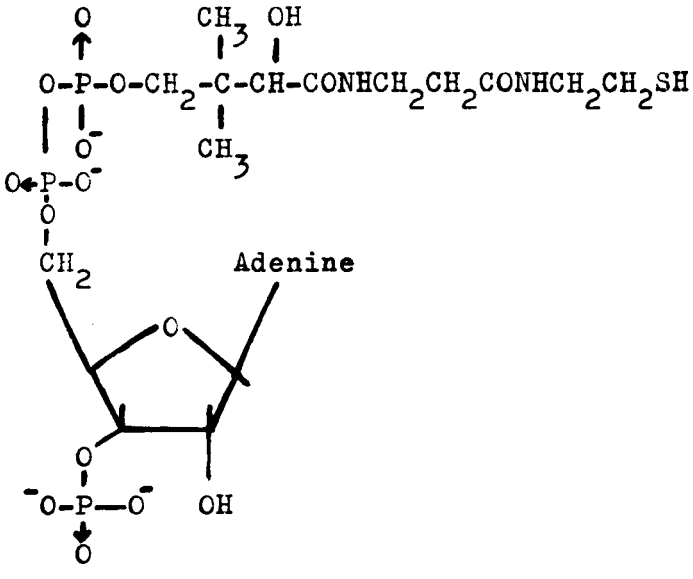


Fig. 41 Structure of Coenzyme A.

In contrast to the fatty acid synthetases from E. coli and C. kluyveri, which have been resolved into various components, the synthetases from S. cerevisiae and pigeon liver have resisted fractionation. As a result of the studies of Lynen and co-workers over the last decade (summarised by Lynen 1967) it is known that the fatty acid synthesising system of yeast comprises a homogeneous, multienzyme complex with a molecular weight of 2.3 million. The system characteristically requires acetyl CoA as a "primer" for fatty acid synthesis; the acetyl

residue forms the methyl end of the fatty acid indicating that the two-carbon units from malonyl CoA are added to the acetyl residue during the synthetic reaction. Homologous, saturated acyl CoA esters were found to replace acetyl CoA as a 'primer', but the oxidised products of the fatty acid degradation process could not prime the synthetic process.

Lynen found that the transformation of malonyl CoA into fatty acids was achieved through intermediates that are covalently bound to thiol groups on the synthetase enzyme. There are two different types of thiol group, designated 'central' and 'peripheral' groups according to their position on the synthetase molecule.

The synthetic process is initiated by the transfer of an acetyl residue to the peripheral thiol group, and the transfer of a malonyl residue to the central thiol group. The next step is a condensation reaction resulting in the formation of an acetoacetyl residue bound to the central thiol group of the enzyme. The β-keto acid residue then undergoes a reduction involving NADPH to form D(-)-β-hydroxybutyryl - enzyme, followed by dehydration to crotonyl-enzyme, and then a second NADPH-linked reduction to form butyryl-enzyme. The butyryl group is transferred to the peripheral thiol group, liberating the

central thiol group for acceptance of the next malonyl residue. The reaction cycle can then proceed again and in this way the fatty acid chain is extended to the appropriate length. In the terminal reaction the long-chain fatty acid-enzyme complex is transferred from the central thiol group to CoA, with the formation of the appropriate acyl CoA and the regenerated enzyme.

Lynen has proposed a hypothetical structure for the multienzyme complex of yeast fatty acid synthetase in which seven different enzymes are arranged around a core containing the central thiol group (Fig. 42).

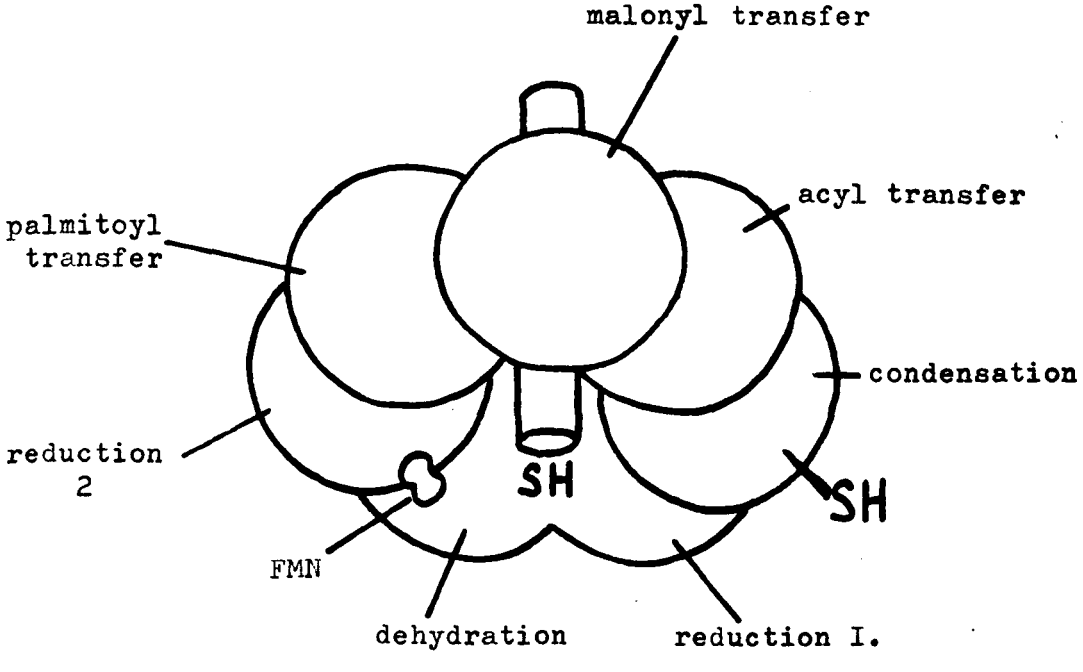


Fig. 42. Hypothetical structure of the multienzyme complex of fatty acid synthetase(Lynen 1967).

This implied high degree of structural order was confirmed by electron microscopy, which also suggested that the purified yeast enzyme may be composed of three sub units (Hagen and Hofschneider 1964). Tracer experiments which showed that the yeast synthetase contains 3 moles of 4'-phosphopantetheine covalently linked to one mole of enzyme (Wells et al. 1966) are taken to provide additional evidence for a three-subunit structure; these results also indicate that the central thiol group of yeast fatty acid synthetase may be analagous with the ACP of bacterial systems. Lynen envisages the 4'phosphopantetheine component as being able to rotate on the central core axis. In this way the acyl intermediates covalently bound to the central thiol group could be conveniently moved from one active site to another on the surrounding enzymes.

There are two distinct mechanisms by which unsaturated fatty acids are synthesised; one mechanism requires molecular oxygen, while the other proceeds anaerobically, acyl carrier protein being involved in both mechanisms.

In the anaerobic pathway, which takes place in bacteria in the order Eubacteriales and Pseudomonadales, desaturation has been shown to occur at the C10 or C12 stages, while the fatty acid chain is still being lengthened.

In the aerobic pathway which has been shown to take place in a small number of eubacteria and widely in members of the protist, plant and animal kingdoms, desaturation occurs after elongation of the fatty acid chain has been completed. In *S. cerevisiae* cell-free extracts have been shown to convert CoA esters of palmitate to palmitoleate, and of stearate to oleate (Bloomfield and Bloch 1961).

$$\underset{\text{palmityl CoA}}{CH_3(CH_2)_{14}CO\text{-}S\text{-}CoA} \xrightarrow{O_2,\,NADPH} \underset{\text{palmitoleyl CoA}}{CH_3(CH_2)_5CH{=}CH(CH_2)_7CO\text{-}S\text{-}CoA} \qquad 9.$$

$$\underset{\text{stearyl CoA}}{CH_3(CH_2)_{16}CO\text{-}S\text{-}CoA} \xrightarrow{O_2,\,NADPH} \underset{\text{oleyl CoA}}{CH_3(CH_2)_7CH{=}CH(CH_2)_7CO\text{-}S\text{-}CoA} \qquad 10.$$

Essentially similar cofactor requirements have been demonstrated in a number of different systems, for the conversion of monounsaturated to polyunsaturated fatty acids; the cofactors so far identified are oxygen, NADPH, CoA, ACP, and in isolated chloroplasts, ferredoxin (Mudd and Stumpf 1961, James 1963, Stumpf and James 1963, Nagai and Bloch 1966). The conversion of oleic to linoleic and linolenic acids has been demonstrated in

cell-free extracts of the fungus Torulopsis utilis (Yuan and Bloch 1961, Meyer and Bloch 1963), the green alga Chlorella vulgaris (Harris and James 1965), and in leaf preparations (James 1963) and isolated chloroplasts (Stumpf and James 1963).

Little is known of the mechanism by which aerobic desaturation proceeds, mainly because it has not been possible to purify the particulate desaturase enzymes. The suggestion that aerobic desaturation proceeds via a hydroxy-acid intermediate (Lennarz and Bloch 1960) has not been supported experimentally (Marsh and James 1962), and neither has the alternate mechanism suggested by Light et al. (1962) that oxygen may serve as an electron acceptor for the removal of hydrogen, without itself being involved in a covalent bond with the fatty acid.

The pathway by which fatty acids are converted to glycerol esters is shown in Fig.43. Esterification of glycerol-3-phosphate, rather than glycerol itself, results in the formation of phosphatidic acid which is important in the biosynthesis of both triglycerides and phospholipids. The biosynthesis of complex lipids by micro-organisms hasbeen reviewed by Kates (1966).

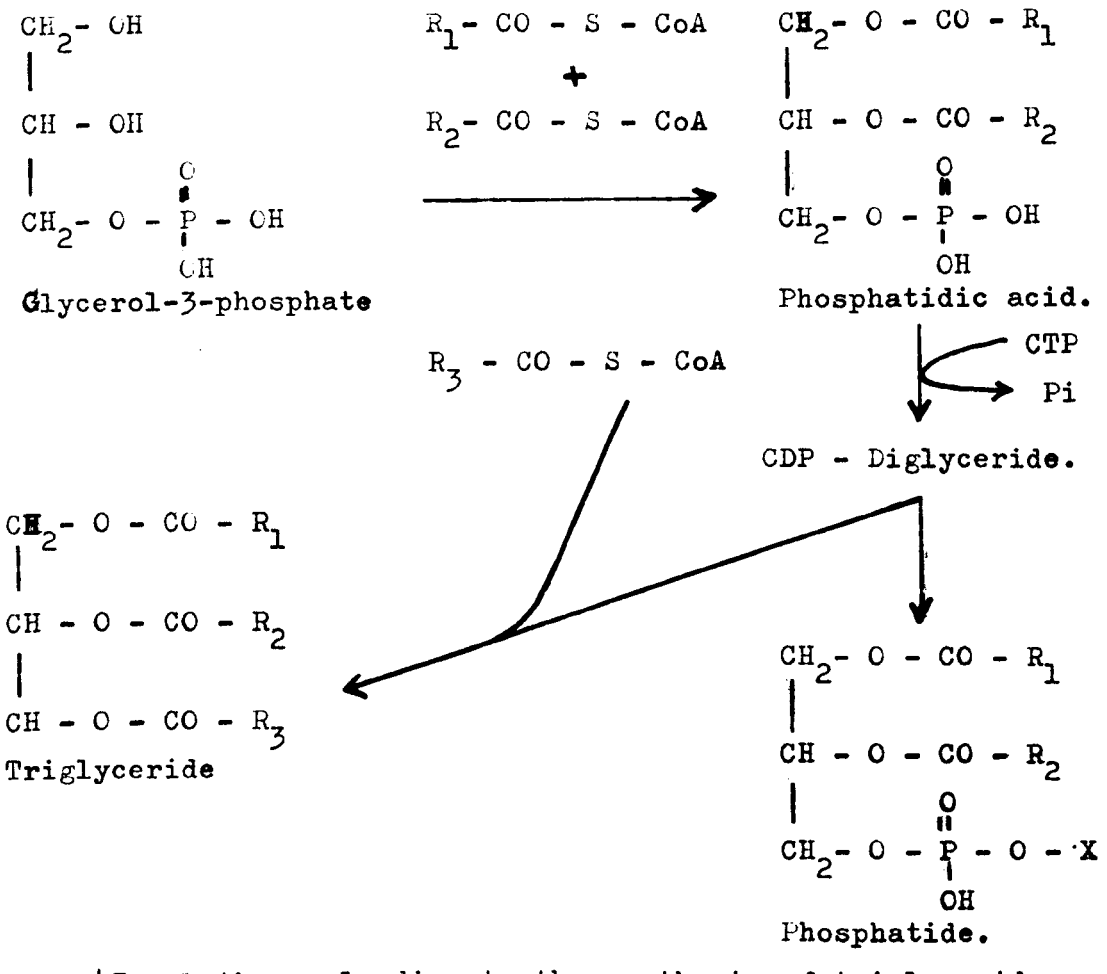


Fig. 43. Pathways leading to the synthesis of triglycerides and phospholipids.
(X = choline, ethanolamine, inositol, ornithine or serine).

The lipids of micro-organisms are separable into a neutral fraction (mainly triglycerides) and a compound fraction (mainly phospholipids); bacteria are exceptional in that their lipids, which probably lack triglycerides, are composed almost entirely of phospholipids. Phospholipids are associated with

the membrane structures of the cell and are considered as "structural" lipids, while triglycerides are looked upon as "storage" lipids.

In the protists there is generally a differential distribution of specific fatty acids between these lipid fractions. In the neutral 'storage' lipids the saturated and monoenoic residues tend to predominate, while the compound 'structural' lipid fraction tends to be rich in polyunsaturated fatty acids. This type of distribution has been demonstrated in blue-green algae (Levin *et al.* 1964) ciliate protozoa (Erwin and Blooh 1963), algae (Schlenk *et al.* 1960 and ascomycete and basidiomycete fungi (Leegwater *et al.* 1962, Talbot and Vining 1963, Bentley *et al.* 1964). However in the phycomycete fungi and certain zooflagellate protozoa there appears little difference in specific fatty acid composition between the two lipid fractions and this is considered as further evidence for supposing that Phycomycetes were evolved from zooflagellate ancestors.

From the lack of differentiation in the fatty acid composition of the various lipid fractions of phycomycete fungi it might be expected that the overall fatty acid composition of the mycelial lipids would remain fairly constant throughout the growth cycle. This was borne out by the present investigation with a number of *Mucor* and *Rhizopus* species, in which the fatty acid composition of the mycelial

lipids was found to fluctuate by only relatively minor amounts. Substantially similar results have been reported for other mucoraceous fungi (Bernhard et al. 1958, White et al. 1962, Shaw 1966). In this connection mucoraceous fungi are useful experimental material, since experimentally-induced modifications are not masked by any "ageing" effects. In ascomycete and basidiomycete fungi, on the other hand, wide variations in fatty acid composition can occur during the various phases of the growth cycle (Leegwater et al. 1962, Salomonowicz and Niewiadomski 1965).

A number of experiments were carried out with M. pusillus, the aim being to define the effect of several environmental factors on lipid synthesis and fatty acid composition of this fungus. The nature of the carbon and nitrogen source in the culture medium markedly influenced the rates of growth and accumulation of mycelial lipids, though the degree of unsaturation appeared little affected by the nature of the carbon and nitrogen source.

The concentration of carbon and nitrogen in the culture medium was found to influence both the lipid content and the degree of lipid unsaturation of the mycelium. When grown on a medium of high C:N ratio the lipid content of the mycelium of M. pusillus was high, lipid comprised over 40% of the mycelial dry weight; in media

of lower C:N ratio the lipid content of the mycelium was reduced. The C:N ratio had a definite effect on the fatty acid composition of the mycelial lipids; as the concentration of nitrogen was increased so was the degree of unsaturation of the lipids. Increased synthesis of unsaturated fatty acids under media conditions of high nitrogen has also been reported for Scenedesmus spp. (Lamonica and Conti 1962, Koelensmid et al. 1962, Erwin and Bloch 1963a, Klenk et al. 1963) and E. coli (Marr and Ingraham 1962).

Two explanations for the "high-nitrogen" effect have been offered. Shaw (1966) considers that in Scenedesmus the C:N ratio modifies the relative amounts of the triglyceride and phospholipid fractions, and since the fatty acid composition of these components is markedly different, changes in C:N ratio result in changes in degree of lipid unsaturation.

In E. coli however, the lipids are composed predominantly of phospholipids, irrespective of culture conditions. Marr and Ingraham (1962) consider that in this organism the increase in degree of lipid unsaturation with increased nitrogen content in the medium is due to the synthesis of certain intermediates of nitrogen metabolism which may stimulate synthesis of unsaturated fatty acids; these intermediates probably limit unsaturated fatty acid synthesis in nitrogen-limited cultures.

In the present investigation, evidence that the "high-nitrogen" effect can occur together with a high rate of storage lipid accumulation was obtained by increasing the concentrations of carbon and nitrogen in the culture medium, while maintaining a constant C:N ratio. It was found that the degree of unsaturation of the mycelial lipids increased as the nutrient status of the medium was increased. Since in M. pusillus the fatty acid compositions of both neutral and compound lipid fractions are practically identical, any increase in degree of unsaturation of the gross mycelial lipids probably occurs in both fractions. It seems likely therefore that increases in the degree of unsaturation of the lipids of micro-organisms under high-nitrogen culture conditions is due to increased synthesis of unsaturated fatty acids per se, rather than to a proportional decrease in the synthesis of saturated fatty acids.

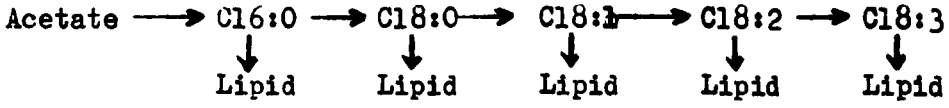
As the result of a number of experiments in which mucoraceous fungi were grown at different temperatures it became clear that fungal lipids become more unsaturated as the incubation temperature is lowered. Several explanations have been advanced for the adaptive desaturation of lipids with decreasing environmental temperature, and these fall into two categories, those concerned with the enzymes involved in fatty acid metabolism (Kates and Baxter 1962, Meyer and Bloch 1963) and those concerned with the cofactor requirements of these enzymes (Harris and James 1968).

The enzymes involved in fatty acid synthesis are the synthetases, which catalyss the elongation of the fatty acid chain, and the desaturases which catalyse the insertion of double bonds into the respective saturated fatty acids; NADPH acts as a reducing agent for both desaturases and synthetases. The degradation of fatty acids (β-oxidation) is catalysed by dehydrogenases, linked with NAD or NADP.

Kates and Baxter (1962), who demonstrated the increased synthesis of unsaturated fatty acids at a lower temperature in the yeast Candida lipolytica, proposed that the catabolic breakdown of linoleic acid probably has a higher temperature coefficient than the synthesis of linoleic acid. Under these conditions linoleic acid would be synthesised more rapidly than it is oxidised resulting in the accumulation of the polyunsaturated acid with consequent increase in degree of lipid unsaturation.

Another explanation is that the temperature effect may be due to a direct modification of the desaturase enzymes. In this connection Meyer and Bloch (1963) compared the desaturase activity of Torulopsis utilis grown at 19° and 30°, and demonstrated that the desaturases were more active when extracted from cells grown at the lower temperature.

An alternative explanation for adaptive saturation is that during incubation at temperatures either above or below the optimum for the growth of an organism, the levels of certain enzymes may be altered. If lipid biosynthesis is represented by the following scheme it can be seen that if, say, linoleic desaturase were present at a significantly lower concentration than the other enzymes, the lipids synthesised would be relatively rich in oleic and linoleic acids, and poor in linolenic acid.



In the incorporation of fatty acid residues into the lipids, therefore, 'competition' exists between on the one hand the synthetases producing saturated acids, and on the other hand the desaturases converting these saturated to unsaturated fatty acids.

While these two classes of enzymes have a number of common cofactor requirements: ACP, CoA, NADPH or NADH, they are markedly different in that synthetases require carbon dioxide and desaturases require oxygen. In aqueous systems the solubility of both oxygen and carbon dioxide is related to temperature, an increase in temperature producing a decrease in solubility and hence of

concentration of both gases. It is possible therefore that an increase in temperature may affect the reaction velocities of the desaturase enzymes by reducing the concentration of one of the cofactors, oxygen, to a level which is rate-limiting.

Evidence that the gaseous environment can influence the relative rates of synthesis of saturated and unsaturated fatty acids was obtained by Light et al. (1962). These authors showed that in aerobic conditions, monoenoic acids comprised 80% of the total fatty acids of Saccharomyces cerevisiae, while under anaerobic conditions the content of unsaturated acids was negligible.

More recently, Harris and James (1968) have shown that in bulb tissue incubated with C^{14} acetate, the "desaturation rate" (which is the ratio of radioactivity of C18 unsaturated acids : radioactivity in stearic acid, and is effectively a measure of the rate of synthesis of unsaturated acids) decreases when the temperature is raised above 10^{o}. The same authors further demonstrated that if the concentration of oxygen in solution was maintained at the same level as that at 10^{o}, the "desaturation rate" remained constant while the temperature was raised to 40^{o}. Temperature was found to have no effect on the synthesis of unsaturated fatty acids in Chlorella vulgaris and in plant leaf tissue, and this

was attributed by Harris and James to the photosynthetic production of excess oxygen. However, the formation of unsaturated fatty acids was inhibited when these tissues were incubated in the dark, this inhibition being removed by incubating the cells with 70% oxygen.

In the present study, an experiment in which the concentration of oxygen dissolved in the medium was measured during the growth of surface cultures of M. pusillus, showed that at 50° the fungus was rapidly subjected to conditions of almost complete anaerobiosis; the oxygen concentration was also reduced at 25° though at this temperature the medium contained at least four times as much oxygen as cultures growing at 50°. In the same experiment the lipids were found to respond in the usual manner to the incubation temperature, the lipids of 25° cultures being more unsaturated than those of 50° cultures. It is considered therefore, that at 50° surface cultures of M. pusillus grew under oxygen-deficient conditions which were probably rate limiting for the fatty acid desaturation reaction; it is thought that the oxygen concentration of 25° cultures was probably not rate-limiting.

A number of experiments were performed in which cultures of M. pusillus were incubated in atmospheres enriched with various

concentrations of oxygen. One aim of these experiments was to attempt to reverse the increased saturation of the lipids at 50° by preventing the oxygen concentration from becoming rate limiting for lipid desaturation. Due to practical difficulties it was not possible to grow M. pusillus in submerged culture, and only surface culture experiments were carried out. In these experiments determination of the oxygen concentration of the medium showed that even when a synthetic gas mixture of 70% oxygen, 30% nitrogen was pumped into the air space above the growing culture, the culture medium still became oxygen-deficient. Evidently the fungal mat prevents adequate diffusion between the culture medium and the gaseous atmosphere within the culture vessel, and since the main bulk of the culture is floating in the medium it is doubtful whether any increase in the oxygen concentration of the gas phase significantly increases the oxygen available to the fungus itself.

It is clear from the experiments of Harris and James (1968) that fatty acid desaturation can be regulated by the concentration of dissolved oxygen. It should be noted however, that their experiments, which were carried out by incubating small quantities of tissue (in the case of Chlorella this was 0.25g. wet weight) in a rotary Warburg apparatus for 5 hours in a stream of gas passing at a

rate of approximately 1.2 l./hour, represent an essentially "experimental" system. No account was taken by Harris and James of the long term effect of the experimental conditions on "natural" factors such as growth, reproduction, morphogenesis. It seemed desirable therefore to show that temperature-induced lipid responses could be compensated by altering the concentration of dissolved oxygen, without any adverse effects on the natural growth characteristics of the organism. In the pressnt study however, wide changes could not be demonstrated in the degree of unsaturation of the lipids during incubation with different concentrations of dissolved oxygen. It is considered more likely that this represents a limitation of the experimental method of aerating the growing culture, rather than the inability of the organism to synthesise highly unsaturated lipids at a high temperature.

Following an investigation of the effect of certain environmental factors on the lipid composition of M. pusillus it is possible to offer some explanation for certain trends which consistently occur during the growth cycle of this fungus. For example the lipids of young (2 or 3 day) cultures are almost invariably more unsaturated than those of mature cultures, and this is attributed to the relatively high nitrogen content of the culture

medium during the early stages of growth; the fact that the oxygen concentration is higher in young cultures also probably promotes the synthesis of unsaturated fatty acids. The high mycelial lipid content of young cultures is probably due to the relatively high concentration of carbon source during the early stages of growth.

It was noted that the mycelial lipid content of thermotolerant and thermophilic fungi differed markedly from that of mesophilic and psychrophilic fungi. In the latter the lipid content remained fairly constant throughout the incubation period and rarely exceeded 20% of the dry weight. In thermotolerant and thermophilic fungi on the other hand, the amount of mycelial lipid varied with the age of the culture, usually reaching a maximum of 30-40% of the dry weight in the early stages of the culture, and falling as the fungus aged. A similar observation has been made with the facultative thermophiles Bacillus stearothermophilus and Bacillus coagulans in which lipids were shown to comprise 8.0% and 12.6% respectively, of the cell wall (Forrester and Wicken 1966). In mesophilic Gram-positive bacteria, similar extraction methods showed the cell wall to contain 1-2% lipid (Salton, 1964). It seems therefore, that in thermophilic fungi and also in thermophilic bacteria (in which the lipid is sited mainly in the cell wall), the thermophilic habit is associated with a

relatively high lipid content, and possible explanations for this are discussed.

A number of factors have been reported as stimulating fatty acid biosynthesis. Martin and Vagelos (1962) have shown that intermediates of the tricarboxylic acid cycle, particularly citrate and isocitrate, stimulate fatty acid biosynthesis in rat and pigeon liver, and in lactating mammary gland preparations. Martin and Vagelos consider that citrate enhances fatty acid synthesis probably by stimulating the activity of acetyl CoA carboxylase, which catalyses the carboxylation of acetyl CoA to malonyl CoA, and which has been shown to be the rate-limiting step in fatty acid biosynthesis (Ganguly 1960, Lynen et al. 1961).

There is also considerable evidence that in pigeon liver and also in E. coli fatty acid synthesis is stimulated by phosphorylated sugars, and to a lesser extent, by inorganic phosphate and pyrophosphate (Wakil et al. 1966). Recently, Plate et al. (1968) showed that the fatty acid synthetase of pigeon liver was sensitive to inhibition by malonyl CoA; however this inhibition could be removed by the addition of phosphorylated sugars, the most effect of which was fructose 1, 6-diphosphate.

It is clear that the respiratory intermediates could influence the biosynthesis of fatty acids in two ways, by stimulating the activity of acetyl CoA carboxylase or by preventing inhibition of fatty acid synthetase. In the case of the latter mechanism the levels of phosphorylated sugars and of NADPH are important, since reduction of these levels would render the fatty acid synthetase more susceptible to inhibition by malonyl CoA, with consequent reduction in the rate of fatty acid synthesis.

The increased accumulation of lipids by thermophilic and thermotolerant as opposed to mesophilic and psychrophilic fungi, might be explained on the basis of the higher respiratory rate of the former fungi. The oxygen concentration of the environment of the thermophilic fungus M. pusillus has been shown to fall to an extremely low level, and it is probable that the carbon dioxide concentration shows a proportional increase. In thermophiles particularly, such conditions might be expected to depress the oxidative phosphorylation reactions which occur mainly in the tricarboxylic acid cycle, causing accumulation of the intermediates of respiratory metabolism, in particular isocitrate, citrate and acetyl CoA; the same conditions would also lead to accumulation of NADPH. In thermophiles therefore, the partially anaerobic conditions may result in the production of

increased levels of acetyl CoA, which is necessary for the carboxylation of acetyl CoA to form malonyl CoA, of NADPH, which provides reducing power for fatty acid synthesis, and of certain respiratory intermediates, which have been shown to stimulate lipid biosynthesis

There seems little doubt that a change in the environmental temperature of the fungus is associated with a change in the degree of unsaturation of the lipids. In the present study this has been demonstrated in experiments when psychrophilic, thermotolerant and thermophilic fungi were grown at temperatures near to their maxima and minima for growth, and also in an experiment where cultures of M. pusillus were transferred either from 50° to 25°, or vice versa. However, the experiments of Harris and James (1968) raise again the important question regarding the lipid composition of poikilothermic organisms, namely: are temperature-induced lipid changes merely a response to the oxygen concentration of the environment, or do they have some functional significance?

Ingraham and his co-workers have provided a considerable amount of evidence which suggests that adaptive desaturation is not important physiologically. Marr and Ingraham (1962)

demonstrated that although the lipids of E. coli became progressively more unsaturated as the growth temperature was decreased from 43° to 10°, the fatty acid composition could be modified at any one temperature by varying the carbon:nitrogen ratio of the medium. Shaw and Ingraham (1965) further showed that the glucose concentration of the medium can reverse temperature-induced desaturation. When cultures of E. coli were transferred from 37° to 10° the proportion of unsaturated fatty acids increased. However if growth of the 37° cultures was continued under glucose-limited conditions, and the 10° cultures under conditions of unlimited glucose, 10° cultures had a fatty acid composition similar to that of organisms grown at 37°.

There is also some evidence to suggest that unlike other lipid components in the cell the main lipid constituents of membrane structures (phospholipids) do not respond to alteration in temperature. Kates and Baxter (1962) showed that while the total lipids of Candida lipolytica grown at 10° were considerably more unsaturated than those of cultures grown at 25°, the degree of unsaturation of the phospholipids was unaffected by temperature.

However the temperature-induced response of the lipids is of extremely widespread occurrence and the possibility of some physiological significance cannot be dismissed. An often-used explanation of adaptive desaturation at lower temperatures is that the membrane lipids must remain in a liquid state, in order to retain activity. Byrne and Chapman (1964) showed that phospholipids can exist in two physical states, liquid or solid, depending on the freezing points of their fatty acid side chains. Unsaturated fatty acids have a lower freezing point than saturated fatty acids, and the presence of unsaturated fatty acid chains has been shown to lower the freezing point of phospholipids (Byrne and Chapman 1964).

Farrell and Rose (1968a, 1968b) have recently obtained some circumstantial evidence for the physiological role of unsaturated fatty acids in the membrane lipids of micro-organisms. An investigation of the resistance of temperature-adapted strains of Pseudomonas aeruginosa to sudden chilling ('cold shock') showed that when mesophilic or psychrophilic strains of P. aeruginosa were grown at 30° they were susceptible to cold shock. However, when these

strains were grown at 10°, at which temperature there was increased synthesis of unsaturated fatty acids in the bacterial membrane, both strains were resistant to cold shock. Farrell and Rose suggest that cold shock results from a sudden release of cellular constituents through 'holes' which occur in the membrane following 'freezing' of the membrane lipids. In cultures of P. aeruginosa grown at 10°, and not susceptible to freezing, they suggest that cold-shock resistance is due to the absence of 'holes' in the membrane because the more unsaturated lipids have a lower melting point and consequently do not 'freeze'.

Farrell and Rose (1968a) in a study of solute uptake in Candida utilis have also obtained preliminary data which indicate that the interaction between membrane proteins and lipids may be affected by the degree of unsaturation of the lipids, and that this in turn may affect membrane activity. It seems therefore that the fatty acid composition of the membrane lipids may be important in maintaining the lipid in a suitable physical state to ensure correct orientation with permeases.

The data obtained by Farrell and Rose (summarised by Rose 1968) that in psychrophiles and mesophiles a relationship

may exist between the fatty acid composition and the lower temperature limit for growth. It is tempting to think that the minimum growth temperature of thermophiles also, may be that temperature below which changes in the molecular architecture of the membrane prevent permease activity.

A number of experiments are required to solve some of the problems discovered in this investigation. For instance, is it possible, by increasing the oxygen concentration of the environment, to induce a thermophile to produce highly unsaturated lipids, or conversely, by reducing the concentration of available oxygen, to induce a psychrophile to produce highly saturated lipids? If the lipids could be modified in this way it might be taken asevidence that the degree of lipid unsaturation is not important in influencing the temperature relations of the organisms.

SUMMARY.

Studies have been made on two aspects of the physiology of thermophilic fungi in the Order Mucorales, their nitrogen nutrition and lipid composition. The main experimental material used in these studies was Mucor pusillus.

Preliminary experiments indicated that growth and sporulation of M. pusillus were affected by a number of environmental factors. No sporulation occurred when the fungus was grown either in shake culture, or in still culture at a temperature just below the maximum (ca. 53°). At the optimum temperature (ca. 45°-48°) and in still culture, the amount of sporulation was inversely proportional to the concentration of nitrogen source in the medium; sporulation was also reduced when the pH of the medium was buffered by the inclusion of an organic acid. Growth of M. pusillus increased as the concentrations of the carbon and nitrogen sources were increased, and when the pH of a glucose-ammonium sulphate medium was stabilised by addition of sodium succinate. The fungus also grew more rapidly in shake culture than in still culture.

Nutritionally, M. pusillus proved remarkably versatile, being able to utilise inorganic nitrogen sources in several oxidation states, and also organic nitrogen sources. Nitrate supported more growth than the other inorganic nitrogen sources

tested, while of 29 amino acids presented as single nitrogen sources the fungus was capable of growing on all but four, p-amino benzoic acid (PABA), hydroxyproline, creatine and thyroxine. Glycine, alanine, proline, aspartic acid and glutamic acid were the most efficient single amino acids for growth, and cysteine, cystine, DOPA, tryptophan and histidine the least efficient. Other organic nitrogen sources which supported good growth were casein, peptone, glycyl-glycine and urea.

Generally, buffering the culture medium by the addition of sodium succinate reduced the amount of growth of M. pusillus; only when ammonia, cystine, methionine or histidine were nitrogen sources was growth increased on a buffered, as opposed to an unbuffered, medium.

M. pusillus spored well on a wide range of inorganic and organic nitrogen sources; high spore counts were obtained when ammonia, glutamic acid, tyrosine, serine, the sulphur-containing and basic amino acids, casein, gelatin, peptone and urea were nitrogen sources. Buffering tended to reduce sporulation both in terms of actual spore counts and of "sporulation indices". The exceptionally high "sporulation indices" obtained on protein and peptone sources were attributed

to the presence of high proportions of those amino acids favourable for sporulation and of low levels of the amino acids favourable for growth.

Though some broad pattern is seen in the effect of different amino acids on vegetative and reproductive growth, and some conclusions made, it is not possible to say why some amino acids stimulate or inhibit different phases of growth.

The fatty acid composition of the lipids of some newly isolated thermophilic and thermotolerant fungi in the Mucorales was compared with that of some psychrophilic and mesophilic Mucor species. The optimum growth temperatures were found to be 40°-50° for the thermophiles and thermotolerants, 20°-25° for the mesophiles and 10°-20° for the psychrophiles.

Thermophiles and thermotolerants tended to accumulate more lipid than related mesophiles and psychrophiles. In the former, lipid usually comprised 30-40% of the total dry weight of the mycelium, though the lipid content fell in the later stages of growth. In mesophiles and psychrophiles however, lipids rarely exceeded 20% of the dry weight of mycelium, and this concentration was generally maintained throughout the incubation period.

The fatty acid composition of all the mucoraceous fungi examined varied with the age of the culture and with the temperature at which it was grown. No significant difference was found between the degree of unsaturation of the lipids of psychrophiles and of mesophiles. When thermophilic and thermotolerant fungi were grown at 48° their lipids were significantly more saturated than those of mesophilic and psychrophilic fungi grown at 25°. At 25° the lipids of thermotolerant and mesophilic fungi had similar degrees of unsaturation, and though the lipids of thermophilic fungi were more unsaturated at the lower incubation temperature they were still significantly more saturated than those of mesophiles and psychrophiles.

As a generality it was noted that in the early stages of growth the lipids were more unsaturated in cultures grown at a higher rather than a lower temperature. It was considered therefore that other factors influence the fatty acid composition of the lipids, and an investigation was carried out to define the effect of some environment factors on the degree of unsaturation of the lipids of M. pusillus. Mucoraceous fungi are particularly suirable experimental material since their

fatty acid composition varies little with the age of the culture, compared with ascomycete and basidiomycete fungi.

It was found that varying the carbon or nitrogen source in the medium affected growth and lipid accumulation, but not the fatty acid composition of the lipids of M. pusillus. The pH of the medium also had no significant effect on lipid composition, except possibly at pH 2, at which pH the lipids were extremely saturated.

The carbon:nitrogen ratio of the medium had a marked effect on lipid synthesis and composition. As the carbon concentration was increased so was the lipid content of the mycelium, while increases in the nitrogen concentration were associated with increases in the degree of lipid unsaturation.

The lipids of M. pusillus were found to respond to the temperature of incubation. The lipids were more unsaturated in cultures grown at a lower compared with a higher temperature; transfer of cultures from a higher to a lower temperature, and vice versa also resulted in changes in lipid unsaturation. Measurement of the partial pressure of oxygen (pO_2) in the medium of cultures growing at 25° and 50° showed that at the higher temperature at least, the fungus was growing under almost completely anaerobic conditions.

The possibility that at high temperatures the oxygen concentration of the medium may limit the production of unsaturated fatty acids was investigated by growing cultures of M. pusillus in atmospheres of enriched oxygen. No significant increases in degree of lipid unsaturation could be demonstrated even when still cultures of M. pusillus were grown in an atmosphere of 70% oxygen and 30% nitrogen. It must be stated however that even under conditions of enriched oxygen the culture medium was still severely oxygen-deficient, indicating that the mycelial mat forms an effective barrier to gaseous diffusion.

A number of experiments were carried out the aim of which was to grow M. pusillus in conditions of submerged, aerated culture, so that all parts of the culture could be effectively aerated with a synthetic gas mixture. All attempts to culture M. pusillus in this way were unsuccessful.

APPENDIX.

Date for the growth and sporulation of *M. pusillus* on amino acid nitrogen sources (see Sections 4.21 and 4.22 for summary).

Tables A - F, growth and sporulation on single amino acid nitrogen sources.

Tables G - L, growth and sporulation on mixtures of amino acids.

TABLE A. Growth of *M. pusillus* on unbuffered media containing a single amino acid as nitrogen source.
(Growth expressed as mycelial dry weight in mg./ml. medium).

Amino Acid Source	Age of Harvest (hours)						Cumulative Growth
	24	36	60	84	108	180	
Glycine	0.64	1.52	2.61*	3.40	4.52	4.76	17.45
Alanine	0.48	1.04	2.42*	3.12	3.80	4.75	15.61
Serine	0	0.88	1.73*	2.49	2.82	3.06	10.98
Threonine	0	0.24	1.30	1.65	2.12*	3.06	8.37
∝-ABA [1]	0	0.20	1.30	1.81*	2.04	3.48	8.83
Valine	0	0.40	1.37	1.81	2.08*	3.48	9.14
Norvaline	0	0.12	0.68	1.48	2.60*	2.84	7.72
Leucine	0	0.52	1.40	1.96	2.72*	3.53	10.13
Norleucine	0	0.28	1.10	1.58	1.84*	3.68	8.43
Isoleucine	0	0.24	1.04	1.36	2.05*	3.76	8.45
Aspartic Acid	0.12	0.80	1.04*	2.00	2.60	3.80	10.36
Glutamic Acid	0.44	1.64	3.33*	3.88	4.60	3.84	17.73
Proline	0.56	1.76	3.47*	3.96	4.70	5.11	19.56
Arginine	0	0.56	2.36*	2.97	3.12	3.24	12.25
Ornithine	0	0.20	1.17*	2.42	3.07	2.68	9.54
Citrulline	0	0.64	1.00*	1.68	2.12	2.72	8.16
Methionine	0	0.08	0.88	1.48	2.03*	2.41	6.88
Cysteine	0	0	0.56	0.68	0.76*	1.10	3.10
Cystine	0	0	0	0.40	0.76*	0.76	1.92
DOPA [2]	0	0	0	0	0	0.32	0.32
Phenylalanine	0	0.36	1.08	1.88*	2.30	3.76	9.38
Tyrosine	0	0.88	1.70*	2.84	3.28	3.72	12.42
Tryptophan	0	0.20	0.36	0.56	1.12	1.12	3.36
Lysine	0	0	0	0.40	0.60*	2.00	3.00
Histidine	0	0	0	0.36	0.44	0.36	1.16
p-Aminobenzoic Acid, Hydroxyproline, Creatine, Thyroxine. No growth.							

1. -Aminobutyric Acid. 2. 3, 4 -Dihydroxyphenylalanine.
* Nitrogen source in the medium exhausted.

TABLE B. Sporulation of M. pusillus on unbuffered media containing a single amino acid as nitrogen source.
(Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Amino Acid Source	Age of Harvest (hours).						Cumulative Sporulation.
	24	36	60	84	108	180	
Glycine	0.37	1.42	0.60*	-	0.39	0.50	3.28
Alanine	0.15	0.36	0.41*	-	0.14	0.25	1.31
Serine	0	1.48	0.98*	0.68	0.89	0.96	4.99
Threonine	0	0.04	0.41	0.34	0.15*	0.13	1.07
∝-ABA	0	0.04	0.45	0.75*	0.67	0.11	2.02
Valine	0	0.15	0.22	0.30	0.16*	0.06	0.89
Norvaline	0	0.11	0.66	0.83	1.07*	0.18	1.85
Leucine	0	0.20	0.07	-	0.24*	0	0.51
Norleucine	0	0.17	0.55	0.96	1.05*	0.29	3.02
Isoleucine	0	0.16	0.45	0.92	0.72*	0.44	2.69
Aspartic Acid	0.08	0.36	0.87*	-	0.17	0.97	2.45
Glutamic Acid	0.15	1.09	1.18*	-	0.36	1.70	4.48
Proline	0.38	0.27	0.78*	-	0.21	0.22	1.86
Arginine	0	0.95	1.29*	1.36	0.14	0.77	4.51
Ornithine	0	0.10	2.21*	1.67	0.81	1.42	6.21
Citrulline	0	1.70	0.67*	0.71	0.27	0.13	3.48
Methionine	0	0.06	0.46	1.49	2.26*	1.77	6.04
Cysteine	0	0	0.46	1.02	1.47*	2.07	5.02
Cystine	0	0	0	0.21	1.23*	1.96	3.40
DOPA	0	0	0	0	0	0.14	0.14
Phenylalanine	0	0.06	0.60	0.65*	0.66	0.26	2.23
Tyrosine	0	1.07	1.71*	0.75	0.63	0.19	4.35
Tryptophan	0	0.78	1.06	1.64*	3.33	0.88	7.69
Lysine	0	0	0	0.21	1.26*	1.96	3.43
Histidine	0	0	0	0.17	0.19	0.21	0.57

* Nitrogen source in the medium exhausted.

TABLE C. pH drift of unbuffered media containing a single amino acid as nitrogen source, following growth of M. pusillus.

Amino Acid Source.	Age of Harvest (hours).					
	24	36	60	84	108	180
Glycine	6.1	5.1	5.0		4.5	4.5
Alanine	6.1	4.9	4.6		5.0	4.6
Leucine	6.0	4.3	3.9		4.1	4.0
Isoleucine	6.3	6.3	4.0	4.0	4.0	4.0
Norleucine	6.3	5.7	3.8	3.8	3.7	3.7
Valine	6.3	5.4	3.9	3.9	3.9	3.9
Norvaline	6.3	6.0	4.0	3.6	3.6	3.6
Serine	6.3	5.5	3.6	3.6	3.6	3.6
Threonine	6.3	6.2	4.3	4.3	4.2	4.2
∝-ABA	6.3	5.6	3.6	3.6	3.6	3.6
Aspartic Acid	3.4	3.5	4.2		4.1	4.1
Glutamic Acid	6.8	7.0	7.0		7.0	7.0
Proline	6.4	5.4	5.4		5.1	5.1
Arginine	6.3	5.9	3.5	3.5	3.5	3.5
Ornithine	6.3	5.8	3.2	2.7	2.7	2.7
Citrulline	6.3	5.7	5.4	5.0	5.0	5.0
Methionine	6.3	6.0	3.4	3.2	3.2	3.2
Cysteine	6.3	6.3	4.7	3.9	3.5	3.2
Cystine	6.3	6.2	6.3	6.6	6.3	6.5
DOPA	6.3	6.3	6.3	6.1	6.0	5.7
Phenyl Alanine	6.3	4.8	3.8	3.8	3.8	3.8
Tyrosine	6.2	6.1	4.6	4.6	4.6	4.6
Tryptophan	6.3	5.7	4.0	3.8	3.8	3.8
Lysine	6.3	6.2	6.0	4.5	3.5	3.1
Histidine	6.3	6.0	6.0	4.2	3.7	3.7

TABLE D. Growth of M. pusillus on buffered media containing a single amino acid as nitrogen source.
(Growth expressed as mycelial dry weight in mg./ml. medium).

Amino Acid Source	Age of Harvest (hours)					Cumulative Growth
	36	60	84	108	180	
Glycine	0.70	2.10	2.64	3.08	3.87	12.39
Alanine	0.84	1.38	1.99	2.49	3.20	9.90
Serine	0.60	1.17	1.50	1.90	2.89	8.06
Threonine	trace	0.53	1.18	1.50	2.08	5.30
∝-ABA	trace	1.02	1.44	1.77	3.29	7.55
Valine	0.13	0.76	1.19	1.60	2.79	6.47
Norvaline	0	trace	1.05	1.60	3.35	6.05
Leucine	0.24	1.06	1.30	1.71	2.85	7.16
Norleucine	0.28	0.99	1.25	1.89	2.89	7.30
Isoleucine	0.24	0.24	0.56	1.22	1.94	4.20
Aspartic Acid	0.61	1.76	1.97	2.64	3.56	10.54
Glutamic Acid	0.72	1.96	2.72	3.43	3.52	12.35
Proline	0.64	1.60	2.36	2.90	3.79	11.29
Arginine	0.12	1.26	1.92	2.69	3.40	9.39
Ornithine	trace	0.58	1.52	2.07	2.49	6.68
Citrulline	0.12	0.66	1.07	1.36	1.60	5.81
Methionine	trace	0.86	1.68	1.93	2.87	8.40
Cysteine	trace	0.56	0.89	0.94	1.56	4.00
Cystine	0	0	trace	0.84	1.20	2.10
DOPA	0	0	0	0	0.37	0.40
Phenylalanine	0.12	1.28	1.96	2.32	3.43	9.11
Tyrosine	0.04	0.50	1.60	1.87	3.08	7.09
Tryptophan	0	trace	0.28	0.73	1.12	2.15
Lysine	0	trace	0.24	0.70	2.01	3.00
Histidine	0	trace	0.16	0.46	0.90	1.55

TABLE E. Sporulation of M. pusillus on buffered media containing a single amino acid as nitrogen source.
(Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Amino Acid Source	Age of Harvest (hours)					Cumulative Sporulation.
	36	60	84	108	180	
Glycine	0.32	1.20	0.40	0.72	0.36	3.00
Alanine	0.56	1.04	0.44	0.44	0.44	2.92
Serine	1.32	0.84	0.96	0.76	0.64	4.52
Threonine	0	0.14	0.54	0.40	0.16	1.24
∝-ABA	0	0.68	0.44	0.28	0.12	1.52
Valine	0.08	0.08	0.60	0.72	0.88	2.36
Norvaline	0	0	0.32	0.40	0.36	1.18
Leucine	0	0.80	1.20	0.96	0.36	3.32
Norleucine	0	0.64	0.48	1.32	0.28	1.72
Isoleucine	0	0.12	0.40	0.84	0.60	1.96
Aspartic Acid	0.44	0.28	1.60	1.12	0.12	3.56
Glutamic Acid	0.24	0.32	0.80	0.28	0.20	1.84
Proline	0.20	0.16	0.60	0.16	0.20	1.32
Arginine	0.08	0.20	0.64	0.52	0.08	1.52
Ornithine	0.12	1.08	1.72	1.56	1.28	5.76
Citrulline	0	1.06	1.85	1.61	1.52	6.04
Methionine	0	0.44	0.80	0.32	0.36	1.92
Cysteine	0	0.14	0.72	0.68	0.24	1.78
Cystine	0	0	0	1.12	1.13	2.25
DOPA	0	0	0	0	trace	trace
Tyrosine	0.16	0.16	1.88	0.54	0.57	3.31
Tryptophan	0	0.20	0.20	0.36	0.48	1.24
Phenylalanine	0.08	0.28	0.76	0.32	0.16	1.50
Lysine	0	0	0.08	0.16	0.36	0.60
Histidine	0	0	0.20	0.28	0.32	0.80

TABLE F. pH drift of unbuffered media containing a single amino acid as nitrogen source, following growth of M. pusillus.

Amino Acid Source.	Age of Harvest (hours).				
	36	60	84	108	180
Glycine	5.97	6.10	6.56	5.60	5.65
Alanine	6.03	6.00	5.58	5.60	5.60
Leucine	6.07	6.00	5.60	5.62	5.60
Isoleucine	6.30	5.90	5.80	5.70	5.70
Norleucine	6.35	5.70	5.10	5.07	5.07
Valine	6.10	5.70	5.60	5.65	5.63
Norvaline	6.30	6.10	5.55	5.45	5.40
Serine	5.90	5.76	5.60	5.45	5.42
Threonine	6.30	5.92	5.65	5.52	5.47
∝-ABA	6.26	5.76	5.50	5.50	5.50
Aspartic Acid	6.22	5.98	5.45	5.53	5.50
Glutamic Acid	6.20	5.92	5.73	6.00	6.02
Proline	5.98	5.62	5.46	5.52	5.56
Arginine	6.22	5.68	5.45	5.53	5.50
Ornithine	6.20	5.83	5.60	5.63	5.63
Citrulline	6.40	6.50	6.90	6.50	6.55
Methionine	5.80	5.46	5.00	4.85	4.80
Cysteine	6.07	5.75	5.60	5.70	5.65
Cystine	6.20	6.10	6.30	6.50	6.60
DOPA	6.40	6.40	6.32	6.00	5.92
Phenylalanine	6.00	5.75	5.43	5.43	5.40
Tyrosine	6.25	6.00	5.80	6.00	5.92
Tryptophan	6.40	6.26	5.75	5.48	5.66
Lysine	6.40	6.16	5.75	5.47	5.16
Histidine	6.40	6.23	5.60	5.26	5.20

TABLE G. Growth of M. pusillus in unbuffered media with mixtures of amino acids as nitrogen sources.
(Growth expressed as mycelial dry weight in mg./ml. medium).

Amino Acid omitted from complete mixture.	Age of mycelial harvest (hours)					Cumulative growth
	24	48	72	120	192	
Glycine	0.16	1.00	1.45	2.12	2.06	4.51
Alanine	0.10	1.10	1.77	2.38	2.25	5.12
Serine	0.13	1.06	1.54	2.26	2.25	4.85
Threonine	0.08	0.85	1.50	2.32	2.06	4.41
∝ -ABA	0.12	1.00	1.65	2.18	2.32	4.97
Valine	0.33	1.06	1.50	1.94	2.05	4.61
Norvaline	0.00	1.06	1.42	1.74	2.18	4.96
Leucine	0.25	1.25	1.48	1.98	2.13	4.86
Norleucine	0.24	1.16	1.55	2.22	2.27	4.98
Isoleucine	0.18	1.12	1.55	2.22	2.03	4.70
Aspartic Acid	0.20	1.05	1.52	2.20	2.05	4.62
Glutamic Acid	0.21	1.02	1.49	2.16	2.12	4.63
Proline	0.12	0.90	1.41	2.28	2.08	4.39
Arginine	0.13	1.16	1.48	2.24	2.04	4.68
Ornithine	0.16	0.98	1.40	2.18	1.94	4.32
Citrulline	0.13	1.13	1.56	2.10	2.25	4.94
Methionine	0.10	1.04	1.50	2.30	2.21	4.75
Cysteine	0.08	1.06	1.50	2.28	2.21	4.77
Cystine	0.26	1.14	1.56	2.28	2.08	4.78
DOPA	0.42	1.61	2.22	2.73	3.03	6.96
Phenylalanine	0.20	1.17	1.54	2.16	2.18	4.89
Tyrosine	0.42	1.18	1.69	2.22	2.18	5.05
Tryptophan	0.32	1.14	1.60	2.16	2.08	4.82
Lysine	0.13	1.09	1.65	2.32	2.42	5.16
Histidine	0.39	1.01	1.56	2.16	2.12	4.79
Complete Mixture 1.	0.34	1.08	1.64	1.74	2.52	5.24
Complete Mixture 2.	0.13	1.14	1.64	1.78	2.29	5.17

TABLE H. Sporulation of M. pusillus in unbuffered media with amino acid mixtures as sources of nitrogen.
(Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Amino Acid omitted from complete mixture.	Age of mycelial harvest (in hours)					Cumulative Sporulation.
	24	48	72	120	192	
Glycine	0	0.48	1.33	0.90	0.59	3.38
Alanine	0	0.44	0.65	0.19	0.59	1.87
Serine	0	0.64	1.67	0.78	0.24	3.33
Threonine	0	0.10	0.70	0.81	0.47	2.08
∝-ABA	0	0.94	0.84	0.44	0.18	2.40
Valine	0	0.31	0.80	0.99	0.40	2.50
Norvaline	0	0.20	0.42	0.44	0.18	1.24
Leucine	0	0.87	0.67	0.94	0.66	3.14
Norleucine	0	1.16	1.50	1.14	0.37	4.17
Isoleucine	0	0.30	1.16	0.71	0.83	3.00
Aspartic Acid	0	0.71	1.26	0.74	0.65	3.36
Glutamic Acid	0	1.14	0.49	0.85	0.50	2.98
Proline	0	0.21	0.68	0.97	0.24	2.10
Arginine	0	0.28	0.21	0.46	0.62	1.57
Ornithine	0	0.40	1.12	0.93	0.89	3.34
Citrulline	0	0.63	1.22	1.04	0.36	3.05
Methionine	0	0.85	1.17	0.84	0.53	3.39
Cysteine	0	0.25	0.71	0.66	0.69	2.31
Cystine	0	0.86	0.84	0.71	0.88	3.29
DOPA	0.18	1.00	1.62	1.22	0.64	4.76
Phenylalanine	0	0.93	0.70	0.59	0.88	3.10
Tyrosine	0.24	2.03	1.75	1.54	0.46	6.02
Tryptophan	0.10	0.90	0.70	1.43	0.26	3.49
Lysine	0	0.36	0.32	0.38	0.14	1.20
Histidine	0	0.44	0.33	0.52	0.40	1.79
Complete Mixture 1	0	0.58	0.34	1.18	0.72	3.82
Complete Mixture 2	0	0.56	0.74	1.24	2.14	4.78

TABLE I. pH drift of unbuffered media with mixtures of amino acids as sources of nitrogen, following growth of M. pusillus.

Amino Acid omitted from complete mixture.	Age of Harvest (hours).				
	24	48	72	120	192
Glycine	5.6	3.9	4.0	3.8	3.5
Alanine	5.8	4.0	4.0	3.8	3.5
Leucine	5.0	3.7	3.8	3.8	3.3
Isoleucine	5.5	3.8	3.9	3.5	3.4
Norleucine	5.0	3.8	4.0	3.7	3.6
Valine	5.0	3.9	3.9	3.7	3.6
Norvaline	6.4	5.8	4.8	5.4	5.3
Serine	5.7	3.9	4.0	3.8	3.6
Threonine	5.7	4.0	3.9	3.7	3.5
α-ABA	5.7	3.9	4.0	3.7	3.6
Aspartic Acid	5.7	4.0	4.0	3.7	3.4
Glutamic Acid	5.6	3.9	4.0	3.6	3.4
Proline	5.7	4.1	4.0	3.7	3.6
Arginine	5.8	4.0	4.0	3.8	3.6
Ornithine	5.6	4.0	3.9	3.9	3.7
Citrulline	5.1	4.0	4.0	3.7	3.5
Methionine	5.8	4.1	4.2	3.9	3.7
Cysteine	5.4	4.2	4.4	4.2	4.0
Cystine	5.4	3.9	3.9	3.6	3.5
DOPA	5.2	4.1	3.8	3.7	3.5
Phenylalanine	5.5	3.9	3.9	3.5	3.4
Tyrosine	3.9	3.6	3.4	3.1	2.9
Tryptophan	5.5	4.1	4.0	3.6	3.5
Lysine	5.6	4.0	4.0	3.8	3.6
Histidine	5.2	4.0	4.0	3.8	3.6
Complete Mixture 1.	5.0	4.0	4.0	3.8	3.5
Complete Mixture 2.	5.2	3.9	3.9	3.6	3.5

TABLE J. Growth of M. pusillus in buffered media with amino acid mixtures as sources of nitrogen.
(Growth expressed as mycelial dry weight in mg./ml. medium).

Amino Acid omitted from complete mixture	Age of Mycelial Harvest (in hours)					Cumulative Growth
	48	72	96	144	192	
Glycine	0.48	1.22	1.52	2.31	3.05	4.75
Alanine	0.52	1.29	1.71	2.41	2.99	4.80
Serine	0.47	1.47	1.57	2.39	3.14	5.08
Threonine	0.30	1.05	1.75	2.55	3.18	4.53
∝-ABA	0.33	0.74	1.54	2.16	3.15	4.22
Valine	0.48	1.22	1.46	2.49	3.14	4.84
Norvaline	0.52	1.27	1.74	2.29	3.07	4.86
Leucine	0.60	1.33	1.54	2.52	2.91	4.84
Norleucine	0.48	1.16	1.59	2.39	3.08	4.72
Isoleucine	0.51	1.41	1.58	2.48	3.01	4.93
Aspartic Acid	0.44	0.96	1.44	2.07	2.77	4.17
Glutamic Acid	0.33	0.80	1.42	2.28	2.88	4.01
Proline	0.40	0.64	1.24	2.29	2.87	3.91
Arginine	0.32	0.70	1.44	2.24	2.95	4.07
Ornithine	0.34	0.84	1.45	2.31	2.99	4.17
Citrulline	0.47	1.15	1.79	2.46	3.10	4.72
Methionine	0.45	1.20	1.48	2.40	2.82	4.57
Cysteine	0.27	0.98	1.49	2.21	3.25	4.50
Cystine	0.60	1.22	1.52	2.30	3.17	4.99
DOPA	1.81	2.78	2.52	3.01	3.60	7.19
Phenylalanine	0.28	0.88	1.50	2.11	2.97	4.23
Tyrosine	0.40	1.33	1.40	2.15	3.12	4.95
Tryptophan	0.43	1.09	1.49	2.40	3.18	4.70
Lysine	0.61	1.43	1.61	2.42	2.75	4.79
Histidine	0.40	0.78	1.32	2.14	3.31	4.49
Complete Mixture 1	0.33	0.68	1.35	2.23	3.15	4.16
Complete Mixture 2	0.29	0.75	1.41	2.39	3.40	4.44

TABLE K. Sporulation of M. pusillus in buffered media with amino acid mixtures as sources of nitrogen.
(Sporulation expressed as number of spores x 10^{-6}/ml. medium).

Amino acid omitted from complete mixture.	Age of mycelial harvest (in hours)					Cumulative Sporulation.
	48	72	96	144	192	
Glycine	0	0	0.20	0.24	0.38	0.82
Alanine	0	0	0.24	0.34	0.32	0.90
Serine	0	0	0.10	0.32	0.18	0.60
Threonine	0	0	0.52	0.48	0.28	1.28
∝-ABA	0	0	trace	0.29	0.26	0.60
Valine	0	0	0.14	0.21	0.44	0.79
Norvaline	0	0	0.15	0.19	0.25	0.59
Leucine	0	0	0.08	0.33	0.34	0.75
Norleucine	0	0	0.25	0.34	0.68	1.27
Isoleucine	0	0	0.08	0.18	0.50	0.76
Aspartic Acid	0	0	0.05	0.30	0.33	0.68
Glutamic Acid	0	0	0.17	0.13	0.37	0.67
Proline	0	0	0.05	0.18	0.22	0.45
Arginine	0	0	0.14	0.20	0.22	0.56
Ornithine	0	0	0	0.29	0.36	0.65
Citrulline	0	0	0.13	0.24	0.20	0.57
Methionine	0	0	0.10	0.36	0.36	0.82
Cysteine	0	0	0.17	0.17	0.24	0.58
Cystine	0	0	0.13	0.65	0.57	1.35
DOPA	1.44	0.92	0.84	0.76	0.38	4.34
Phenylalanine	0	0	trace	0.12	0.26	0.40
Tyrosine	0	0	trace	0.13	0.24	0.40
Tryptophan	0	0	0.17	0.29	0.37	0.83
Lysine	0	0.09	0.13	0.21	0.34	0.77
Histidine	0	0	trace	0.21	0.28	0.55
Complete mixture 1	0	0	0.05	0.26	0.17	0.48
Complete mixture 2	0	0	0.08	0.26	0.26	0.60

TABLE L. pH drift of buffered media containing mixtures of amino acids as sources of nitrogen, following growth of M. pusillus.

Amino Acid omitted from complete mixture.	Age of Harvest (hours)				
	48	72	96	144	192
Glycine	5.95	5.80	5.85	6.20	6.70
Alanine	5.95	5.85	5.75	6.15	6.65
Leucine	5.95	5.85	5.75	6.50	6.65
Isoleucine	6.05	5.85	5.70	6.30	6.65
Norleucine	6.10	5.80	5.70	6.00	6.45
Saline	6.00	5.75	5.80	6.20	6.55
Norvaline	6.05	5.80	5.50	6.35	6.55
Serine	6.00	5.80	5.50	6.30	6.65
Threonine	6.13	5.80	5.60	6.20	6.55
∝-ABA	6.10	5.90	5.50	5.80	6.65
Aspartic Acid	6.00	5.85	5.65	6.05	6.55
Glutamic Acid	6.15	5.85	5.70	6.05	6.55
Proline	6.10	5.95	5.70	6.05	6.60
Arginine	6.05	6.00	5.75	5.95	6.50
Ornithine	6.03	5.90	5.65	6.05	6.50
Citrulline	5.96	5.80	5.65	6.05	6.60
Methionine	5.97	5.80	5.70	6.20	6.50
Cysteine	6.18	5.90	5.70	6.05	6.60
Cystine	5.78	5.80	5.70	6.30	6.60
DOPA	5.85	6.05	6.35	6.45	6.65
Phenylalanine	6.03	5.80	5.60	5.85	6.50
Tyrosine	5.95	5.90	5.05	5.85	6.60
Tryptophan	5.90	5.80	5.60	6.10	6.65
Lysine	5.95	5.80	5.50	6.30	6.65
Histidine	6.06	5.85	5.80	5.80	6.45
Complete Mixture 1.	6.05	5.85	5.55	5.70	6.25
Complete Mixture 2.	6.10	5.90	5.50	5.80	6.60

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