ENVIRONMENT-MEDIATED CHANGES

IN THE

CHEMICAL COMPOSITION AND PHYSIOLOGY

OF

ASPERGILLUS NIDULANS

A thesis

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ABSTRACT

A study was made of the chemical composition of <u>Aspergillus nidulans</u> strain 13 mel in glucose limited chemostat culture under varying conditions of growth rate ($D = 0.02$ h^{-1} to 0.175 h^{-1}), temperature (22°C to 50°C), pH (3.1 to 7.9) and NaCl concentration (0 to 8% w/v).

Asexual differentiation occurred at a dilution rate of 0.02 h^{-1} (10% μ_{max}) following a progressive reduction in the glucose feed rate, and was preceded (at $D = 0.05$ h^{-1}) by major variations in the levels of intracellular and wall components. Peak values for RNA and magnesium, and a minimal amino acid pool content were observed at $D = 0.05$ h^{-1}, while walls isolated from mycelia grown at this rate contained double the normal content of α-glucan. Measurements of wall thickness in association with determinations of the proportion of wall material present in the mycelium indicated that these walls were less dense than normal.

Increasing the temperature between 25°C and 50°C produced a rise in the intracellular carbohydrate content while, over the same range, protein reached a maximal value at 38°C. The amino acid pool content was reduced at temperatures above 30°C. Increases of 47% and 139% in the protein and total mycelial carbohydrate levels, respectively, followed a reduction in temperature from 25°C to 22°C, while the RNA content doubled between 30°C and 22°C. Magnesium concentration also rose over the latter temperature range. The wall content of protein, phosphorus, galactose and uronic acid was increased and mannose reduced at elevated temperatures. Notable changes in glucose and hexosamine levels occurred below 30°C.

Growth in the presence of increasing H^{+} ion concentrations was accompanied by increased synthesis of RNA. Mycelial magnesium and potassium levels were reduced below pH 4.1 when a pH differential could not be maintained. The major influence of pH, however, was on wall composition. The content of individual neutral sugar components

and hexosamine increased with increasing pH, indicating an inhibitory effect of H^+ ions on the extracellular polymerisation reactions concerned with wall biosynthesis. Protein, phosphorus and uronic acid contents were reduced at increased pH values.

Accumulation of intracellular carbohydrate was noted in mycelia grown in the presence of NaCl and coincided with reduced levels of magnesium, potassium, phosphorus and the amino acid pool. Wall composition was only slightly affected by changes in culture salinity.

The protein synthesising activity of RNA (protein/RNA X D) was strongly dependent upon cultivation conditions, activity (1) increasing 8-fold between D = 0.02 h^{-1} and 0.175 h^{-1}; (2) doubling within the temperature range 20°C to 30°C; and (3) falling by 50% between 40°C and 50°C. A slight reduction in activity was noted at decreased pH values and increased NaCl concentrations.

The molar stoichiometry between RNA and magnesium, unlike that of bacteria, was also influenced by the nature of the environment but remained steady in face of changing temperature.

A positive correlation between the level of mycelial potassium and the efficiency with which glucose is utilised for cell synthesis was revealed. High levels of potassium restricted the efficiency of oxygen utilisation.

Of the wall components, protein, glucose, mannose and phosphorus were the most variable, values ranging from 8.5 to 20.5% w/w (protein), 17.4 to 43.6% w/w (glucose), 1.6 to 4.0% w/w (mannose) and 0.15 to 0.78% w/w (phosphorus). Under all conditions wall phosphorus and protein contents varied in like manner, indicating the presence of a phosphoprotein component. Calcium binding to the wall is probably effected through the latter.

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1.1. ENVIRONMENTAL CONTROL OF FUNGAL GROWTH

Many environmental factors are known to influence the physiological behaviour of fungi. The effects of medium composition, pH value and growth temperature on various aspects of fungal development have been well documented, but, as many of these observations have been derived from batch culture studies it is possible that factors, other than the one(s) under study, do contribute to the overall growth response. The use of controlled environmental conditions in continuous culture, however, permits valid interpretations of the effects of individual environmental parameters on microbial metabolism. The expediency of this technique for physiological studies of unicellular prokaryotes and eukaryotes is well established but, because of the need to refine fermenter equipment, specifically to cope with mycelial growth, similar studies of filamentous fungi are few in number.

1.1.1. The continuous culture technique

When a microbial population is cultivated in a closed or batch system, several different phases of growth can be observed. Initially, a lag phase or period of adaptation to the culture conditions exists followed by a period of unrestricted growth, in which the rate of growth is limited by the intrinsic characteristics of the organism. This latter phase represents a period of nutrient excess when the growth rate is the maximum possible for the organism under the specified conditions. In such circumstances growth is exponential and the increase in cell mass can be expressed mathematically:

$$x_t = x_o e^{\mu t} \tag{i}$$

or
$$\frac{x_t}{x_o} = e^{\mu t} \tag{ii}$$

where x_t, x_o = biomass at time t and time 0, and μ = specific

growth rate.

Taking natural logarithms, Equation (ii) becomes:

$$\log_e \frac{x_t}{x_o} = \mu t \qquad \text{(iii)}$$

and rearranging

$$\log_e x_t - \log_e x_o = \mu t \qquad \text{(iv)}$$

$$\mu = \frac{\log_e x_t - \log_e x_o}{t} \qquad \text{(v)}$$

Unrestricted growth ceases when the supply of a limiting nutrient decreases and eventually becomes depleted and a decelerating growth phase ensues. Once net growth stops the culture enters the stationary or maximum population phase.

In the batch system growth-associated changes in the environment occur throughout the fermentation and in consequence only transient growth states are possible. The chemostat method of continuous culture provides a valuable technique by which constant conditions can be maintained indefinitely and by which steady state growth can be achieved. The underlying principle stems from the Monod (1942) equation relating the rate of growth to the concentration of the growth-limiting substrate:

$$\mu = \mu_{max} \frac{S}{K_s + S} \qquad \text{(vi)}$$

where μ_{max} = maximum specific growth rate, S = concentration of growth-limiting substrate and K_s, the substrate saturation constant = the value of S when $\mu = \mu_{max}/2$.

Thus, by regulating the supply of an essential nutrient it is possible to control the growth rate of the organism. The essential

features of a continuous culture system are represented in
Figure 1.1. Nutrient medium is fed from a reservoir (A) at a flow
rate F ($1\ h^{-1}$), into a fermenter (B) which holds a constant volume
V (l). Spent medium leaves the fermenter, also, at flow rate F,
through an overflow and is collected in a receiving vessel (C). In
such a system:

Increase in organisms due to growth,

$$\frac{dx}{dt} = \mu x \qquad\qquad\qquad \text{(vii)}$$

The dilution rate D is the fractional dilution of the culture per
unit time:

$$D = \frac{F}{V}\ (h^{-1})$$

Loss of microorganisms by dilution,

$$\frac{dx}{dt} = -\ Dx \qquad\qquad\qquad \text{(viii)}$$

Thus, the actual change in the concentration of organisms can be
expressed by the equation:

$$\frac{dx}{dt} = \mu x - Dx \qquad\qquad\qquad \text{(ix)}$$

A steady state condition is established when the increase in biomass
due to growth is balanced by the loss due to dilution. Thus, at
steady state:

$$\frac{dx}{dt} = 0$$

and $\qquad\qquad \mu = D = \dfrac{F}{V} \qquad\qquad\qquad$ (x)

Gas eg Oxygen

B

Culture vessel

A

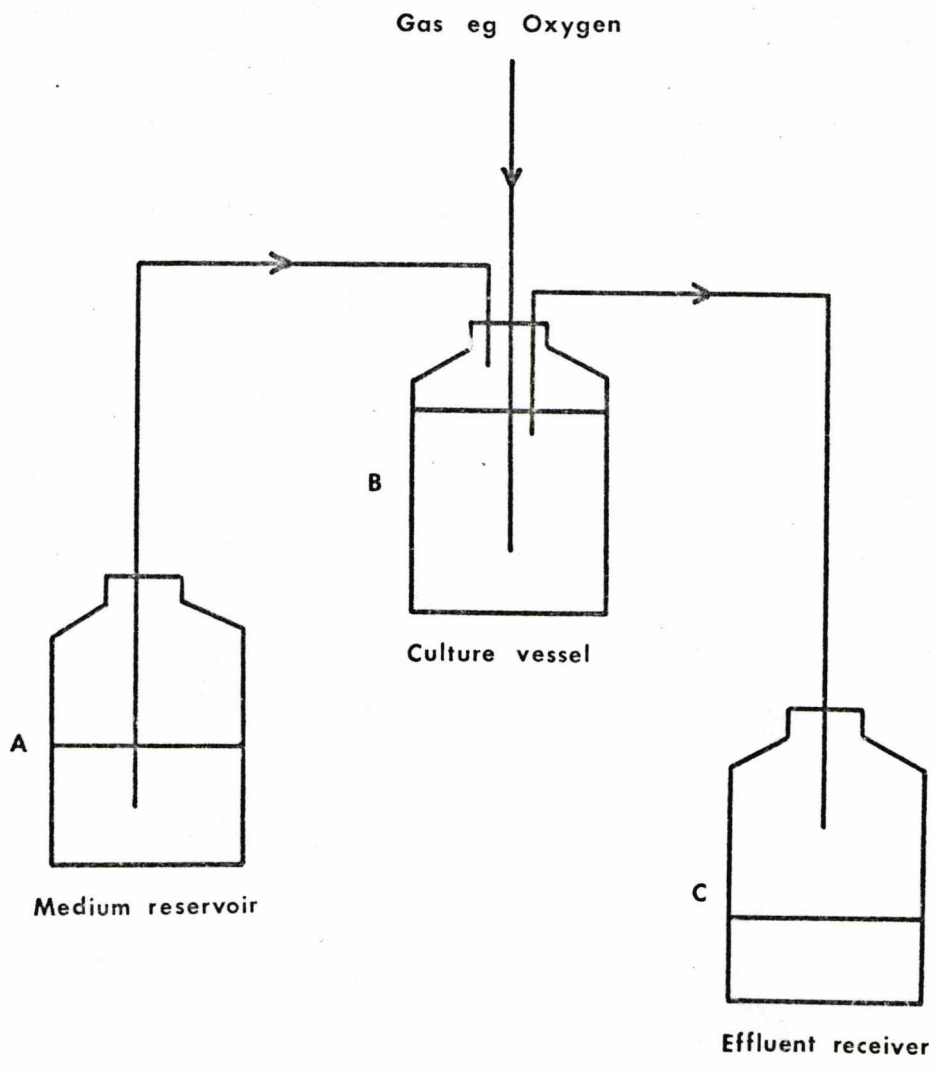Medium reservoir

C

Effluent receiver

Figure 1.1. Simplified diagram of a continuous culture system.

The growth rate can be expressed as a function of the input rate of the limiting nutrient:

$$\frac{dx}{dt} = - Y \frac{ds}{dt} \qquad \text{(xi)}$$

where Y is the yield coefficient (Monod, 1942) with respect to S.

Substitution of the combined Equations (vi) and (vii) into Equation (xi) gives:

$$- \frac{ds}{dt} = \mu_{max} \frac{x}{Y} \left(\frac{S}{K_s + S} \right) \qquad \text{(xii)}$$

which expresses the relation between substrate utilisation and biomass production.

For a steady state chemostat culture the substrate balance is defined thus:

Increase of = input — output — utilisation of = 0
substrate   substrate by
                                 microorganisms

$$\frac{ds}{dt} = DS_R - DS - \mu_{max} \frac{x}{Y} \left(\frac{S}{K_s + S} \right) = 0 \qquad \text{(xiii)}$$

where S_R = growth-limiting substrate concentration of inflowing medium, S = growth-limiting substrate concentration in fermenter.

Rearranging, Equation (xiii) becomes:

$$D (S_R - S) = \frac{x}{Y} \mu_{max} \left(\frac{S}{K_s + S} \right) \qquad \text{(xiv)}$$

and since it follows from Equations (vi) and (x) that in the steady state

$$D = \mu_{max} \frac{S}{K_s + S} \qquad \text{(xv)}$$

$$x = Y (S_R - S) \qquad \text{(xvi)}$$

The biomass balance for the steady state is defined by the following equation:

| Increase of biomass | = | growth of microorganisms in fermenter | — | loss of microorganisms by dilution | = | 0 |

$$\frac{dx}{dt} = \mu_{max} \; x \left(\frac{S}{K_s + S} \right) - Dx = 0 \qquad \text{(xvii)}$$

$$x \left(\mu_{max} \; \frac{S}{K_s + S} - D \right) = 0 \qquad \text{(xviii)}$$

$$D = \mu_{max} \; \frac{S}{K_s + S} \qquad \text{(xix)}$$

and hence

$$S = \frac{K_s \; D}{\mu_{max} - D} \qquad \text{(xx)}$$

The steady state is thus characterised by a constant organism and substrate concentration as designated by Equations (xvi) and (xx).

The steady state may be disturbed by altering one or more environmental parameters, for example, the composition of the inflowing medium, the dilution rate or the temperature. A new steady state may then be established after a period of time, during which the culture is in a transient state. As long as the dilution rate is controlled at a level which gives a growth rate below μ_{max} steady state conditions are possible. However, once the critical dilution rate D_c is reached (when $D = \mu_{max}$) or exceeded, culture "washout" occurs.

Because the chemostat extends to the experimenter a unique

means of manipulating and controlling the culture environment, it offers distinct advantages over other cultural methods.

1.1.2. Application of continuous culture to filamentous fungi: Aim of the present investigation

The traditional surface culture method for filamentous fungi has severe limitations for most physiological studies. The development of aerial, surface and submerged hyphae, each with its particular physiological condition (Smith & Galbraith, 1971) produces a heterogeneous growth form which is difficult to analyse in terms of precise environmental effects. The application of submerged liquid culture and in particular, the continuous flow technique has enabled this problem to be resolved and has also facilitated progress in areas, hitherto neglected. This technique, however, poses additional problems not encountered during cultivation of unicellular micro-organisms.

One of the difficulties concerns the concept of exponential growth in filamentous fungi. Since individual hyphae grow by linear extension, exponential growth requires the production of new branches at a rate proportional to the rate of increase in cell mass. Several workers have shown that filamentous fungi do grow exponentially in submerged culture (Zalokar, 1959a; Pirt & Callow, 1960; Borrow, Brown, Jeffreys, Lessell, Lloyd, Lloyd, Rothwell, Rothwell & Swait, 1964; Trinci, 1969) but the frequent aggregation of hyphae into compact masses or pellets produces a rate of growth which is cube root in nature (Pirt, 1966). Because pellet growth is normally restricted to a peripheral zone (Trinci, 1970), a culture which contains pellets is heterogeneous and is therefore, undesirable for most physiological and metabolic studies. Prevention of pellet formation, in part, is dependent upon the shearing action of the agitation system which breaks up large aggregates of hyphae.

However, since certain conditions, such as the frequency of hyphal branching and the composition of the medium may influence the sensitivity of the organisms to shear, and hence the degree of pellet formation (Righelato, 1974), it may be impossible to obtain filamentous growth under all cultivation conditions.

The accumulation of mycelial masses on the internal surfaces of culture vessels, which is thought to arise from an electrostatic attraction between the organism and the surfaces involved (Munson & Bridges, 1964), constitutes a major problem in growing filamentous organisms. Such surface growth is to be avoided because of the possible errors it may introduce into mathematical analyses of continuous culture systems. Adequate design of the culture apparatus (see Figure 1.2) can effectively reduce surface growth (Rowley & Bull, 1973). Internal surfaces coated with Teflon or consisting of highly polished stainless steel discourage mycelial accretions and the use of the paddle-type impeller operating close to the fermenter base provides an extremely efficient agitation system which again is vital for the control of surface growth. In addition, by operating the fermenter nearly full all internal surfaces are subjected to continuous washing, a procedure which tends to dislodge any hyphal aggregates that develop.

Now that the major obstacles have been overcome the use of continuous flow techniques for mycelial cultivation has become more widespread. The work of Pirt and his associates (Pirt & Callow, 1959, 1960; Righelato, Trinci, Pirt & Peat, 1968) especially opened the way for critical analysis of fungal development, in particular the vegetative phase. For example, glucose limited growth of Aspergillus nidulans in chemostats has been obtained at different dissolved oxygen tensions and growth rates, and various aspects of intermediary metabolism analysed (Carter & Bull, 1969, 1971). Production of secondary metabolites, such as melanin, has been
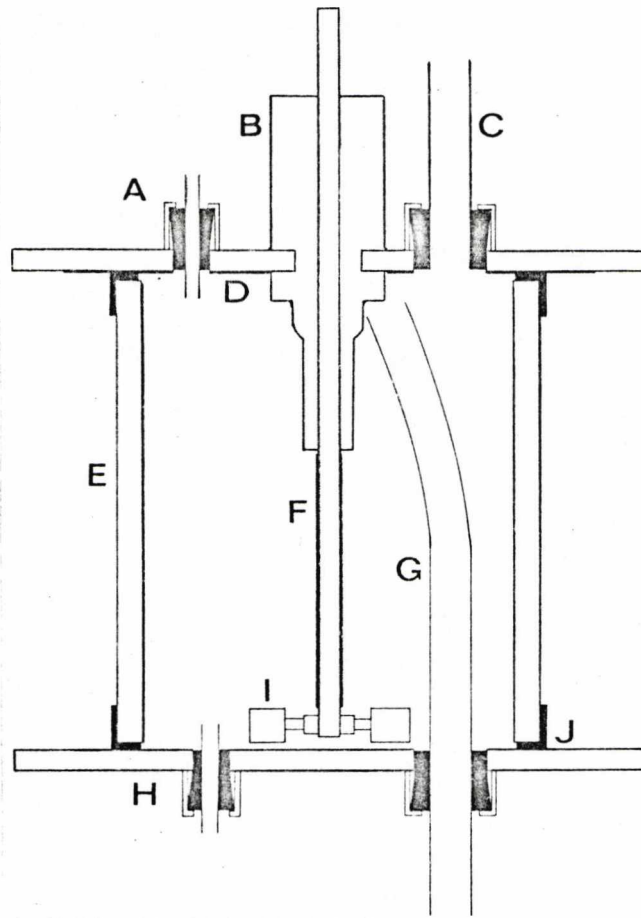
Figure 1.2. Chemostat vessel for mould cultivation. A, inlet
(teflon tube) for air or medium; B, stirring
gland; C, outlet (teflon tube) for sampling
effluent gas; D, stainless steel top-plate of
vessel against the underside of which is fitted a
teflon sheet (thickness, 1 mm); E, pyrex glass
pipe section; F, stirrer shaft covered by teflon
tube; G, teflon overflow pipe (normally a common
effluent line for gas and spent culture); H,
culture sampling line (teflon tube); I, paddle
type stirrer; J, neoprene rubber gasket.
(From Rowley & Bull, 1973).

studied in the same organism under different environmental conditions, also using the continuous culture technique (Rowley & Pirt, 1972). Although considerable attention has been focussed on specific metabolic processes few attempts have been made to study systematically the structure and composition of the cell wall under rigidly controlled conditions. This is surprising when one considers that wall polysaccharides constitute major cellular products. In addition, because the wall is the structure which immediately restricts and hence controls cell extension our understanding of hyphal growth could be greatly enhanced by studies of wall synthesis under various cultural conditions.

To date the only detailed chemostat investigation of fungal wall composition is confined to the yeast Saccharomyces cerevisiae which has been examined at different growth rates under varying substrate limitations (McMurrough & Rose, 1967). The apparent lack of information of this kind regarding mycelial walls prompted the present study, which explores the phenotypic variability of wall composition in Aspergillus nidulans in response to a changing external environment. In the absence of comparable work with other moulds or even yeast this investigation must be regarded as of a purely preliminary nature.

A review of the current status of the fungal wall follows.

1.2. THE FUNGAL WALL

1.2.1. Structure and morphogenesis

The fungal cell wall is a composite biological material comprising a microfibrillar skeletal mesh embedded in a continuous matrix of amorphous material. In filamentous fungi the microfibrillar components of the wall are composed of (1) chitin, a polymer of unbranched chains of β-1,4 linked 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine) residues, (2) chitosan, a non-acetylated

polymer of D-glucosamine (in some Phycomycetes), or (3) cellulose, a polymer of β-1,4 linked D-glucose units. The microfibrillar element of yeast walls consists of non-cellulosic glucans comprised of main chains of β-1,6 linked D-glucose residues to which linear side chains of β-1,3 linked residues are attached. Polysaccharides constitute the major portion of the matrix together with lesser amounts of protein and lipid. Melanin, polyphosphate and inorganic ions may also be present (Bartnicki-Garcia, 1968) and although some evidence exists for the presence of nucleic acids (Bartnicki-Garcia & Nickerson, 1962) this is likely to derive from cytoplasmic contamination. A variety of sugars has been found in wall polysaccharides and these include hexosamines, hexoses, hexuronic acids, methylpentoses and pentoses (Table 1.1). Glucose and N-acetylglucosamine are the most common building blocks while D-mannose occurs in small amounts in most mycelial fungi and is abundant in yeast walls. Less frequently found are the following monosaccharides, which are restricted to certain groups of fungi (Bartnicki-Garcia, 1968): D-galactose and D-galactosamine (Ascomycetes), L-fucose (Mucorales and Basidiomycetes), D-glucosamine (Mucorales), xylose (Basidiomycetes) and D-glucuronic acid. Other sugars such as rhamnose, ribose and arabinose have been reported as minor components of fungal walls. The chemical association between individual wall components has received scant attention. However, isolated reports indicate the existence of glycopeptides (Emiliani & Ucha De Davie, 1962; Ballesta & Villanueva, 1971; Babczinski & Tanner, 1973; Wessels & De Vries, 1973; Le'John, Cameron, Stevenson & Meuser, 1974), mannolipids (Barr & Hemming, 1972) and phosphomannans (Bretthauer, Wilkens & Hansen, 1963; Mill, 1966).

While conventional techniques have revealed the complexity of hyphal wall chemistry, relatively little is known concerning the distribution and arrangement of the individual polymers. Electron

Table 1.1. Polysaccharide components of fungal walls.

Polymer	Linkages	Monomers
Aminopolysaccharides		
Chitin	β-1,4	N-acetylglucosamine
Chitosan	β-1,4	D-glucosamine
Neutral polymers		
Cellulose	β-1,4	D-glucose
β-glucan	β-1,3; 1,6	D-glucose
α-glucan	α-1,3; 1,4	D-glucose
Glycogen	α-1,4	D-glucose
Mannan	α-1,2; 1,6; 1,3	D-mannose
Polyuronides		
Pullularia	α-1,6; 1,5-	D-glucose,
heteropolysaccharide	β-1,3; 1,6-	D-glucuronic acid,
		D-galactose,
		D-mannose.
Mucoran		L-fucose, D-mannose,
		D-galactose,
		D-glucuronic acid

From Bartnicki-Garcia (1970).

microscope observations have shown multi-layered walls and the localisation of certain components has been achieved by using sequential enzymic digestion methods combined with electron micros- copy. Such analyses have led to the proposal that the innermost region of the wall is microfibrillar (Mahadevan & Tatum, 1967; Livingston, 1969; Hunsley & Burnett, 1970) but some controversy exists regarding the nature of the outer layers. Some workers believe that an inner chitinous layer is covered by a layer of glucan (Mahadevan & Tatum, 1967; Livingston, 1969) while others hold the view that chitin and glucan are separated by an intervening layer of protein fibrils (Hunsley & Burnett, 1970). The observations of Hunsley & Burnett (1970) have also revealed structural differ- ences between the walls of young (growing) and mature (non-growing) hyphae. In Neurospora crassa for example, the older walls are characterised by glycoprotein reticulum development and thickening of the outermost glucan region, while in apical walls of Schizophyllum commune and Phytophthora parasitica less S-glucan (an α-1,3 glucan; Bacon, Jones, Farmer & Webley, 1968) and protein, respectively, are found than in sub-apical regions.

That the wall is the key determinant of cell shape has been inferred from the universal demonstration that the fungal protoplast has a spherical configuration. Studies of wall biochemistry have enhanced our understanding of fungal development, with the result that morphological changes can be correlated with alterations in wall composition (Bartnicki-Garcia & Nickerson, 1962; Bartnicki- Garcia, 1968). The role of individual components in the shape- determining process, however, is not well documented and may vary from organism to organism. Mutants of Aspergillus nidulans with chitin deficient walls exhibit normal hyphal morphology (Katz & Rosenberger, 1970a) and chemical inhibition of chitin synthetase in Neurospora does not affect the overall hyphal shape (Endo, Kakiki &

Misato, 1970). However, a phosphohexoseisomerase mutant of Neurospora shows an altered phenotype when grown on glucose, while growth on glucose plus fructose reverses the morphological effects (Murayama, 1969). The accompanying differences in the wall composition of glucose-grown cells (Murayama, 1969) may be the result of a deficiency in chitin formation since the first reaction in the pathway utilises fructose-6-phosphate. The tight colonial morphology shown by the wild-type strain of Neurospora when grown in the presence of high concentrations of L-sorbose has been correlated with a reduced level of β-1,3 glucan (Mahadevan & Tatum, 1965). Since L-sorbose is known to inhibit an extracellular polysaccharide degrading enzyme in Schizophyllum (Wilson & Niederpruer, 1967) the implication is that it induces phenotypic variation by inhibiting an enzymic process involved in the extracellular assembly of glucan fibrils in the wall. The importance of β-1,3 glucan synthesis in vegetative morphogenesis is also demonstrated by the grossly altered phenotypes of mutants partially blocked in the synthesis of this polymer (Brody & Tatum, 1966, 1967).

Although wall polysaccharides play a decisive role in vegetative morphogenesis, other components may also be important. The triangular cells of the yeast _Trigonopsis variabilis_, induced by growth in choline or methionine media contain a much higher level of phospholipid in their wall fraction than the ellipsoidal form (Sentheshanmuganathan & Nickerson, 1962). Using chemostat cultures, Johnson (1975) has further demonstrated that morphogenesis in this organism is influenced by the rate of growth as well as by the availability of carbon and nitrogen. Variations in wall lipid content have also been noted in different cellular forms of the yeasts _Candida albicans_ (Bianchi, 1968) and _Saccharomyces cerevisiae_ (McMurrough & Rose, 1967). Control of normal growth and morphology in _Neurospora crassa_ may be determined by the quantity of peptide in

the wall (Wrathall, Richard & Tatum, 1974), while specific changes
in wall protein, such as the differences in the amino acid composit-
ion of Saccharomyces cerevisiae walls, elicited by growth in
inositol deficient medium, produce walls which are structurally
different from and weaker than the normal type (Power & Challinor,
1969).

1.2.2. Biogenesis

Hyphal growth involves the deposition of new wall material at the
apex. Complete understanding of this process requires a definition
of the sites where wall precursors are synthesised and where poly-
merisation of monomeric units and their assembly occur. Ultra-
structural studies have shown an increased degree of vesicular diff-
erentiation in the apical region of cells which extend by tip growth
(Zalokar, 1959b; Moor, 1967; Brenner & Carroll, 1968; McClure, Park
& Robinson, 1968; Girbardt, 1969; Grove & Bracker, 1970; Grove,
Bracker & Morré, 1970; Matile, Cortat, Wiemken & Frey-Wyssling,
1971) and the involvement of these apical vesicles in wall synthesis
has been frequently suggested. Generation of vesicular material for
wall expansion may reside ultimately with the endoplasmic reticulum
(Marchant, Peat & Banbury, 1967; Moor, 1967) and in some cases may
involve the mediation of the Golgi (dictyosome) complex (Figure 1.3).
Chemical analysis of dictyosome membranes has indicated that they
have a composition intermediate between that of the endoplasmic
reticulum and that of the plasmalemma (Keenan & Morré, 1970). Thus,
it would appear that the dictyosome is the site where membrane
material is modified to resemble that of the plasmalemma so that
exocytosis may readily occur. In fungi lacking dictyosomes little is
known of the membrane transformation process.

While autoradiographic studies have provided direct evidence
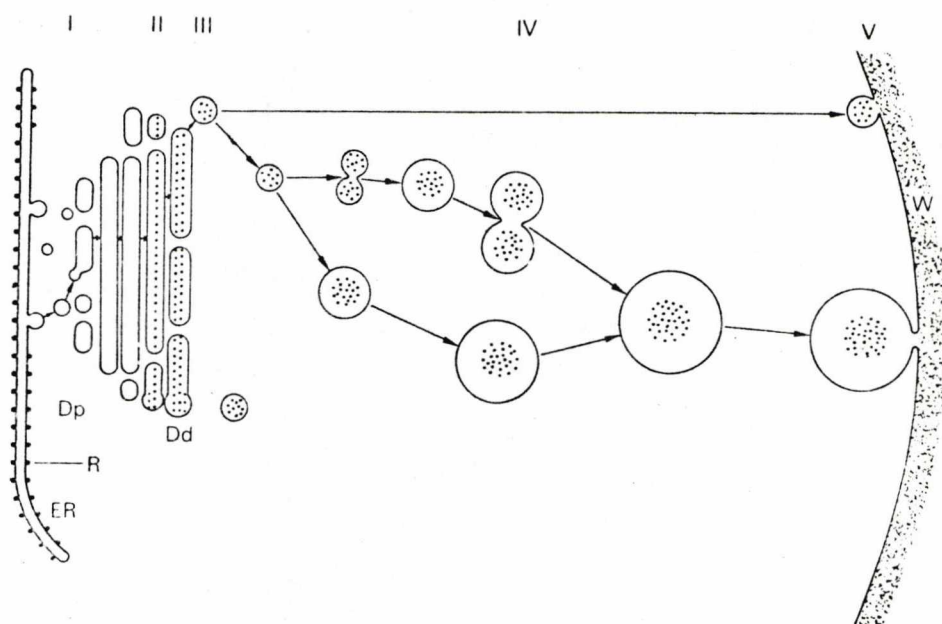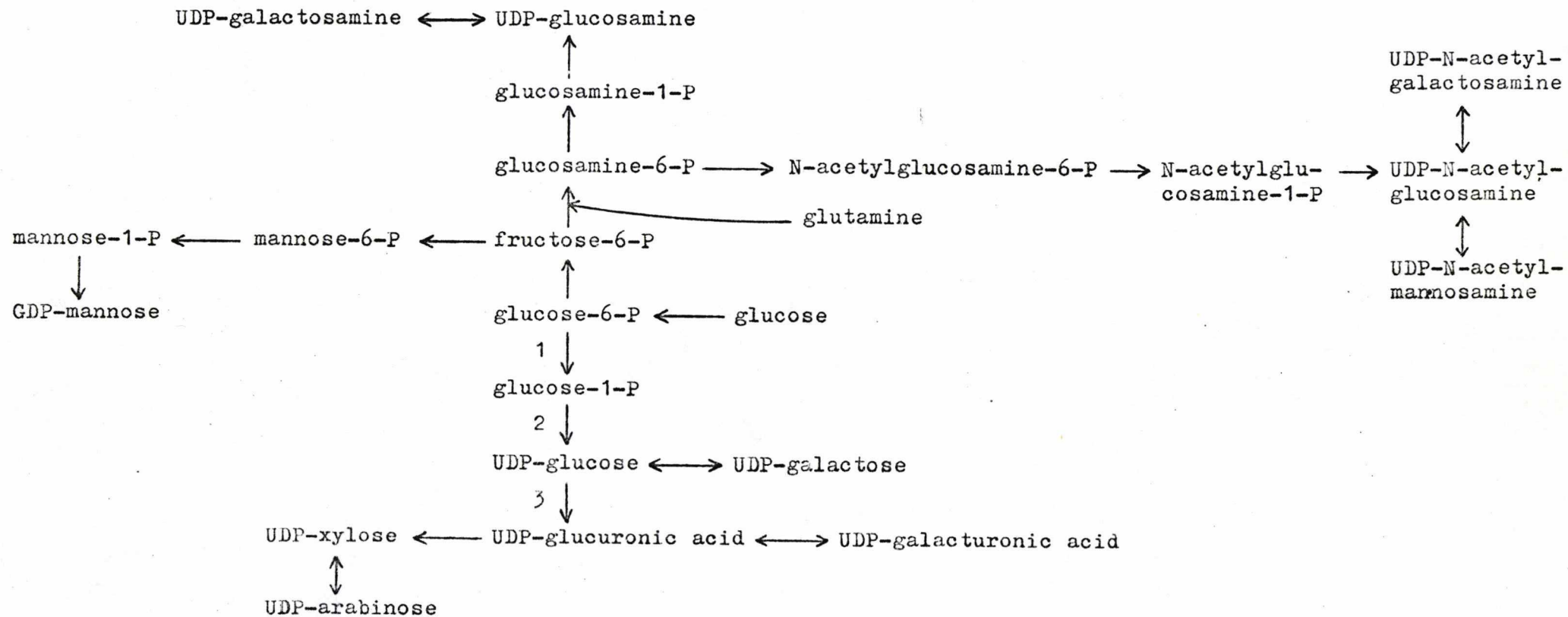for the flow of wall precursor material from the interior to the

Figure 1.3. Diagrammatic interpretation of the sequence leading
to expansion of a hypha at the apex. I. Material is
transferred from ER to dictyosome by blebbing of ER
and refusion of vesicles to form a cisterna at the
proximal pole of the dictyosome (Dp). II. Cisternal
contents and membranes are transformed as the
cisterna is displaced to the distal pole (Dd) by
the continued formation of new cisternae. III.
Cisternae vesiculate to form secretory vesicles as
they approach and reach the distal pole. IV.
Secretory vesicles migrate to the hyphal apex. Some
may increase in size or fuse with other vesicles to
form large secretory vesicles, while others are
carried directly to the cell surface. V. Vesicles
accumulate in the apex and fuse with the plasma
membrane, liberating their contents into the wall
region. Key to labelling: endoplasmic reticulum, ER;
ribosome, R; cell wall, W.
(From Grove, Bracker & Morré, 1970).

exterior of cells of higher plants (Northcote & Pickett-Heaps, 1966; Northcote & Wooding, 1966; Wooding, 1968) little definitive information is available concerning this process in fungi. There is much circumstantial evidence, however, for the view that some wall polymers are formed in the cytoplasm and then transported to their final destination in the wall, while others are synthesised in situ, either at the outer plasmalemma surface or within the wall fabric. Katz & Rosenberger (1970b) isolated a cytoplasmic polysaccharide containing the neutral sugars, glucose, galactose, mannose and arabinose from Aspergillus nidulans and found that the ratio of these sugars resembled closely the ratio of sugars present in the wall. Electron microscopic observations of the apical vesicles of Gilbertella persicaria have shown them to contain amorphous material similar in appearance to the amorphous matrix of the cell wall (Bracker, 1971), while investigations of encysting zoospores of Phytophthora preclude the existence of preformed microfibrillar glucan in the "peripheral" vesicles which fuse with the plasmalemma (Bartnicki-Garcia, 1973a). Fractionation of hyphae of Phytophthora cinnamomi (Wang & Bartnicki-Garcia, 1966) and Neurospora crassa (Mishra & Tatum, 1972) have revealed the presence of sugar nucleotide transferases for the synthesis of a β-1,3; 1,6 linked glucan and a β-1,3 glucan respectively, mainly in the wall fraction. The finding that Saccharomyces fragilis protoplasts release uridine-diphospho-glucose, the substrate for these enzymes, across the plasmalemma (Rost & Venner, 1968) is consistent with the theory that wall glucan synthesis occurs outside the plasmalemma. In Mucor rouxii chitin synthetase activity has been demonstrated in the apical region of the hypha and corresponds to the sites of N-acetyl-D-glucosamine deposition (McMurrough, Flores-Carreon & Bartnicki-Garcia, 1971). Some activity was also found associated with the particulate fraction of the hyphae and was considered to represent

enzyme en route to its site of operation. Studies of freeze-etched yeast cells further indicate that wall material arises from an organised part of the plasmalemma. Regular hexagonal areas have been observed on the outer plasmalemma surface of Saccharomyces cerevisiae and these consist of particles which are connected by fibres to the inner layer of the wall (Moor & Muhlethaler, 1963; Northcote, 1968). Freeze fracture studies of the unicellular green alga Oocystis have also revealed an intimate association between extending glucan chains and organised areas within the membrane (interpreted as microfibrillar enzyme complexes) during cellulose biosynthesis (Brown & Montezinos, 1976), an observation which may be applicable also to fungal cells.

The biosynthesis of wall polymers involves the formation of the appropriate precursors (Figure 1.4), their subsequent polymerisation and, in some cases, modifications of oligomeric subunits by substitution or other reactions. Fungal studies have been restricted to a few individual polysaccharides, one of which, chitin, is synthesised from the same precursor by all the fungi so far examined (Bartnicki-Garcia, 1968). Enzyme preparations from a variety of organisms have been found to catalyse the sequential transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to an endogenous acceptor (Glaser & Brown, 1957; Jaworski, Wang & Carpenter, 1965; Porter & Jaworski, 1966; Camargo, Dietrich, Sonneborn & Strominger, 1967; Keller & Cabib, 1971) thereby producing an insoluble reaction product which can be chemically characterised as chitin. In addition, polyoxin D, a specific competitive inhibitor of the chitin synthetase of Neurospora crassa, inhibits incorporation of N-acetylglucosamine into wall chitin and leads to the accumulation of UDP-N-acetylglucosamine (Endo, Kakiki & Misato, 1970). Whether the polymerisation of N-acetylglucosamine monomers requires a template-termination mechanism, as has been suggested for cellulose biosynthesis in

UDP-galactosamine ⟷ UDP-glucosamine

UDP-N-acetyl-galactosamine

↑

glucosamine-1-P

↑

glucosamine-6-P ⟶ N-acetylglucosamine-6-P ⟶ N-acetylglu-cosamine-1-P ⟶ UDP-N-acetyl-glucosamine

↕

UDP-N-acetyl-galactosamine

glutamine

mannose-1-P ⟵ mannose-6-P ⟵ fructose-6-P

↕

UDP-N-acetyl-mannosamine

↓

GDP-mannose

↑

glucose-6-P ⟵ glucose

1 ↓

glucose-1-P

2 ↓

UDP-glucose ⟷ UDP-galactose

3 ↓

UDP-xylose ⟵ UDP-glucuronic acid ⟷ UDP-galacturonic acid

↕

UDP-arabinose

1   phosphoglucomutase

2   UDP-glucose pyrophosphorylase

3   UDP-glucose dehydrogenase

Figure 1.4.   The biosynthesis and interconversion of nucleoside diphosphate sugars important in fungal wall biosynthesis.

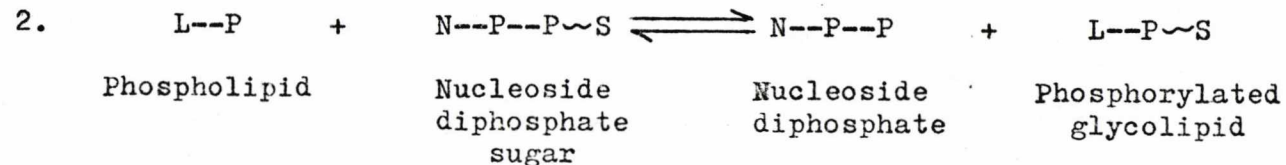higher plants (Marx-Figini, 1969), has yet to be elucidated.

Uridine diphosphate sugars serve as the glycosyl donors for the synthesis of a wide range of polysaccharides, including the β-1,3; 1,6 linked glucan of Phytophthora cinnamomi (Wang & Bartnicki-Garcia, 1966) and the polyuronides of Mucor rouxii (Bartnicki-Garcia, 1968). However, nucleoside diphosphate sugars with bases other than uracil are functional in glycosyl transfer. Mannan synthesis in Saccharomyces carlsbergensis (Algranati, Carminatti & Cabib, 1963) and phosphomannan synthesis in Hansenula holstii (Bretthauer, Wilkens & Hansen, 1963) require GDP-diphosphomannose as precursor.

The transglycosylation of mono- or oligomeric subunits into fungal wall polysaccharides may involve phosphorylated glycolipids as intermediates. Such lipid-linked sugar carriers have been demonstrated in bacterial systems (Robbins, Bray, Dankert & Wright, 1967; Lennarz & Scher, 1972; Strominger, Higashi, Sandermann, Stone & Willoughby, 1972) where the lipid moiety has been characterised as a C_{55}-polyisoprenoid alcohol. The modes of synthesis of the functional glycolipids in bacteria is summarised in Table 1.2. Among the fungi, glycoprotein biosynthesis in yeast has received most attention. The carbohydrate component of this polymer is mannan and the catalytic transfer of mannose from GDP-D-mannose into dolichol monophosphate has been shown to produce a mannosyl phosphoryl dolichol, which is functional in mannosyl transfer to the peptide portion of the mural glycoprotein (Babczinski & Tanner, 1973). A mannosyl polyprenyl phosphate has also been implicated in mannolipid and mannan synthesis in Aspergillus niger (Barr & Hemming, 1972). Whether lipid intermediates are specifically concerned in facilitating reactions which occur within the hydrophobic environment of the membrane system (dictyosomes, vesicles and plasmalemma) has need of investigation.

Table 1.2. Modes of synthesis and properties of glycolipid intermediates functional in the formation of carbohydrate-containing polymers.

1.   L--P   +   N--P--P$\sim$S $\rightleftharpoons$ N--P   +   L--P--P$\sim$S

Phospholipid   Nucleoside   Nucleoside   Phosphorylated
                      diphosphate   monophosphate   glycolipid
                      sugar
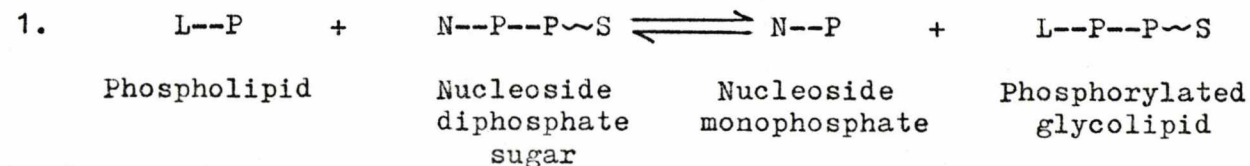
(Salmonella O-antigen; Robbins, Bray, Dankert & Wright, 1967; Staphylococcus aureus peptidoglycan; Strominger, Higashi, Sandermann, Stone & Willoughby, 1972).

2.   L--P   +   N--P--P$\sim$S $\rightleftharpoons$ N--P--P   +   L--P$\sim$S

Phospholipid   Nucleoside   Nucleoside   Phosphorylated
                      diphosphate   diphosphate   glycolipid
                      sugar

(Micrococcus lysodeikticus mannan; Lennarz & Scher, 1972).

3.   L--P--P$\sim$S'   +   N--P--P$\sim$S" $\longrightarrow$ L--P--P--S'$\sim$S"   +   N--P--P
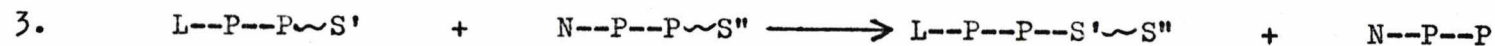
(Acetobacter xylinum cellulose, where S' = S" = glucose; Kjosbakken & Colvin, 1973).

From Kauss (1974a).

Regulation of wall biosynthesis in fungal cells has received little attention and so any consideration of this process must, inevitably, remain largely speculative. However, it is probable that control can be exerted at one or more levels along the biosynthetic route. The flow of precursor material into the pathways leading to polymer formation (Figure 1.4) is dependent upon the synthesis of appropriate hexose phosphate intermediates. Specific impedance of the reactions which produce these intermediates may, therefore, affect the polymeric composition of the wall. Mutants of Neurospora with a defective phosphoglucomutase accumulate glucose-1-phosphate and have a reduced level of β-1,3 glucan in the wall as compared to the wild type (Brody & Tatum, 1967), while in Aspergillus nidulans phosphoglucomutase activity determines the level of α-1,3 glucan (Zonneveld, 1975). Wall glucan synthesis is also impaired in a Neurospora mutant which accumulates glucose-6-phosphate dehydrogenase (Brody & Tatum, 1966) the enzyme which catalyses the first step in the pentose phosphate shunt. Allosteric control of N-acetylglucosamine synthesis in liver is effected by UDP-N-acetylglucosamine (Kornfeld, Kornfeld, Neufeld & O'Brien, 1964) and the formation of certain nucleotide sugars may be restricted by the levels of others (Bernstein & Robbins, 1965; Young & Arias, 1967): similar regulatory controls may operate in fungal cells. The enzymes, UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase from lily pollen are inhibited in vitro by one or more sugar nucleotides and, may, therefore be likely sites of in vivo feedback inhibition (Dickinson, Hopper & Davies, 1973). The synthesis of chitin in fungi necessitates a strict regulatory mechanism since this polymer is deposited only in specific regions of the cell wall (see for example, Cabib & Keller, 1971; Katz & Rosenberger, 1971). Proteinaceous inhibitors of chitin synthetase activity have been located in the cytoplasm of Saccharomyces (Cabib & Farkas, 1971) and Mucor rouxii (McMurrough &

Bartnicki-Garcia, 1973) and it would appear that the enzyme is produced in a zymogen state and acquires an active configuration at its site of operation by means of an "activating factor" which removes the masking protein. The final stages of wall synthesis, secretion and assembly into the wall also constitute potential rate-limiting steps. In the latter case it is conceivable that a localised enzyme needed for the assembly of one polymer would actually require the presence of a different polymer for activity at the site of wall growth or would require the presence of a particular site on the wall that was not masked by an inhibitory protein (Yabuki & Fukui, 1970).

1.2.3. Hyphal growth: Apical extension

It has long been established that fungal hyphae extend by synthesising new wall material at the apex (Robertson, 1965; Marchant & Smith, 1968; Bartnicki-Garcia & Lippman, 1969; Gooday, 1971; Katz & Rosenberger, 1971). Vesicles carrying materials and enzymes for growth of the plasmalemma and wall are believed to originate from a subapical region and are transported to the apex where the incorporation of material into the wall fabric occurs by reverse pinocytosis. The mechanism for the movement of the vesicles seems to be part of the general cytoplasmic streaming process but electrophoretic movement has been suggested as a plausible mechanism (Jaffe, 1968). Although maximum incorporation of wall precursors occurs at or within 1μm of the apex, a gradient of wall deposition exists along the hyphal filament (Gooday, 1971) and a subapical segment of length 100 - 200μm may be involved in growth (Trinci, 1969). Localised sites of wall synthesis may also form at subapical positions giving rise to lateral branches in which apical dominance of wall synthesis again prevails. Although the determinant(s) of branch formation has yet to be identified, the observations of Katz and coworkers (Katz &

Rosenberger, 1971; Katz, Goldstein & Rosenberger, 1972) indicate
that localised accumulation of wall precursor material in the cyto-
plasm may trigger branching at predetermined sites along the hyphal
filament.

The process of hyphal elongation is seemingly dependent upon
the internal turgor pressure (Robertson, 1968). Turgor may be re-
quired solely to maintain the plasmalemma tightly appressed against
the apical wall, thus permitting the flow of materials to the apical
wall. However, it is unlikely that turgor alone is responsible for
wall expansion. The insertion of new wall material at the apex
necessitates a mechanism for "opening up" the existing wall
structure. The role of wall-bound autolytic enzymes in bacterial and
plant wall biosynthesis is well established (Morré & Eisinger, 1968;
Ray, 1969; Fan & Beckman, 1971; Forsberg & Rogers, 1971; Higgins &
Shockman, 1971), while presumptive evidence exists for a similar
situation in fungal cells. Increased levels of wall-bound proteases
and glucanases are associated with hyphal branching (Mahadevan &
Mahadkar, 1970) and conidial germination (Mahadevan & Rao, 1970) in
Neurospora crassa and cleistothecial production in Aspergillus
nidulans is dependent upon the activity of a wall degrading α-1,3
glucanase (Zonneveld, 1972). In addition, endo-β-1,3 glucanases have
been demonstrated specifically in the wall fraction of yeast cells
(Barras, 1972; Fleet & Phaff, 1973). Such observations lead to the
inference that fungal walls have the capacity for localised diss-
olution which would allow intussusception of new material.

A hypothetical scheme for cell wall growth, involving wall
degrading enzymes has been proposed by Bartnicki-Garcia (1973b). The
scheme considers that net wall growth results from the cumulative
action of minute units of wall growth, where each unit represents
the contribution from either a single vesicle or a minimum combinat-
ion of vesicles. For simplicity the model (Figure 1.5) depicts the
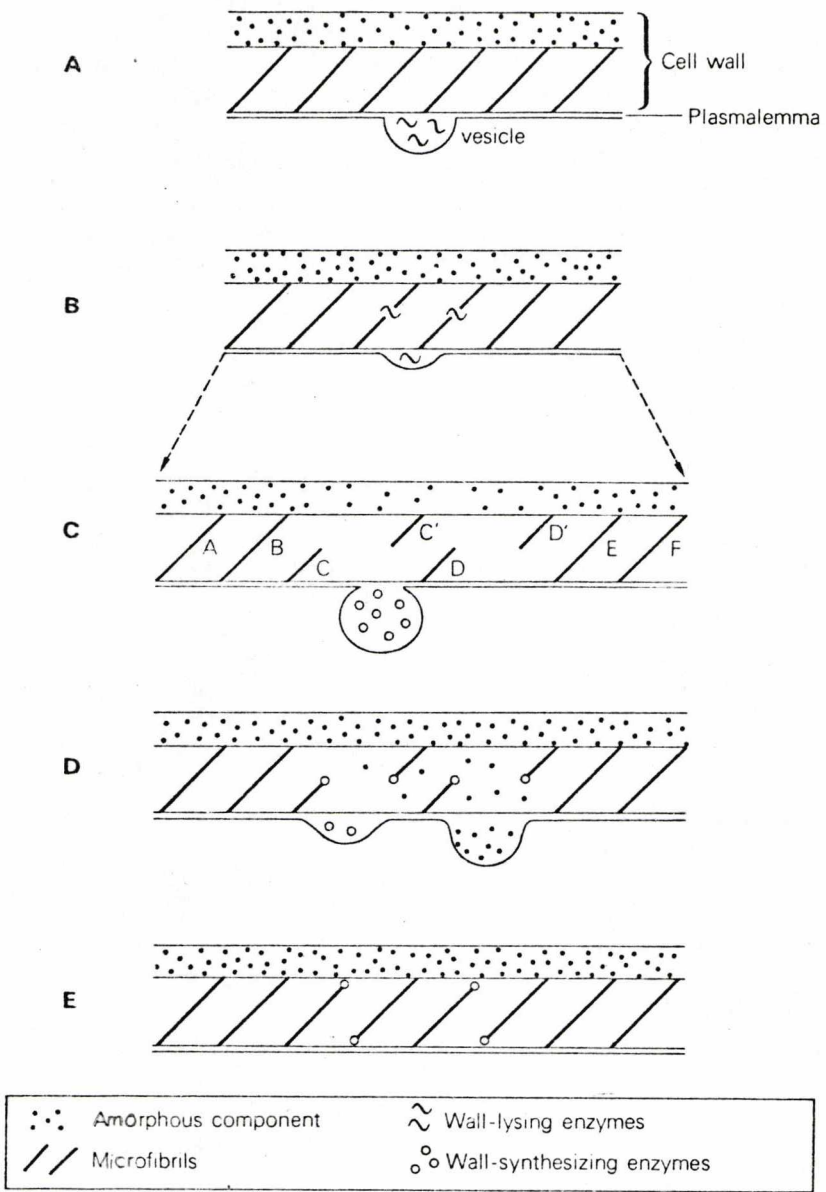
Figure 1.5. Hypothetical representation of the events in
a unit of cell wall growth.
(From Bartnicki-Garcia, 1973b).

wall as a two component system, comprising amorphous and micro-
fibrillar elements. The process of wall growth is initiated by the
secretion of lytic enzymes from a vesicle into the wall fabric (A).
Subsequent enzymic cleavage of either inter- or intramolecular bonds
in the microfibrillar skeleton produces a weakened complex (B) which
cannot withstand the high turgor pressure of the cell and, in con-
sequence, becomes stretched and less integrated, thereby allowing an
increase in surface area to occur (C). The dissociated microfibrils
are reconstructed by synthetases situated in the wall or on the
outer plasmalemma surface, and preformed amorphous material is
forced into the microfibrillar network by turgor pressure (D). As a
result the cell wall has expanded by one unit area, and the co-axial
arrangement of the polymers remains intact (E). Although in the
model the enzymes and matrix material are shown arriving at the
plasmalemma in separate vesicles it is possible that these compon-
ents may be carried in one or two types of vesicle only. The finding
by Cortat and coworkers (Cortat, Matile & Wiemken, 1972) of vesicles
containing both exo- and endo-β-1,3 glucanases, as well as mannan,
protein and mannan synthetase in budding yeast provides support for
this view and also demonstrates that wall synthetases and wall lytic
enzymes may be transported and discharged in a similar manner.

If this model of apical growth is correct, accumulation of the
growth units at the region of the cell surface that was to become
the apical pole of the hyphal tube would be predicted. The ultimate
shape and diameter of the hypha would be determined by the spatial
distribution of the postulated growth units and by the relative
ratios of biosynthetic and lytic components in these units. A wide
range of environmental conditions might be expected to influence
such a complex interaction with possible morphological manifestat-
ions. Thus, orderly growth of the wall necessitates a harmonious
balance between the factors for wall synthesis and wall lysis, as

well as a mechanism for rendering inactive synthetic and lytic enzymes present in the non-growing regions of the wall. Wall synthetases may become inactivated through depletion of wall precursor material (Bartnicki-Garcia, 1973b) or by means of endogenous inhibitory proteins of the type found in yeast (Cabib & Keller, 1971) and Mucor rouxii (McMurrough & Bartnicki-Garcia, 1973). Hydrolases, however, may be inherently unstable or become actively destroyed by proteases associated with the wall. It is interesting to note in this respect that endogenous enzymes which hydrolyse plant cell walls are unstable (Morré & Eisinger, 1968; Cleland, 1970).

CHAPTER 2. MATERIALS AND METHODS

2.1. GROWTH PROCEDURES

2.1.1. Organism

A hyaline mutant, 13 mel (originally designated 13. 1. OL) of
Aspergillus nidulans (Eidam) Winter (Bull & Faulkner, 1965) (ATCC
28270) was used throughout this work.

2.1.2. Culture medium

A chemically defined liquid growth medium (Carter & Bull, 1969),
consisting of glucose, nitrate, inorganic salts chelated with EDTA
and phosphate buffer was used for all experiments. Medium constit-
uents were of AR quality and were as follows (g/l): glucose, 10 to
15; $NaNO_3$, 6.0; KH_2PO_4, 10.0; $Na_2HPO_4.12H_2O$, 9.5; EDTA. $Na_2.2H_2O$,
0.60; NaOH, 0.10; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.05; $ZnSO_4.7H_2O$, 0.02;
$MnSO_4.4H_2O$, 0.02; $CuSO_4.5H_2O$, 0.005; $FeSO_4.7H_2O$, 0.10; Na_2SO_4, 0.50;
$Na_2MoO_4.2H_2O$, 0.005.

For solid media the concentrations of the buffer components
i.e. KH_2PO_4 and $Na_2HPO_4.12H_2O$ were reduced to 2.5 and 2.4 g/l
respectively, and agar (Oxoid agar No. 3, Oxoid Ltd., London, S.E.1)
was added to a final concentration of 1.5% (w/v).

Phosphate buffer, nitrate and inorganic salts were autoclaved
together at 15 lb pressure/in^2 for 30 min. Glucose was autoclaved
separately at 10 lb pressure/in^2 for 30 min.

Where NaCl was used, this was incorporated into the phosphate
buffer-nitrate-inorganic salts reservoir.

2.1.3. Preparation of inoculum

Cultures of the organism were routinely maintained at $30^{o}C$ on
defined medium agar and used to provide spore inocula for shake
flask cultures. After a 24 to 48 h growth period the contents
(normally 200 ml) of the flask cultures were blown aseptically into
the chemostat vessel. Inocula prepared in this manner consisted of

exponentially growing, vegetative hyphae.

2.1.4. Fermenter design and operation

(a) Basic apparatus

For all experiments an L. H. Engineering (Stoke Poges, England) fermenter unit was used in conjunction with a glass culture vessel holding at full capacity a working volume of 3.5 l. A schematic figure of the fermenter assembly is depicted in Figure 2.1. The vessel was provided with stainless steel top and base plates. All necessary additions to the growing culture were made via ports situated in the top plate, through which air was also supplied and through which overflowing culture and effluent gases escaped. Vortex aeration was effected by means of a motor-driven, stainless steel, six-bladed paddle-type impeller operating 2 to 3 mm from the fermenter base at a speed of 1100 rev/min. Rapid culture sampling was achieved via a port located at the base of the vessel.

Accumulation of fungal growth on internal stainless steel surfaces was overcome by coating the surfaces with Teflon tubing (Polypenco Ltd., Welwyn Garden City, Herts). This was necessary in order to minimise possible errors in culture dry weight determinations which could have arisen by loss of biomass from the immediate culture environment.

The chemostat vessel and attachments were sterilised by autoclaving at 15 lb pressure/in^2 for 1 h. Medium reservoirs, control agents (acid, alkali and antifoam) and a culture overflow device were connected aseptically to the culture vessel, which was later half filled with defined medium and subsequently inoculated with vegetative hyphae. The culture was allowed to grow batchwise for 24 h before medium flow was commenced.
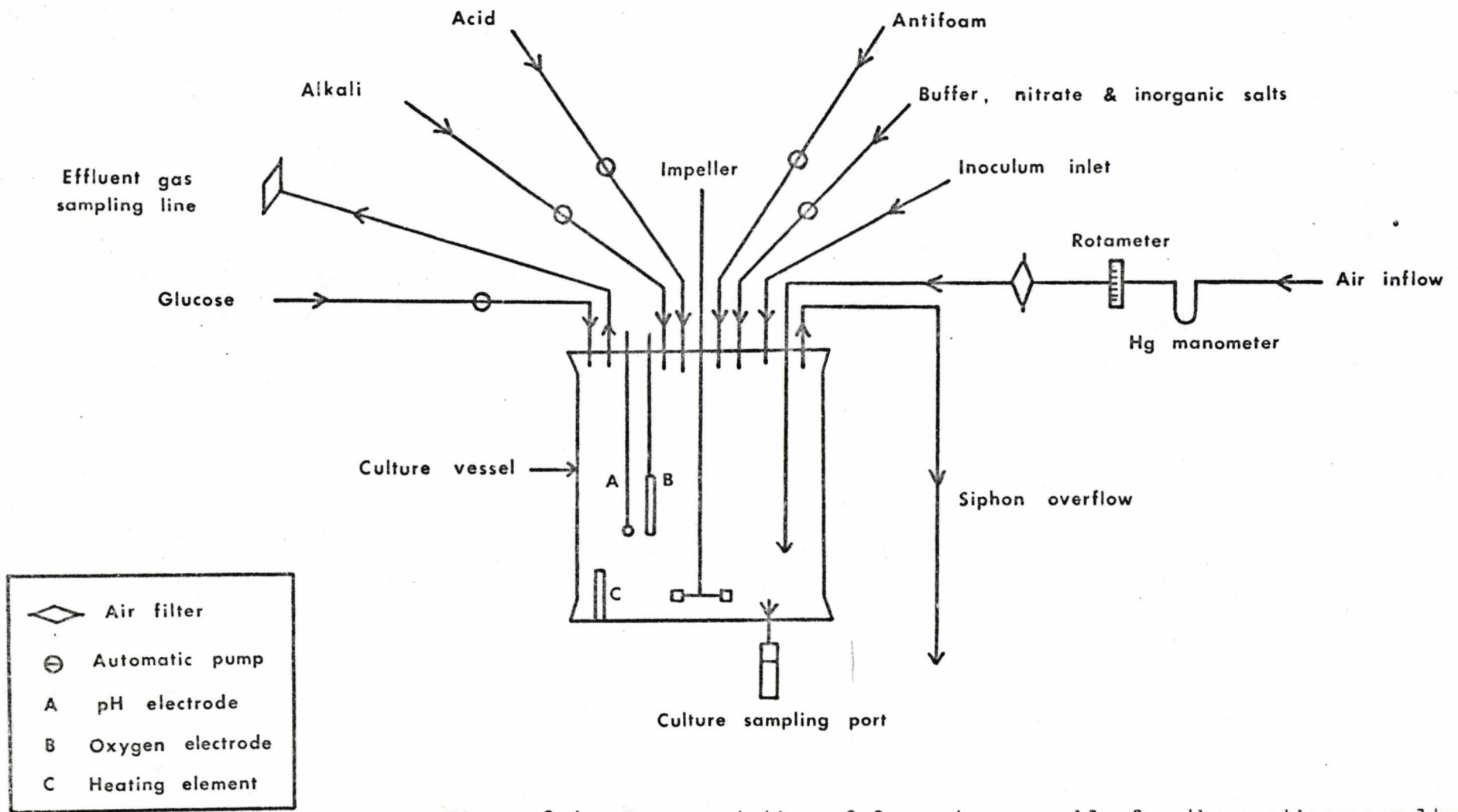
Figure 2.1. Representation of fermenter assembly for the continuous culture of <u>Aspergillus</u> <u>nidulans</u>.

(b) Control systems

(1) Medium

The flow rates of media entering the chemostat vessel were control-
led by means of Watson-Marlow H. R. Flow Inducers (Falmouth, Corn-
wall, England). Glucose, at a concentration of 400 g/l, was supplied
as a separate feed from all other constituents in order to minimise
growth on the medium inlet tubes and prevent blockage.

(2) Oxygen

Air entering the fermenter vessel was sterilised by passage through
air filters (E. N. Mackley & Co., Gateshead, Durham) and the air
flow rate measured by a rotameter (M. F. G. Co. Ltd., England). Aer-
ation was achieved by means of an impeller operating at 1100 rev/min
and aeration efficiency was enhanced by sparging air directly into
the culture through a Teflon tube submerged in the culture fluid.
Dissolved oxygen tension was monitored using an oxygen electrode
(Pfizer Ltd., Sandwich, Kent) and was maintained at 40% saturation
by adjusting the air inflow rate.

(3) Temperature

The culture was heated by an electric element incorporated into the
base of the culture vessel. The temperature was maintained to within
$\pm$ 0.5°C of a fixed value by a device consisting of a resistance
thermometer immersed in the culture fluid and connected to a temp-
erature control unit (Nobel Instruments Ltd.).

(4) pH

A pH control unit connected to an EIL glass electrode (EIL, Rich-
mond, Surrey) actuated peristaltic pumps for the automatic addition
of 2 M H_2SO_4 or 2 M KOH to maintain the culture pH at the desired
level. The maximum fluctuation was 0.05 pH units.

(5) Antifoam

The automatic addition of 0.1 to 0.2 ml of polypropylene glycol P2000 (Dow Chemical Co. (U.K.) Ltd.) per litre of culture medium every 4 h overcame the problem of foaming.

2.1.5. Culture treatments

(a) Sampling

Samples for analyses were removed aseptically from the chemostat vessel via a silicone rubber tube fitted with a sampling hood. For dry weight determinations and glucose analyses samples were collected in 20 ml aliquots. The first 10 ml was discarded as being unrepresentative of the bulk material, i.e. dead space material. Samples of 1.5 to 2.0 l were collected and lyophilised for large scale analyses once a steady state had been established. A steady state was assumed on the basis of the constancy of measured culture parameters (biomass, residual glucose concentration, rates of oxygen uptake and carbon dioxide evolution), usually after allowing 4 to 5 culture volumes to pass through the culture vessel.

(b) Culture purity checks

During the course of fermenter experiments daily checks were carried out on culture purity and strain stability by streaking samples onto nutrient and defined medium agar and incubating at 30°C. Cultures showing contamination were immediately abandoned.

(c) Mycelial dry weight

Culture samples (10 ml) were filtered through sintered glass filters, porosity 2 (SintaGlass, A. Gallenkamp & Co. Ltd., London) and washed twice with distilled water. The mycelial mat was enclosed in preweighed aluminium foil and dried to constant weight at 110°C.

(d) Glucose

Non-metabolised glucose in the culture filtrate was determined with

the glucose oxidase/peroxidase reagent (Boehringer Mannheim GMBH).

(e) Gas analyses

Effluent gas from the culture was analysed for its oxygen and carbon

dioxide contents. Carbon dioxide was collected and measured after

washing with 3% (w/v) KOH in an Orsat apparatus (A. Gallenkamp & Co.

Ltd., London). The oxygen content was determined after passage

through a paramagnetic oxygen analyser (Mark II, Servomex Controls

Ltd., Crowborough, Sussex); the instrument was first standardised

with oxygen-free nitrogen (British Oxygen Co. Ltd., North Wembley,

Middlesex) and the inflow air (oxygen content 20.95%) to the

culture.

2.1.6. Calculations

(a) Respiratory parameters

(1) Specific rate of oxygen consumption (Q_{O_2})

The recorded air inflow rate was first corrected for temperature,

pressure and water vapour content:

Corrected air inflow = recorded air inflow X a X b X c
rate (l/h)   rate (l/h)

where a = temperature correction factor

$$= \frac{273}{273 \;+\; temp \; (^{\circ}C) \; of \; inflowing \; air}$$

b = air pressure correction factor

$$= \frac{(atmospheric \; press. \;+\; culture \; back \; press.) \; (mm \; Hg)}{760}$$

(Culture back pressure was measured by means of a manometer

connected to the air inlet line).

$$c = \text{water vapour correction factor} = 0.984$$

$$
\begin{array}{ccc}
O_2 \text{ consumed by culture} & = & O_2 \text{ in air supply} & - & O_2 \text{ in effluent gas} \\
(1/h) & & (1) & & (1)
\end{array}
$$

$$= O_2 \text{ inflow rate} - \frac{nx}{1 - (z + x)}$$

where n = throughput of N_2 plus inert gases (1/h)

= corrected air inflow rate X 0.7905

x = fractional O_2 content of effluent gas

z = fractional CO_2 content of effluent gas

and O_2 inflow rate = corrected air inflow rate X 0.2095

Q_{O_2} (mmol/g biomass/h)

$$= \frac{\text{mmol } O_2 \text{ consumed/h}}{\text{total culture biomass (g)}}$$

$$= \frac{O_2 \text{ consumed (1/h)}}{22.4} \quad X \quad \frac{1000}{\text{culture vol (1) X biomass(g/1)}}$$

(2) Specific rate of carbon dioxide production (Q_{CO_2})

$$
\begin{array}{ccc}
CO_2 \text{ produced by culture} & = & CO_2 \text{ in effluent} & - & CO_2 \text{ in inflowing} \\
(1/h) & & \text{gas (1)} & & \text{air (1)}
\end{array}
$$

The CO_2 content of inflowing air is too small to measure in the Orsat apparatus and is therefore ignored.

Therefore,

$$
\begin{array}{ccc}
CO_2 \text{ produced by culture} & = & CO_2 \text{ in effluent gas} \\
(1/h) & & (1)
\end{array}
$$

$$= \frac{nz}{1 - (z + x)}$$

Q_{CO_2} (mmol/g biomass/h)

$$= \frac{\text{mmol } CO_2 \text{ produced/h}}{\text{total culture biomass (g)}}$$

$$= \frac{CO_2 \text{ produced (1/h)}}{22.4} \quad X \quad \frac{1000}{\text{culture vol (1) X biomass (g/1)}}$$

(3) Respiratory quotient (RQ)

$$RQ = \frac{Q_{CO_2} \text{ (mmol/g biomass/h)}}{Q_{O_2} \text{ (mmol/g biomass/h)}}$$

(b) Specific rate of glucose consumption ($q_{glucose}$)

The rate of consumption of substrate in the culture at any given time is given by

$$\frac{ds}{dt} = qx \qquad (xxi)$$

where x is the biomass and q, the specific metabolic rate.

In a small time interval, dt, the substrate consumed for growth is

$$ds = \frac{\mu x}{Y} dt \qquad (xxii)$$

where μ is the specific growth rate and Y, the growth yield.

Hence

$$\frac{ds}{dt} = \frac{\mu x}{Y} \qquad (xxiii)$$

and comparison of Equations (xxi) and (xxiii) shows that

$$q = \frac{\mu}{Y} \qquad (xxiv)$$

Therefore, $q_{glucose}$ (g glucose/g biomass/h) is given by

$$\left[\frac{\text{glucose concentration in feed (g/1)} \quad - \quad \text{glucose concentration in culture (g/1)}}{\text{biomass (g/1)}} \right] \quad X \quad D \qquad (xxv)$$

$(\mu = D \ (h^{-1})$ in the steady-state).

(c) <u>Determination of the maximum specific growth rate from washout kinetics</u>

In the chemostat the organism balance is given by

Increase in organism = growth resultant — overflow loss
concentration   biomass   of biomass

$$\frac{dx}{dt} = \mu x - Dx \qquad (xxvi)$$

and rearranging

$$dt = \frac{dx}{x(\mu - D)} \qquad (xxvii)$$

During culture washout x varies with time and $\mu = \mu_{max}$,

$$\int_0^t dt = \frac{1}{(\mu_{max} - D)} \int_0^x \frac{1}{x} dx \qquad (xxviii)$$

which upon integration becomes

$$t = \frac{1}{(\mu_{max} - D)} \log_e(x - x_o) \qquad (xxix)$$

and

$$\mu_{max} = \frac{1}{t} \log_e(x - x_o) + D \qquad (xxx)$$

The slope of the washout graph ($\log_e x$ vs. t) is

$$- \frac{1}{t} \log_e(x - x_o)$$

Therefore

$$\mu_{max} = D - slope \qquad (xxxi)$$

2.2.   WHOLE CELL ANALYSES

2.2.1.   Nucleic acids

(a)   Extraction

Hyphal material was extracted by the method of Herbert, Phipps &
Strange (1971). Lyophilised mycelium (50 mg) was suspended in 5 ml
of ice-cold 0.25 N $HClO_4$ for 30 min., with occasional shaking, to
remove acid-soluble compounds.

To extract RNA the residue was suspended in 4 ml of 0.5 N $HClO_4$
for 2 h at 37^oC. After centrifugation the residue was washed once
with 0.5 N $HClO_4$ and the combined extracts made up to 10 ml with
0.5 N $HClO_4$. RNA in the extract was determined with the orcinol
reagent.

DNA was extracted, after the initial removal of acid-soluble
material, by adding 4 ml of 0.5 N $HClO_4$ to the centrifuged residue
and heating at 70^oC for 15 min. with occasional shaking. The
suspension was centrifuged and the extraction repeated twice more
with 3 ml volumes of 0.5 N $HClO_4$. The extracts were pooled, brought
to 10 ml with 0.5 N $HClO_4$ and DNA determined with the diphenylamine
reagent.

(b)   Determination

(1)   RNA

The orcinol method of Dische (Herbert, Phipps & Strange, 1971) was
followed to estimate RNA. To 1 ml of sample, 3 ml of freshly pre-
pared orcinol reagent (1 vol. of 1% w/v aqueous orcinol: 4 vols. of
0.09% w/v $FeCl_3.6H_2O$ in concentrated HCl) were added. The solutions
were heated in a boiling water bath for 20 min., cooled and made up
to 15 ml with n-butanol. Absorbances were measured at 672 nm and
compared with the values obtained with standard RNA (Baker's yeast
RNA, type XI, Sigma Chemicals).

(2) DNA

DNA was measured with the diphenylamine reagent of Burton (Herbert, Phipps & Strange, 1971). To 2 ml of sample, 2 ml of diphenylamine reagent (10 vols. of 1.5% v/v steam distilled diphenylamine in redistilled glacial acetic acid: 0.15 vol. of concentrated H_2SO_4) containing 0.08 mg acetaldehyde/ml were added. The solutions were incubated for 16 to 20 h at $30^{\circ}C$ after which absorbances were measured at 600 nm. A standard curve of DNA (Calf thymus DNA, type I, Sigma Chemicals) was prepared after mixing equal volumes of DNA solution (400 µg DNA/ml 5 mM NaOH) and 1 N $HClO_4$ and heating for 15 min. at $70^{\circ}C$.

2.2.2. Protein

The method of Lowry, Rosebrough, Farr & Randall (Herbert, Phipps & Strange, 1971) was used to determine protein, with bovine serum albumin, fraction V (Sigma Chemicals) as a standard.

2.2.3. Total carbohydrate

Total cell carbohydrate was assayed by the anthrone procedure (Herbert, Phipps & Strange, 1971). Cell suspensions (1 ml) were cooled to $0^{\circ}C$ in an ice-water bath and 5 ml of ice-cold anthrone reagent (200 mg anthrone in 5 ml of absolute ethanol, made up to 100 ml with 75% v/v H_2SO_4) added. The solutions were mixed and allowed to stand for 5 min at $0^{\circ}C$ before being transferred to a boiling water bath for 10 min. The solutions were then cooled and absorbances measured at 625 nm. Glucose was used as a standard.

2.2.4. Minerals

(a) Wet ashing

Whole mycelia were wet-ashed by the method of Chen, Toribara & Warner (1956). Mycelium (10 mg) was digested with 1 ml of

concentrated H_2SO_4 and 72% (v/v) $HClO_4$ was added dropwise to clarify
the digest. Ashed solutions were diluted to an appropriate volume
with deionised water and analysed for total phosphorus, potassium
and magnesium.

(b) Determination

(1) Phosphorus

Total phosphorus was estimated by the method of Dryer, Tammes &
Routh (1956) using the n-phenyl-p-phenylene-diamine monohydrochlor-
ide (semidine) chromogen (Kodak-Eastman). To 1 ml of sample 0.2 ml
of 0.008 M ammonium molybdate was added and the solutions mixed.
Semidine reagent (50 mg % semidine in 1% v/v $NaHSO_3$) (2 ml) was then
added and the solutions allowed to stand at room temperature for
10 min. Absorbances were read at 770 nm and related to a standard
curve of phosphorus (prepared from KH_2PO_4 of AR quality) in the
range 0 to 10 µg/ml.

(2) Potassium and magnesium

Wet-ashed solutions were analysed in an atomic absorption spectro-
photometer (Pye Unicam SP90A Series 2) using air-acetylene mixtures.
Potassium standards were prepared from AR quality KCl and measure-
ments made at 766.5 nm. A stock magnesium solution (from which
standards were made up by further dilution) was prepared by dissolv-
ing 0.10 g of oxide-free magnesium ribbon (Fisons Ltd.) in a minimum
quantity of concentrated HCl (AR grade) and making up to 1 l with
deionised water. Magnesium measurements were made at 285.2 nm. All
measurements were made in the absorption mode and operating condit-
ions for each element were as described in the Pye Unicam SP90A
handbook.

2.2.5. Amino acid pool

Lyophilised mycelium (10 mg) was extracted with 2.5 ml of 0.25 N $HClO_4$ at $0°C$ for 30 min, centrifuged and the supernatant retained. The residue was re-extracted with 1 ml of 0.25 N $HClO_4$. Both extracts were combined, brought to pH 5.0 with 1 N NaOH and diluted with water to 5 ml. The amino acid content of the extract was determined by the method of Yemm & Cocking (1955).

Neutralised extract (1 ml) was mixed with 0.5 ml of 0.2 M citrate buffer, pH 5.0 (21.008 g citric acid monohydrate dissolved in 400 ml of 0.5 N NaOH and diluted to 500 ml), 0.2 ml of ninhydrin reagent (5 g ninhydrin (Fisons Ltd.) in 100 ml of redistilled 2-methoxyethanol (B.D.H. Ltd.)), and 1 ml of KCN-2-methoxyethanol (5 ml of 0.01 M KCN made up to 250 ml with 2-methoxyethanol). The solutions were heated in a boiling water bath for 15 min and cooled. Aqueous ethanol (60% v/v) (3 ml) was added and the solutions well mixed before the absorbances were read at 570 nm. Analytical grade alanine (Fisons Ltd.) was used as a standard.

2.3. ANALYSES OF HYPHAL WALLS

2.3.1. Preparation of hyphal walls

Hyphal walls were obtained from lyophilised mycelia by the method of Bull (1970). The dry mycelial powder (1.5 g) was suspended in 50 ml of 0.033 M sodium phosphate buffer, pH 6.8 containing 0.01% (w/v) merthiolate (Eli Lilly & Co., Ltd., Basingstoke, England) to prevent microbial growth and macerated in a glass-teflon homogeniser (Jencons "Uniform", Hemel Hempstead) driven by an electric motor at maximum speed. The supernatant was removed by centrifugation at 800 g for 10 min and the residue resuspended in buffer. Aliquots (15 ml) of the homogenised mycelial suspension were disrupted by 2 X 1 min bursts from an ultrasonic disintegrator (M.S.E., 150 Watt) operating at full power, the suspension being maintained below $12°C$

in an ice-salt bath. Wall material was recovered by centrifugation
(800 g for 10 min) and subjected to two further cycles of sonic
oscillation. The final residue was collected at 1400 g for 10 min
and washed with chilled water until the supernatant fluid became
clear. The wall material was then freeze-dried and stored in a
vacuum desiccator over P_2O_5 prior to analyses. Microscopic examin-
ation of the wall material after staining with lactophenol cotton
blue confirmed the absence of cytoplasmic contamination.

2.3.2. Infra-red spectrophotometry

Samples of wall material were finely powdered with infra-red quality
KBr (Fisons Ltd.) to a concentration of 1% (w/w) and pressed into
12 mm discs under vacuum. Spectra were recorded in a Perkin-Elmer
model 457 spectrophotometer over the range 4000 to 250 cm^{-1}.

2.3.3. Carbohydrate

(a) Acid hydrolyses of wall material

Hydrolyses in HCl were performed as described by Bartnicki-Garcia
(1966) using 2 mg wall/ml acid in tubes sealed under N_2. After
hydrolysis insoluble residues were removed by filtration and the
solutions neutralised with NaOH and diluted to an appropriate
volume.

For neutral sugar determinations hydrolysis proceeded for 3 h
in 3 N HCl at $100^{\circ}C$; for amino sugars 6 h in 6 N HCl at $100^{\circ}C$; and
for uronic acid 3 h in 1 N HCl at $100^{\circ}C$.

(b) Chromatography

Neutralised solutions from acid hydrolysates were concentrated by
lyophilisation, dissolved in pyridine and centrifuged to remove
NaCl. Samples were applied to Whatman No. 1 chromatography paper in
100 μl aliquots and subjected to a descending chromatographic

system, the paper being irrigated with n-butanol-pyridine-water
(6: 4: 3 v/v) for 26 h. Neutral sugars were located with alkaline
silver nitrate (Trevelyan, Proctor & Harrison, 1950) and amino sugar
with the Elson-Morgan reagent (Smith, 1960). Mobilities were related
to glucose.

(c) Quantitative determination of neutral sugars

Total neutral sugar was determined by the anthrone procedure (see
Section 2.2.3.).

Glucose and galactose were measured enzymically with Biochemica
Test Combinations (Boehringer Mannheim GMBH): glucose with the
glucose oxidase/peroxidase reagent; galactose with galactose dehy-
drogenase, the $NADH_2$ formed from galactose and NAD being measured at
340 nm.

Mannose was determined from paper chromatograms by measuring
spot areas (Fisher, Parsons & Morrison, 1948): spot area was propor-
tional to log_{10} mannose concentration.

(d) Total hexosamine

The basic procedure of Rondle & Morgan (1955) for hexosamine, as
modified by Herbert, Phipps & Strange (1971), was adopted. To 1 ml
of sample was added 1 ml of acetylacetone reagent (2.5 vols. of re-
distilled acetylacetone: 50 vols. of 1 M $NaHCO_3$/1 M Na_2CO_3 buffer,
pH 9.6). The solution was mixed, heated for 20 min in a boiling
water bath and cooled. Ethanol (5 ml) was added, followed by 1 ml of
p-DMAB reagent (0.8 g p-dimethylaminobenzaldehyde dissolved in 30 ml
of ethanol and made up to 60 ml with concentrated HCl) and finally
2 ml of ethanol. The solutions were mixed gently and transferred to
a water bath at 65°C for 10 min. The absorbances of cooled solutions
were measured at 530 nm; α-D-glucosamine HCl (Koch-Light) was used
as a standard.

(e) Hexuronic acid

The carbazole reagent described by Bitter & Muir (1962) was used for the determination of hexuronic acid. Sodium tetraborate solution (0.025 M sodium tetraborate in concentrated H_2SO_4) (5 ml) was added to 1 ml of sample, the solutions mixed and placed in a boiling water bath for 10 min. After cooling, 0.2 ml of 0.125% (w/v) carbazole in ethanol was added to the mixture, which was then heated for 15 min in a boiling water bath. The samples were allowed to stand for 16 h at room temperature prior to measurement of absorbance at 530 nm; sodium glucuronate (Koch-Light) was used as a standard.

(f) Extraction of alkali-soluble glucan

Lyophilised wall material (50 mg) was heated for 30 min at $75^{o}C$ with 0.5 M acetic acid (3 ml), centrifuged and washed with acetic acid and then, extracted with 5% (w/v) KOH (4 ml) for 18 h at $37^{o}C$ (Zonneveld, 1971). After centrifugation the residue was washed once with 5% (w/v) KOH and both extracts were combined. The extract was adjusted to pH 7 by the addition of glacial acetic acid and the resultant white precipitate centrifuged, washed 3 times with water and dried to constant weight.

2.3.4. Protein

See Section 2.2.2.

2.3.5. Phosphorus

See Section 2.2.4.(b)(1).

2.3.6. Cation binding capacities

Freeze-dried walls (2 mg) were suspended in 2 ml volumes of 1 mM "cation chloride" ($MgCl_2$, $CaCl_2$, KCl and NaCl) solution and left to equilibrate for 2 h at room temperature (method modified from Meers

& Tempest, 1970). Walls were separated by centrifugation for 15 min at 3000 g and washed repeatedly with deionised water until the bound cation content was constant. The walls were then wet-ashed, diluted to an appropriate volume and adsorbed cation measured by atomic absorption spectrophotometry (see Sections 2.2.4.(a) and 2.2.4.(b)(2)). Calcium and sodium standards were prepared with $CaCO_3$ and NaCl, and measurements were made at 422.7 nm and 594 nm, respectively.

2.4. ELECTRON MICROSCOPY

2.4.1. Electron microscopy of hyphae

Mycelial material, freshly harvested from chemostat cultures was fixed with 2.5% (w/v) glutaraldehyde and centrifuged to remove culture liquor. The material was then resuspended in a phosphate buffered, 2.5% (w/v) glutaraldehyde solution, pH 7, containing 2% (w/v) sucrose and fixation allowed to proceed for 2 h. After washing 3 times in water, the material was post-stained in 1% (w/v) OsO_4 for 1 h. The material was then washed in 3 changes of water, each of 5 min duration and dehydrated by a graded ethanol series, changes being made at 15 min intervals. The fixed material was embedded initially in 30% (v/v) Spurr's resin (Spurr, 1969) and then in a series of resin dilutions up to 100% (v/v) resin. The resin was polymerised overnight at $70^{\circ}C$ and the embedded material sectioned with an L.K.B. Ultrotome III 8800 (L.K.B. Produkter AB, Sweden) using glass knives. Sections were mounted on 3 mm copper grids and stained with 5% (v/v) aqueous uranyl acetate in 1% (v/v) acetic acid for 30 min, followed by alkaline lead citrate (Reynolds, 1963) for 10 min. Sections were viewed in an AEI EM6G electron microscope and photographs taken at an instrument magnification of X 2500. Measurements of hyphal diameter and wall thickness were made directly from photographic plates.

2.4.2. Shadowing of wall preparations

Aqueous suspensions of wall material were dried onto formvar coated copper grids and then shadowed with gold-palladium (Polaron, Finchley, London) at an angle of 45° using an Edwards 306 Coater.

CHAPTER 3.   THE EFFECT OF STEADY STATE GROWTH RATE

ON THE PHYSIOLOGY OF <u>ASPERGILLUS</u> <u>NIDULANS</u>

3.1. INTRODUCTION

Before the advent of continuous flow techniques for cell cultivation various methods were adopted for altering the specific growth rate of an organism. The most common of these methods involved manipulation of the environmental temperature or the nutritional status of the culture medium. However, the use of such methods precluded a separate assessment of the influence of growth rate and other cultural factors on microbial physiology. The development of continuous culture techniques and their application to a vast range of cell types has rendered it possible to examine growth at any desired rate, within a range defined by the inherent characteristics of an organism, in a constant culture environment.

Investigations of bacterial and yeast growth in chemostats have demonstrated that growth rate exerts a considerable influence on metabolic activity and gross cellular composition (Tempest & Herbert 1965; Tempest, Hunter & Sykes, 1965; Harder & Veldkamp, 1967; McMurrough & Rose, 1967). Variations, also, in the chemical composition of the cell wall have been noted in response to changes in the growth rate (Collins, 1964; McMurrough & Rose, 1967; Tempest & Ellwood, 1969; Ellwood, 1970; Ellwood & Tempest, 1972), thus substantiating earlier findings of altered wall composition at different stages of batch growth (Sud & Schaechter, 1964; Young, 1965). Similar investigations with mycelial fungi, however, are relatively few in number and relate to the effects of growth rate on morphology (Righelato, Trinci, Pirt & Peat, 1968), growth kinetics (Righelato, Trinci, Pirt & Peat, 1968; Carter, Bull, Pirt & Rowley, 1971; Fiddy & Trinci, 1975), intermediary carbon metabolism (Carter & Bull, 1969) and secondary metabolite production (Rowley & Pirt, 1972). The present report presents information relating to the effect of growth rate on the mycelial and hyphal wall composition of the mould, Aspergillus nidulans 13 mel.

3.2. RESULTS

3.2.1. Hyphal growth

As a preliminary to chemostat experiments, Aspergillus nidulans
13 mel was grown from a vegetative inoculum in stirred 3 l batch
culture. The defined nutrient medium containing glucose as the car-
bon and energy source was used and pH and temperature were maintain-
ed at pH 6.8 and $30^{o}C$ respectively. Growth took the form of homo-
geneous, filamentous mycelium and lag, exponential and decline
phases of growth were clearly defined (Figure 3.1a). The maximum
specific growth rate, μ_{max}, attained during the exponential phase of
growth (Figure 3.1b), was 0.201 h^{-1} (t_{d} = 3.5 h).

For chemostat experiments, glucose, the growth limiting sub-
strate, was present in the culture medium at a concentration of
15 g/l and pH and temperature were controlled at pH 6.8 and $30^{o}C$
respectively; culture pH remained steady independently of automatic
control. Steady state growth was achieved over the range 0.02 h^{-1} to
0.175 h^{-1}. However, at a dilution rate of 0.20 h^{-1}, corresponding to
μ_{max} in batch culture, steady state conditions were not established
and culture washout occurred. Over the entire range of dilution
rates studied growth was filamentous and the mycelium was only
sparsely branched; however, at the lowest dilution rate, D = 0.02 h^{-1},
vegetative hyphae were branched, and conidia were present in the
culture. Under these conditions conidia were produced from phialides
borne terminally or laterally on undifferentiated hyphae.

Microscopic examination of mycelia grown at different rates in-
dicated that hyphal size and wall thickness were independent of D
(Table 3.1).

Figure 3.2a shows the variation in mycelial production, growth
limiting substrate concentration and growth yield as a result of
changes in the dilution rate. Mycelial production assumed a steady
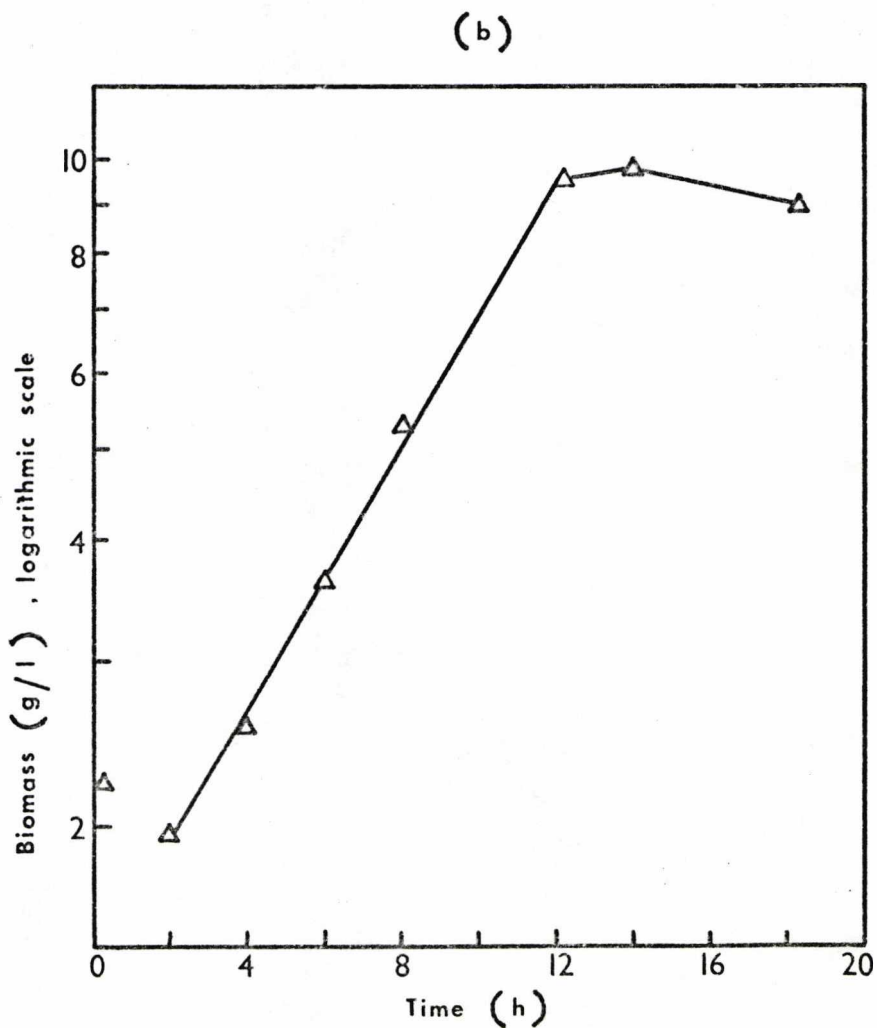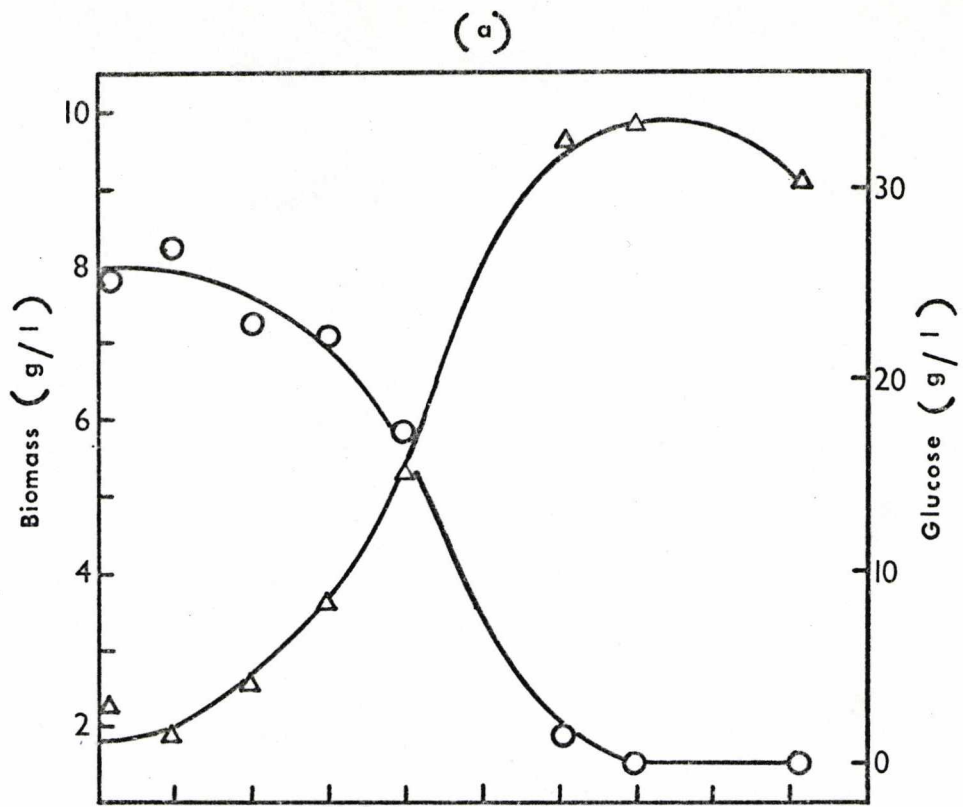level at values of D between 0.07 h^{-1} and 0.15 h^{-1}, but declined

Figure 3.1. Batch culture of <u>Aspergillus</u> <u>nidulans</u> 13 mel.
(a) biomass, Δ ; residual glucose concentration,
O ;— linear scale; (b) biomass — logarithmic
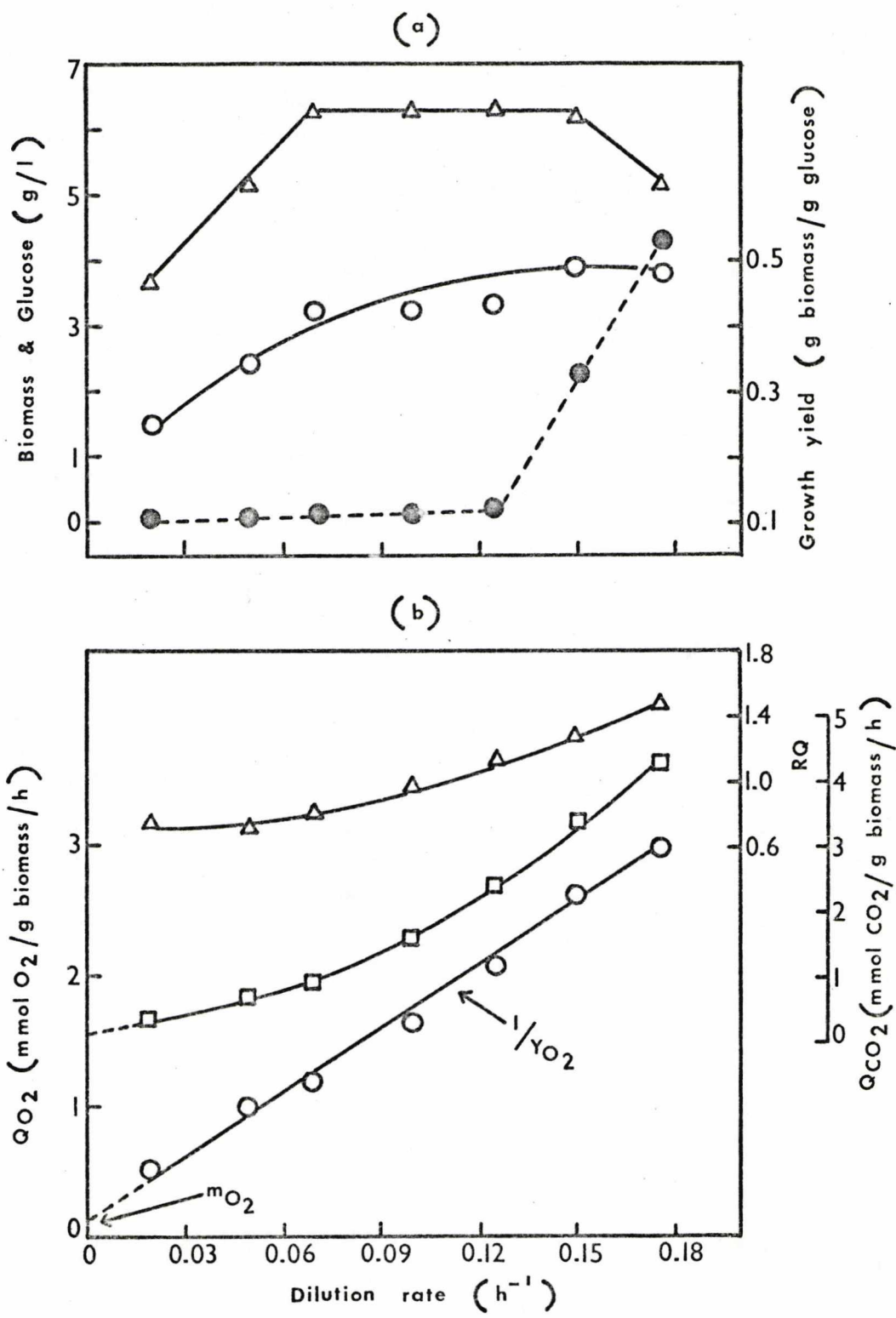scale.

Figure 3.2.   Effect of dilution rate on: (a) biomass production,
△ ; growth limiting substrate concentration, ● ;
growth yield, ○ ; (b) Q_{O_2}, ○ ; Q_{CO_2}, □ ; RQ, △ .

Table 3.1.   Hyphal size and wall thickness at different dilution
rates.

Dilution rate (h^{-1})	Hyphal diameter (μm)	Wall thickness (μm)
0.02	0.94 ± 0.17^a	0.061 ± 0.005^a
0.05	1.17 ± 0.14	0.067 ± 0.010
0.07	1.11 ± 0.16	0.066 ± 0.006
0.10	0.93 ± 0.13	0.064 ± 0.005
0.125	1.08 ± 0.16	0.062 ± 0.003
0.15	1.10 ± 0.03	0.061 ± 0.006
0.175	1.11 ± 0.01	0.058 ± 0.007

Each result is the mean of 10 measurements.
^{a}Standard deviation.

towards extreme values outside this range. The concentration of non-
metabolised glucose in the culture medium rose significantly once D
had exceeded a value of 0.125 h^{-1}. The growth yield, which increased
steadily as D was raised, maintained a constant value over the range
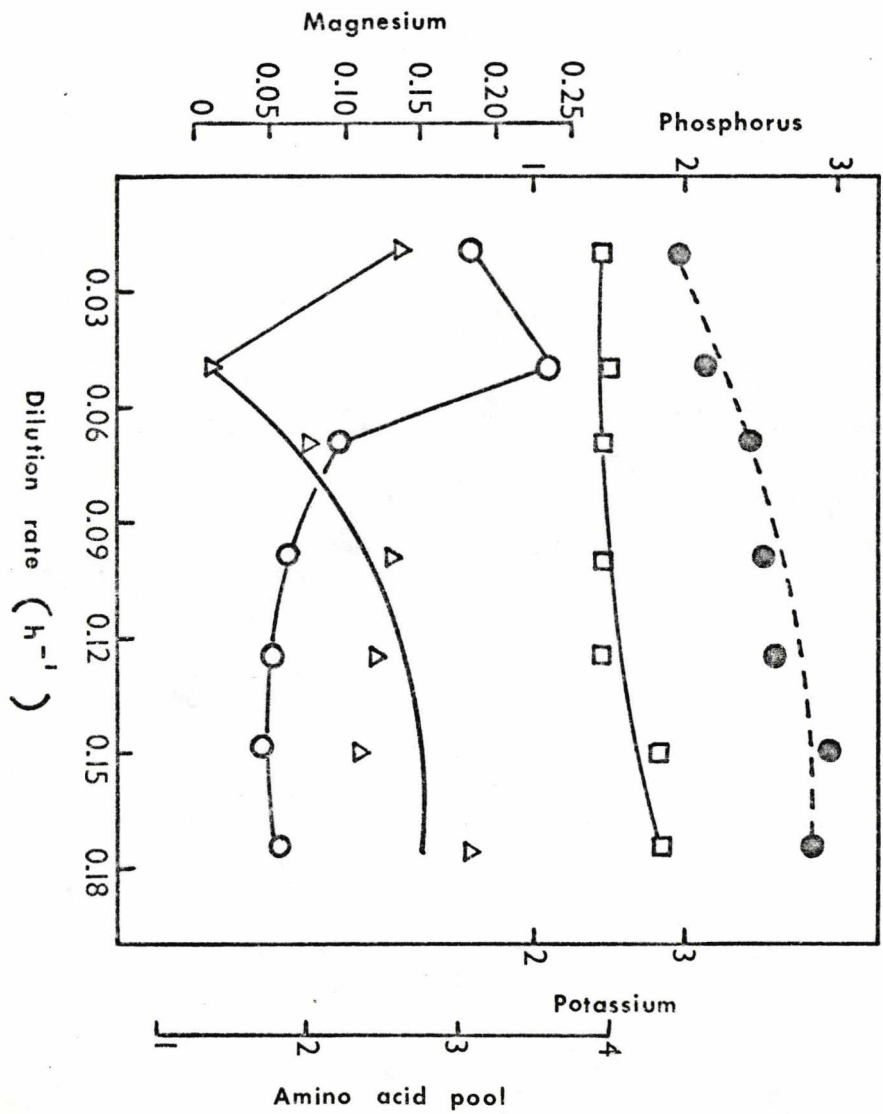D = 0.125 h^{-1} to 0.175 h^{-1}.

The enhanced respiratory activity observed with increases in D,
was accompanied by an increase in carbon dioxide evolution (Figure
3.2b); the respiratory quotient (RQ) also exhibited a positive cor-
relation with D.

3.2.2.   Hyphal composition

The variation in macromolecular content is shown in Figure 3.3a.
Altering the dilution rate had little effect on the DNA, protein and
carbohydrate contents. However, the level of RNA attained a peak
value at D = 0.05 h^{-1}; this value was almost twice that observed at
any other dilution rate.

The cellular magnesium content was maximal at D = 0.05 h^{-1} and
fell over the range 0.07 h^{-1} to 0.175 h^{-1} (Figure 3.3b). Cellular
potassium showed little variation with D, but the level of

Figure 3.3. Effect of dilution rate on hyphal contents of:
(a) DNA, O ; protein, ▲ ; carbohydrate, ☐ ;
RNA, △ ; (b) amino acid pool, △ ; magnesium, O ;
potassium, ☐ ; phosphorus, ● . All values are
quoted as % w/w of mycelium.
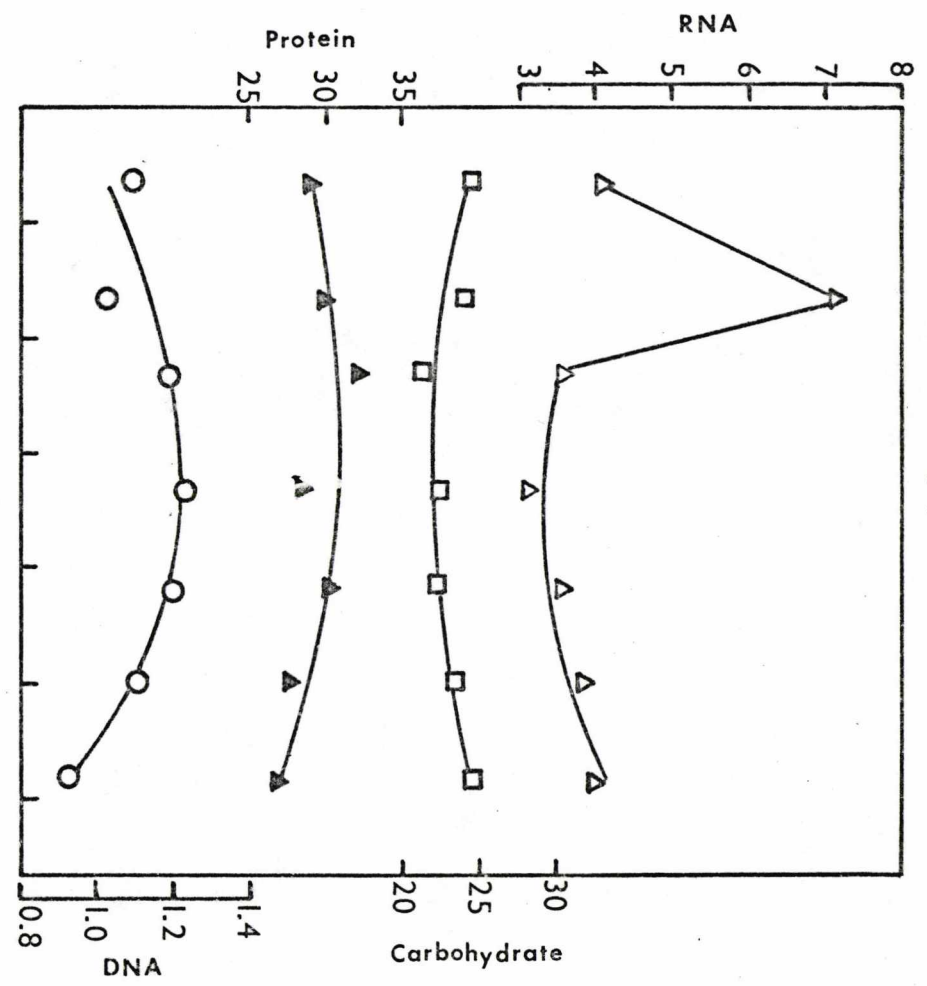
phosphorus increased progressively over the entire dilution rate range studied. Increases in the amino acid pool content were observed between $0.05 \, h^{-1}$ to $0.175 \, h^{-1}$ and $0.05 \, h^{-1}$ to $0.02 \, h^{-1}$.

The amount of wall material present in the mycelium remained fairly constant; however, at $D = 0.05 \, h^{-1}$ the content dropped to approximately 50% of the average value (Table 3.2).

Table 3.2. Effect of dilution rate on the proportion of
wall material present in the mycelium.

Dilution rate (h^{-1})	Wall content (% w/w of mycelium)
0.02	17.6
0.05	9.8
0.07	18.3
0.10	18.3
0.125	17.6
0.15	16.9
0.175	16.6

3.2.3. Composition and properties of the hyphal wall

Hyphal walls from mycelia grown in submerged liquid culture at different dilution rates, under glucose limiting conditions had the appearance shown in Figure 3.4. Fibrillar organisation can be seen at both the inner and outer wall surfaces.

Figure 3.5a,b shows the effect of culture dilution rate on wall chemistry. In general, the variation in the levels of wall components displayed a consistent trend over the range $0.07 \, h^{-1}$ to $0.175 \, h^{-1}$. However, below $0.07 \, h^{-1}$ the changes in wall composition were less predictable. At values of D above $0.07 \, h^{-1}$ there was a fall in the levels of galactose, hexosamine, phosphorus and protein. The drop in the galactose and hexosamine contents amounted to 10% in each case, while the levels of protein and phosphorus decreased by 37% and 29% respectively. Between $0.07 \, h^{-1}$ to $0.02 \, h^{-1}$ the variation

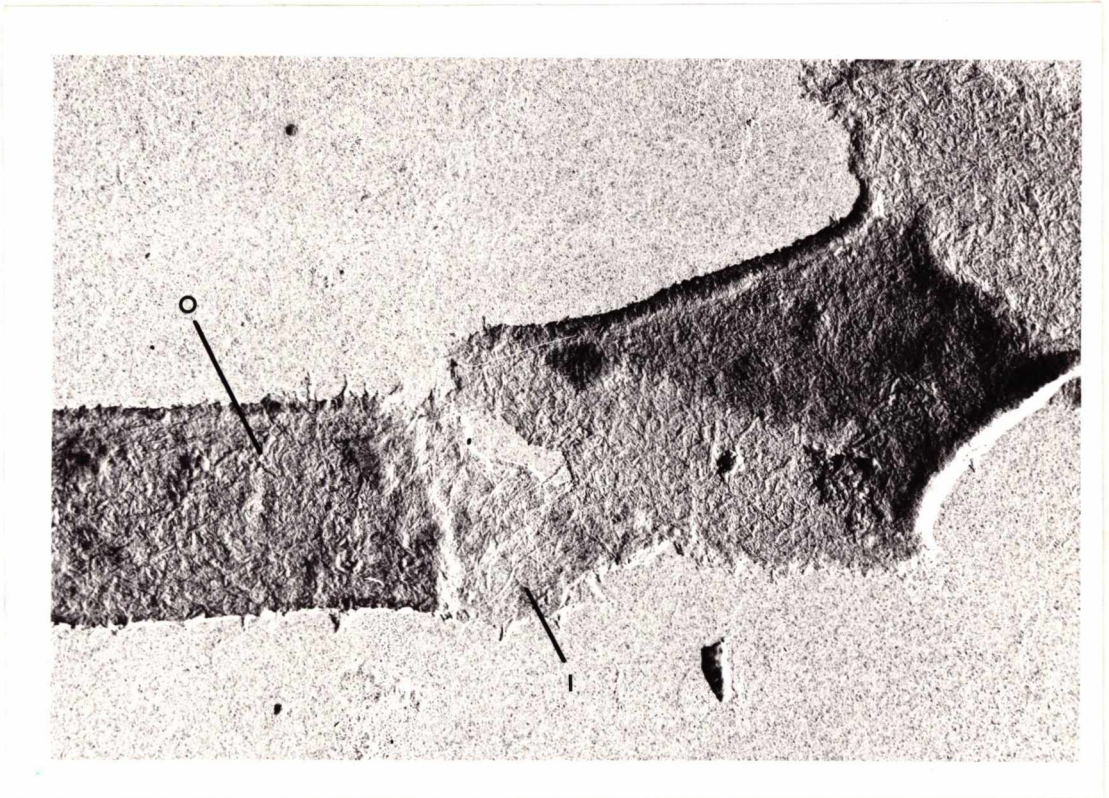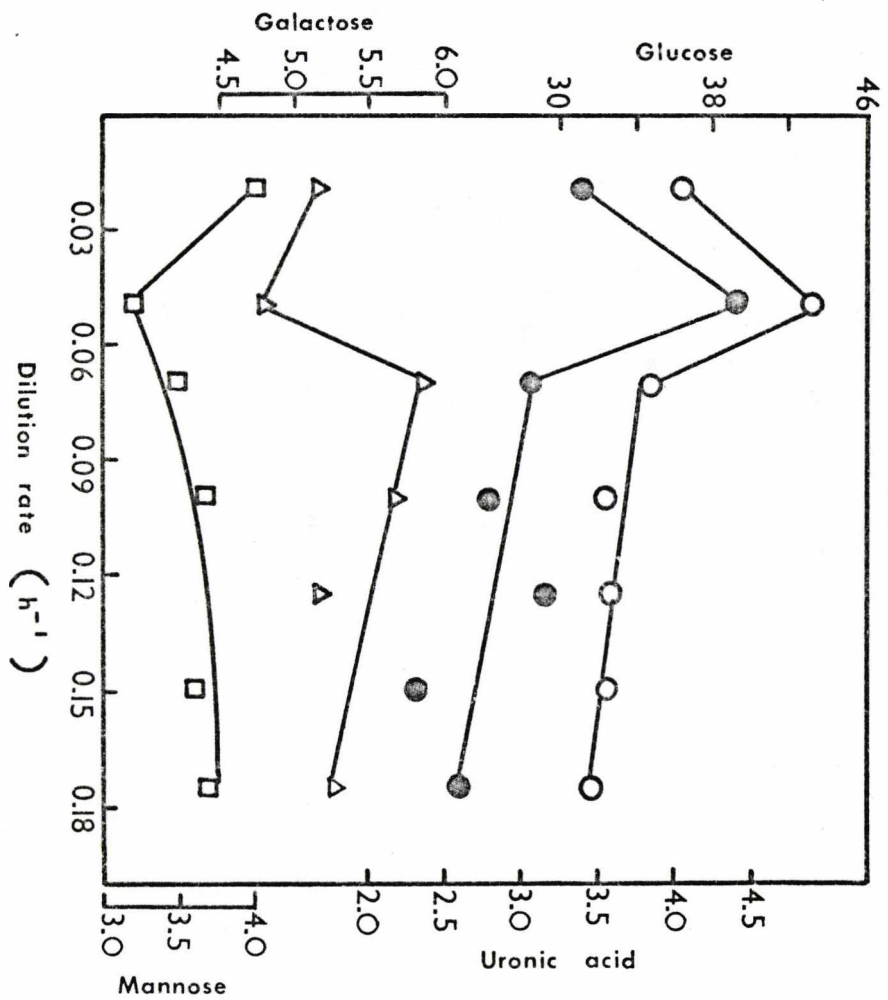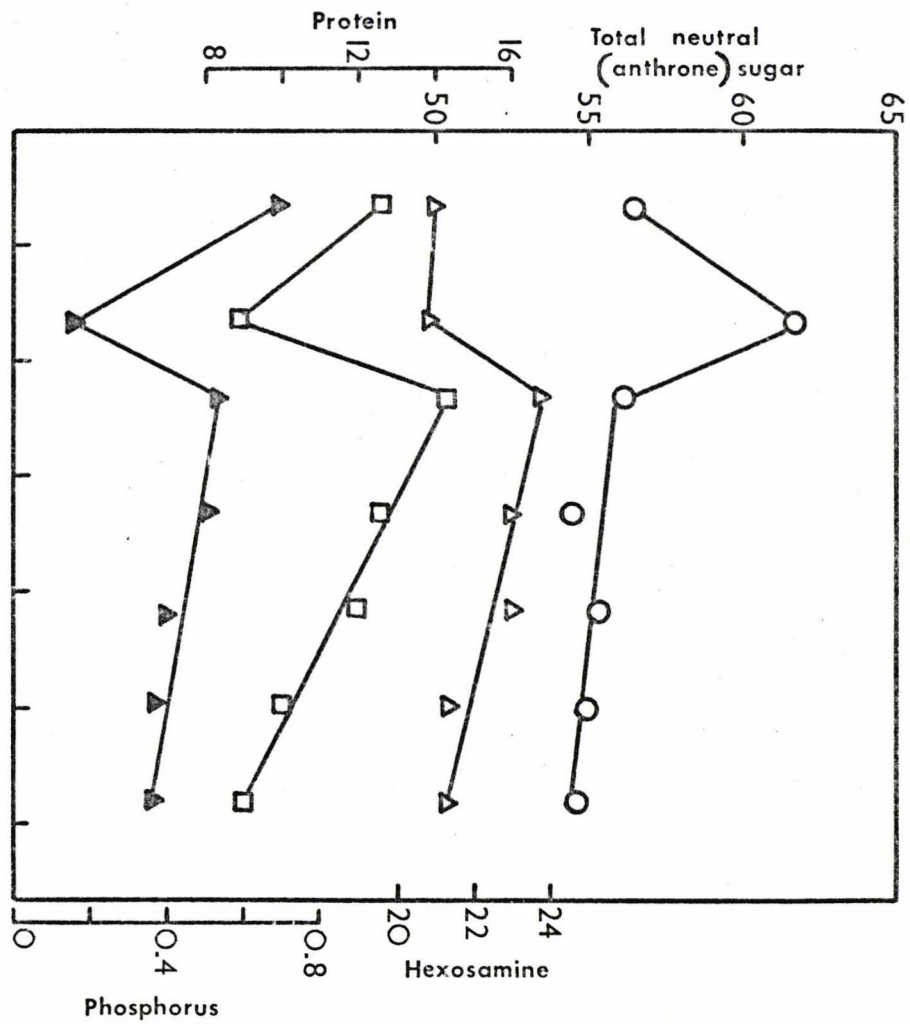Figure 3.4. Electron micrograph of <u>Aspergillus</u> <u>nidulans</u> cell
walls shadowed with gold/palladium. O, outer
surface; I, inner surface. X 20,000.

Figure 3.5.   Effect of dilution rate on hyphal wall contents
of: (a) phosphorus, ▲ ; protein, □ ; hexosamine,
△ ; total neutral (anthrone) sugar, ○ ; (b)
mannose, □ ; galactose, △ ; uronic acid, ● ;
glucose, ○ . All values are quoted as % w/w of
wall.

in the contents of galactose and hexosamine followed a similar pattern, each component experiencing a reduction in value over the range $0.07 \ h^{-1}$ to $0.05 \ h^{-1}$ and then maintaining this value between $0.05 \ h^{-1}$ and $0.02 \ h^{-1}$. There was a significant drop in the levels of protein and phosphorus also over the range $0.07 \ h^{-1}$ to $0.05 \ h^{-1}$, but unlike galactose and hexosamine, these rose again between $0.05 \ h^{-1}$ and $0.02 \ h^{-1}$. The proportion of mannose in the wall showed a small increase over the range $0.05 \ h^{-1}$ to $0.175 \ h^{-1}$, but between $0.05 \ h^{-1}$ and $0.02 \ h^{-1}$ an increase of 25% was observed. Maximal levels of neutral (anthrone) sugar, glucose and uronic acid were realised at a dilution rate of $0.05 \ h^{-1}$; above this rate the levels of all three components fell slightly. The total carbohydrate content of the wall remained fairly constant as D was altered (Table 3.3).

Table 3.3. Total carbohydrate content of the hyphal wall at different dilution rates.

Dilution rate (h^{-1})	Total carbohydrate (% w/w of wall)
0.02	77.5
0.05	82.7
0.07	80.6
0.10	77.5
0.125	78.4
0.15	76.4
0.175	75.9

A simple wall fractionation procedure based on the method of Zonneveld (1971) was carried out in order to determine whether the additional glucose present in the walls of hyphae grown at a dilution rate of $0.05 \ h^{-1}$ could be attributed to a specific fraction. The fraction obtained from the initial treatment with cold 5% (w/v) KOH and subsequent precipitation with glacial acetic acid was subjected to acid hydrolysis (2 N HCl for 2 h at $100^{o}C$). The resulting

hydrolysate was analysed by paper chromatography; the only detectable component was glucose. Infra-red spectroscopy of this alkali-soluble fraction (Figure 3.6) suggested the predominance of an α-linked polyglucan. The absorption bands observed at 815 cm^{-1}, 840 cm^{-1} and 920 cm^{-1} in spectra of the alkali-soluble fraction are characteristic of the α-1,3 linked S-glucan of walls of <u>Aspergillus nidulans</u> (Zonneveld, 1971); Figure 3.6 shows the infra-red spectrum of this glucan (kindly supplied by B. J. M. Zonneveld) for comparison. Using this extraction procedure it was found that the α-glucan content of the wall was maximal at a dilution rate of 0.05 h^{-1} (Figure 3.7).

The cation-binding properties of an isolated wall preparation are shown in Table 3.4. The binding capacity for Ca^{2+} was consider-

Table 3.4. Cation-binding capacities of hyphal walls isolated from hyphae grown at different rates.

Dilution rate (h^{-1})	Cation-binding capacity (μg cation bound/mg wall)			
	Ca^{2+}	Mg^{2+}	K$^+$	Na$^+$
0.02	3.48	0.039	0.10	
0.05	0.98	0.057		
0.07	2.95	0.060		0.125
0.10	1.87	0.065	0.18	0.090
0.125	1.07	0.044	0.27	0.125
0.15	1.57	0.067	0.18	
0.175	1.38	0.067	0.33	

All measurements were made at pH 5.8.

ably greater than for Mg^{2+}, K$^+$ or Na$^+$. Progressively less Ca^{2+} was bound at increased dilution rates, although when D was 0.05 h^{-1} a minimal Ca^{2+}-binding capacity was observed. The amount of Mg^{2+} bound by the walls rose steadily with dilution rate to a constant value at 0.10 h^{-1}, while K$^+$ binding increased over the complete range studied.
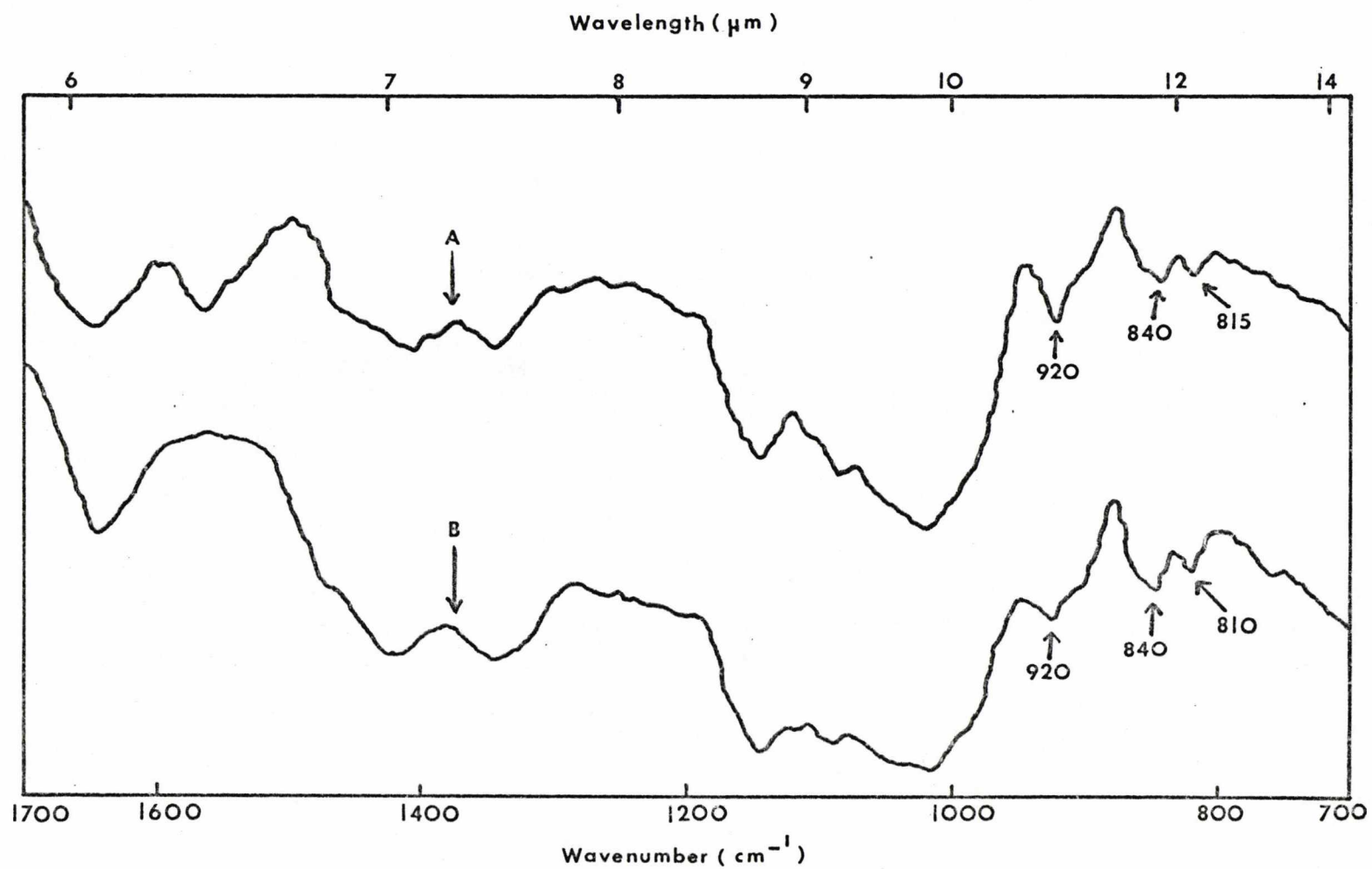
Figure 3.6. Infra-red spectra of cold alkali soluble fraction of _Aspergillus_
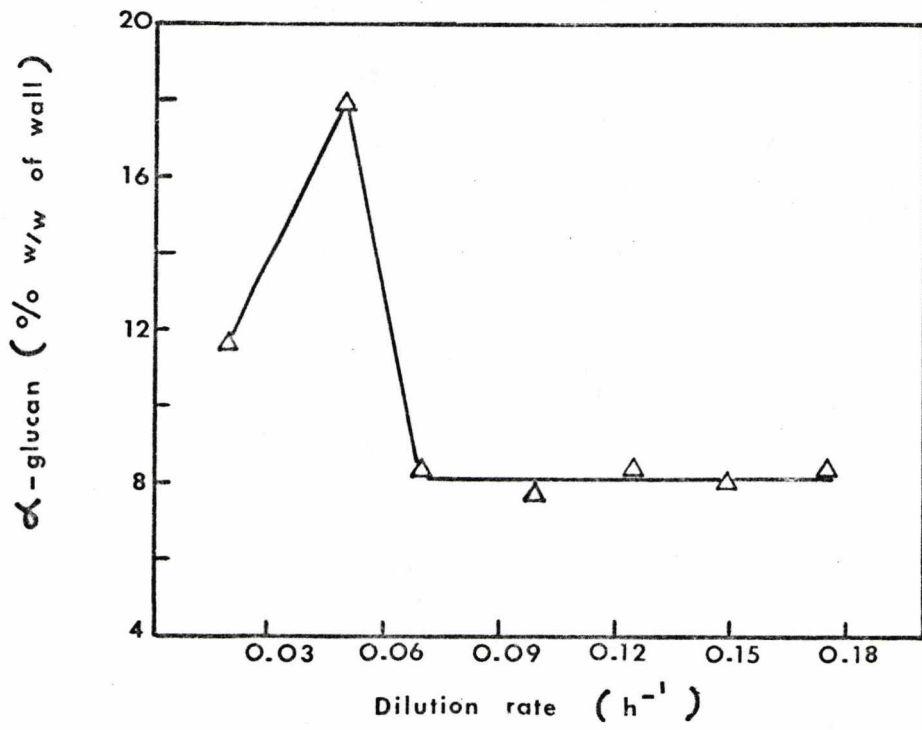_nidulans_ mel 13 wall (A) and the S-glucan of _Aspergillus_ _nidulans_
bi A_1 (B).

Figure 3.7. Effect of dilution rate on α-glucan content of hyphal wall.

3.3. DISCUSSION

3.3.1. Kinetic parameters: Maintenance coefficients, yield coefficients, and substrate saturation constant

The concept of a maintenance energy requirement in relation to growing cells was developed by Pirt (1965). Such processes as the turnover of cellular macromolecules, osmoregulation and pH control were considered to consume a constant proportion of the ATP generated from energy substrate. Thus, of the total energy source, S_E, consumed by a microorganism a portion, S_G, is utilised for growth and a portion, S_M, for maintenance. Hence,

$$Y_E = \frac{x}{S_E} = \frac{x}{S_G + S_M} \qquad \text{(xxxii)}$$

where Y_E is the growth yield and x, the biomass produced.

For a given amount of biomass, x, it is assumed that the rate of consumption of energy source specifically for maintenance functions is constant, i.e.,

$$\left(\frac{ds}{dt}\right)_m = mx, \quad \text{a constant} \qquad \text{(xxxiii)}$$

where m is the maintenance coefficient.

The balance for energy source utilisation is given by,

Total rate of energy consumption	=	rate of energy consumption for growth	+	rate of energy consumption for maintenance

$$\frac{\mu x}{Y_E} = \frac{\mu x}{Y_{EG}} + mx \qquad \text{(xxxiv)}$$

where μ is the specific growth rate and Y_{EG}, the "true" growth yield i.e. the value of Y_E when S_M is zero.

Thus,

$$q_E x = \frac{\mu x}{Y_{EG}} + mx \qquad \text{(xxxv)}$$

where q_E is the specific rate of energy substrate utilisation (see
Chapter 2, Section 2.1.6.(b) for calculation of this function).
A plot of q_E against μ therefore, enables m to be determined.
The oxygen maintenance coefficient, m_{O_2} can be determined in a simi-
lar manner using the function q_{O_2} i.e. the specific rate of oxygen
utilisation (see Chapter 2, Section 2.1.6.(a)(1) for calculation of
this function),

$$q_{O_2} = \frac{\mu}{Y_{O_2}} + m_{O_2} \qquad \text{(xxxvi)}$$

where Y_{O_2} is the yield coefficient for oxygen.
The values of Y_{EG} and Y_{O_2} are given by the inverse of the slopes of
the graphs of q_E against μ and q_{O_2} against μ, respectively.

Estimation of the glucose saturation constant, K_s from the
Monod (1942) equation,

$$\mu = \frac{\mu_m S}{S + K_s} \qquad \text{(xxxvii)}$$

and rearranging,

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m S} \qquad \text{(xxxviii)}$$

where μ_m is the maximum specific growth rate and S, the concentra-
tion of growth limiting substrate in the fermenter, requires a plot
of $1/\mu$ against $1/S$. The intercept on the $1/S$ axis is thus equal to
$-1/K_s$, i.e. K_s is the value of S when $1/\mu$ is zero.

The kinetic parameters for Aspergillus nidulans 13 mel, derived
in the above manner (Figures 3.2b, 3.8, 3.9) are listed in Table 3.5.
The values of the glucose maintenance coefficient, "true" growth
yield and oxygen yield coefficient are of the same order as those
reported for other filamentous fungi. Thus, Bainbridge and coll-
eagues (Bainbridge, Bull, Pirt, Rowley & Trinci, 1971) found an m
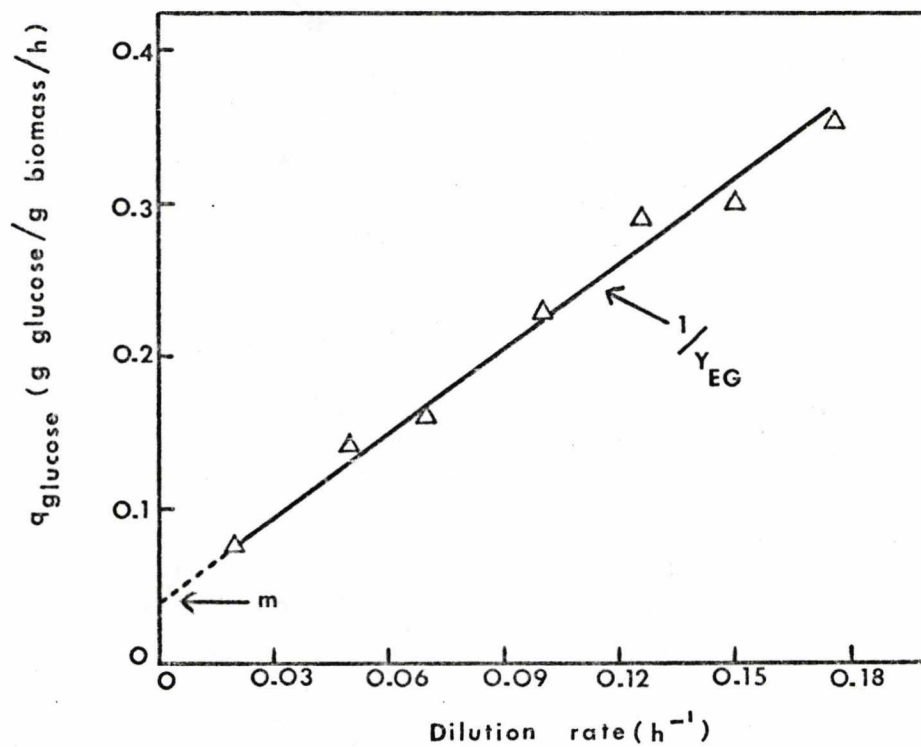
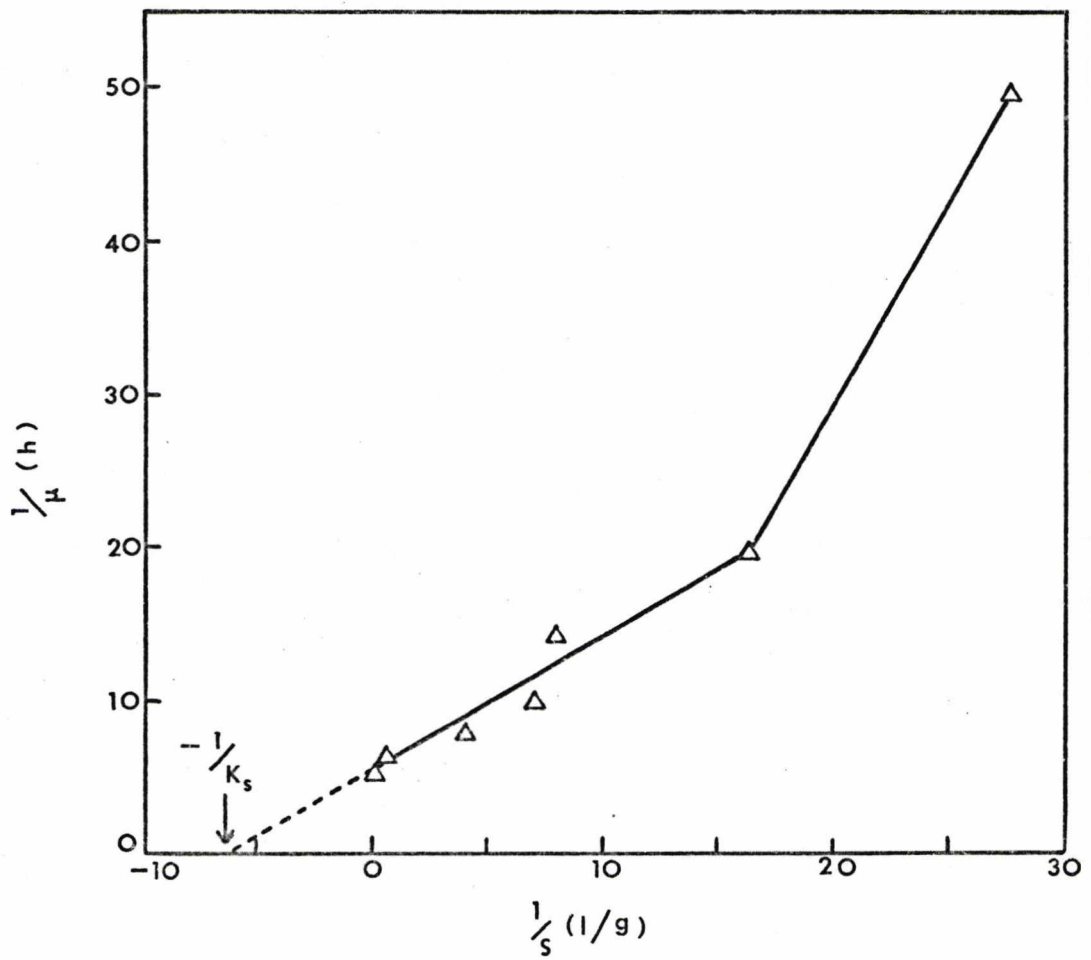Figure 3.8. Effect of dilution rate on specific rate of glucose utilisation ($q_{glucose}$).

Figure 3.9. Plot of reciprocal glucose concentration (1/S) against reciprocal growth rate (1/μ) for K_s determination.

Table 3.5. Kinetic parameters for <u>Aspergillus</u> <u>nidulans</u> 13 mel.

Glucose maintenance coefficient (m)	0.041 g glucose/g biomass/h
Oxygen maintenance coefficient (m_{O_2})	0.13 mmol O_2/g biomass/h
"True" growth yield (Y_{EG})	0.53 g biomass/g glucose
Oxygen yield coefficient (Y_{O_2})	0.060 g biomass/mmol O_2
Glucose saturation constant (K_s)	0.166 g glucose/l (9.2 X 10^{-4} M)

value of 0.029 g glucose/g biomass/h for <u>Aspergillus</u> <u>nidulans</u> BWB 224 grown at 30^oC while for <u>Penicillium</u> <u>chrysogenum</u> at 25^oC the glucose maintenance coefficient is 0.022 g glucose/g biomass/h (Righelato, Trinci, Pirt & Peat, 1968). Values of 0.45 g biomass/g glucose and 0.050 g biomass/mmol O_2 for Y_{EG} and Y_{O_2}, respectively, have also been obtained for <u>Penicillium</u> <u>chrysogenum</u> (Righelato, Trinci, Pirt & Peat, 1968) and a Y_{EG} value of 0.47 g biomass/g glucose for <u>Aspergillus</u> <u>niger</u> (Ng, Smith & McIntosh, 1974). The oxygen maintenance coefficient for <u>Aspergillus</u> <u>nidulans</u> 13 mel, however, is a great deal lower than the 0.74 mmol O_2/g biomass/h reported for <u>Penicillium</u> <u>chrysogenum</u> (Righelato, Trinci, Pirt & Peat, 1968) and the 0.55 mmol O_2/g biomass/h for <u>Aspergillus</u> <u>nidulans</u> BWB 224 (Carter, Bull, Pirt & Rowley, 1971). The stoichiometric relationship:

$$1 \text{ mole glucose} + 6 \text{ moles } O_2 \longrightarrow 6 \text{ moles } CO_2 + 6 \text{ moles } H_2O$$
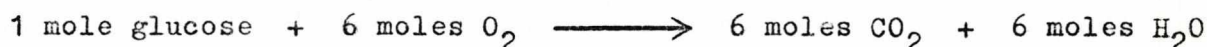
would predict an m_{O_2} value of 1.37 (mmol O_2/g biomass/h) for complete oxidation of the maintenance glucose ration i.e. 0.041 g (0.228 mmol) glucose/g biomass/h. Thus, it would appear that only 10% of the maintenance glucose supply is actually used for the production of energy; the maintenance rate of CO_2 evolution (obtained

by extrapolation of Q_{CO_2} values to zero growth rate, Figure 3.2b) is equivalent to the maintenance rate of O_2 utilisation. The fate of the additional "maintenance" glucose is not clear. Unexplained anomalies regarding maintenance parameters have also been reported for unicellular organisms. Shepherd & Sullivan (1976), for example, found that the oxygen maintenance coefficient for Candida albicans was more than three times that needed for the complete oxidation of maltose and, in tryptophan limited cultures of Aerobacter aerogenes only a small portion of the energy derived from maintenance metabolism was utilised for true maintenance purposes (Stouthamer & Bettenhaussen, 1975).

Compared with most other filamentous fungi, the K_s value of 0.166 g glucose/l for Aspergillus nidulans 13 mel is high. Thus, Pirt (1973) estimated K_s values of 5.0 and 4.24 mg glucose/l for Aspergillus nidulans BWB 224 and Mucor hiemalis, respectively, on solid media; the colony radial growth rate was used as a substitute for μ in the Monod equation. The sewage fungus, Fusarium aquaeductuum, also has a high affinity for glucose with a K_s value of 0.32 mg/l (Steensland, 1973). Neurospora crassa, however, has two transport systems (high and low affinity) for glucose (Scarborough, 1970a,b) and Fiddy & Trinci (1975) have reported a K_s value of 440 ± 200 mg/l (determination made on solid medium) for the low affinity system. Chemostat derived affinity constants for yeast, however, are more directly comparable with the value obtained for Aspergillus nidulans. For example, K_s(glucose) values of 108 mg/l (Leuenberger, 1971) and 129 mg/l (Jones & Hough, 1970) have been calculated for Saccharomyces cerevisiae.

3.3.2. Morphology

Asexual spore differentiation in Aspergillus nidulans 13 mel occurred at a dilution rate of 0.02 h^{-1} (10% μ_{max}), corresponding to a

glucose feed rate of 1.9 times the maintenance ration i.e. 0.078 g glucose/g biomass/h. Conidiation in glucose limited chemostat cultures of Penicillium chrysogenum has also been observed at a glucose feed rate of 1.7 times the maintenance requirement (Righelato, Trinci, Pirt & Peat, 1968). A reduction in the intensity of the conidial apparatus (as compared with that obtained on solid media) similar to that observed in Aspergillus nidulans has also been noted during carbon limited chemostat culture of Penicillium chrysogenum (Righelato, Trinci, Pirt & Peat, 1968) and Aspergillus niger (Ng, Smith & McIntosh, 1973) and indicates only a partial switch on of the conidiation mechanism.

3.3.3. Efficiency of RNA in protein synthesis

Several continuous culture studies of unicellular organisms have revealed a close correlation between cellular RNA content and growth rate (Tempest, Hunter & Sykes, 1965; Harder & Veldkamp, 1967; McMurrough & Rose, 1967; Aiking & Tempest, 1976). Such a correlation has been attributed to growth rate dependent variations in the cellular ribosome concentration (Tempest, Hunter & Sykes, 1965). Maaløe & Kjeldgaard (1966) proposed that the rate of protein synthesis per ribosome is constant, maximal and independent of growth rate. Based on this assumption, therefore, an increase in ribosomal concentration with growth rate would enable the overall rate of protein synthesis to increase in synchrony with the rate of growth. A detailed investigation of chemostat grown Candida utilis, however, demonstrated an increase in the efficiency of nucleic acid in protein synthesis, defined as protein/nucleic acid X D, as the growth rate was increased (Alroy & Tannenbaum, 1973). Growth of bacteria at increased rates has also been shown to involve an increase in ribosomal efficiency (Rosset, Julien & Monier, 1966; Sykes & Young, 1968).

Although the variation in the RNA content of Aspergillus nidulans, in response to changes in growth rate was not as extensive as that observed with unicellular organisms, the efficiency of RNA in protein synthesis was considerably enhanced at increased growth rates (Figure 3.10). Thus, between 0.02 h^{-1} and 0.175 h^{-1} RNA efficiency increased almost 8-fold. However, at values of D exceeding 62.5% μ_{max} the efficiency remained relatively constant. The increased RNA efficiency at increased growth rates may be attributed to either an increase in the fraction of ribosomes engaged in protein synthesis or an increase in the polypeptide chain growth rate or a combination of both factors. Batch culture studies with Escherichia coli have shown an increase in the fraction of ribosomes in polysomes after a shift up of the specific growth rate, following transfer of the organism from a simple to a complex nutrient medium (Godson & Sinsheimer, 1967; Harvey, 1970), while Koch & Deppe (1971) noted that only 15% of the protein translational machinery in slow growing glucose limited cells is actually utilised. Additional analyses of protein synthesis in Escherichia coli (Forchhammer & Lindahl, 1971) and Saccharomyces cerevisiae (Boehlke & Friesen, 1975) have revealed that the rate of peptide chain elongation, also increases with growth rate, although in the former case chain elongation assumes a constant rate at 40% μ_{max}.

3.3.4. Phosphorus, potassium, magnesium and RNA contents

An increase in the potassium content of prokaryotic microorganisms has been observed in association with an increase in growth rate (Dicks & Tempest, 1966; Tempest, 1970). In the Gram-negative bacterium, Aerobacter aerogenes increases in cellular magnesium, phosphorus and RNA also accompany the increased levels of potassium (Dicks & Tempest, 1966). An analysis of the interrelationship between these components has revealed a constant molar stoichiometry
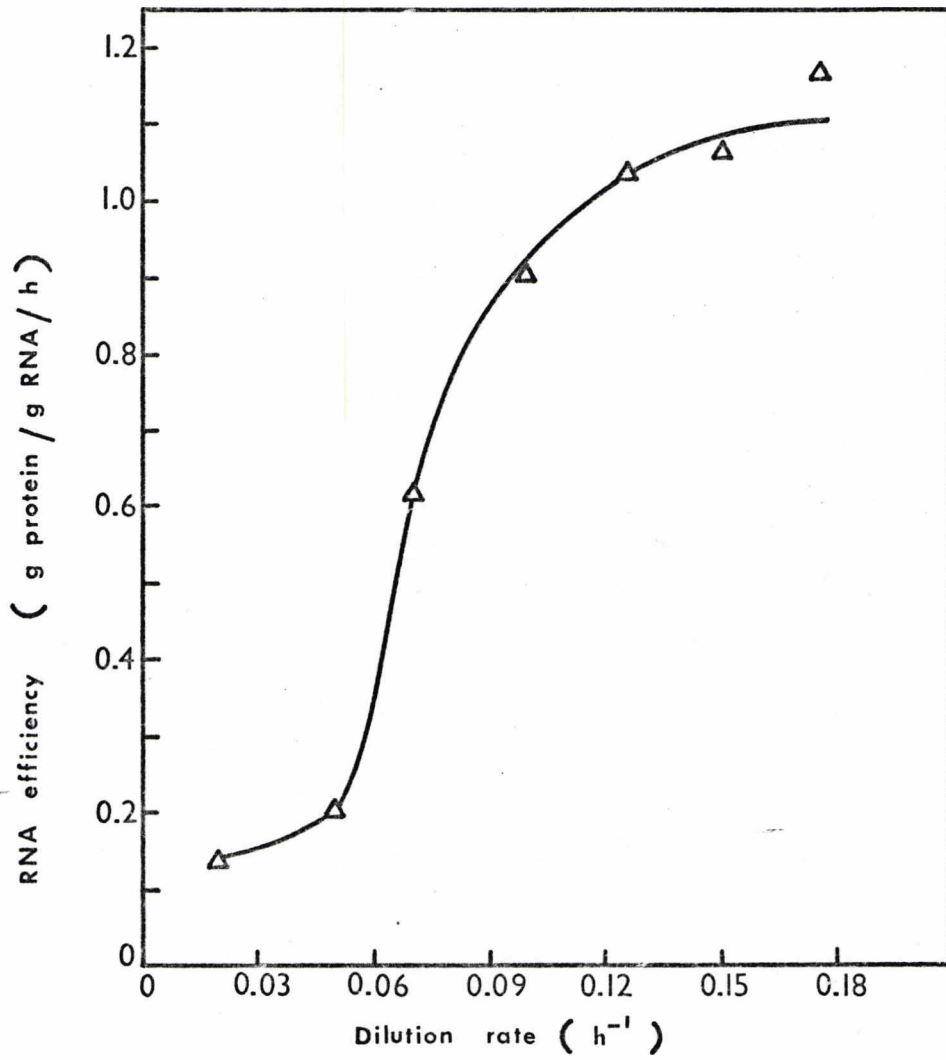
Figure 3.10. Effect of dilution rate on the efficiency of
RNA in protein synthesis.

between magnesium, potassium, RNA and phosphorus, which is indepen-
dent of growth rate. This finding has led to the suggestion that in
bacteria, potassium, in addition to magnesium, is involved in the
stabilisation of ribosomal complexes (Tempest, Dicks & Hunter, 1966).

A similar analysis has been performed for Aspergillus nidulans.
As can be seen from Figure 3.3a,b the variation in the levels of RNA
and phosphorus were totally dissimilar, indicating the presence of
large quantities of non-nucleic acid phosphorus. Although the
potassium content (average value 2.5% w/w), which falls within the
range reported for other mycelial fungi (Dalby & Gray, 1974), is
similar to that of Aerobacter aerogenes (Dicks & Tempest, 1966), the
RNA content, which averaged between 3 and 4% (w/w) over most of the
dilution rate range studied, is considerably lower, (values for
Aerobacter varied with dilution rate from 9.5 to 18% under glycerol
limiting conditions; Tempest, Hunter & Sykes, 1965). Hence, it is
unlikely that environmentally induced variations in the RNA content
of Aspergillus nidulans would be accompanied by parallel changes in
the level of potassium, if this cation were involved in maintaining
a functional ribosomal configuration. Moreover, a recent investiga-
tion of the physiology of Candida utilis has indicated that the cel-
lular potassium requirement of eukaryotes, in contrast to prokary-
otes, may not be associated chiefly with the anionic groups of RNA
(Aiking & Tempest, 1976). In agreement with the results obtained for
Aspergillus nidulans, Aiking & Tempest (1976) found that the cellu-
lar potassium content of glucose limited cultures of the yeast was
not affected by variations in growth rate. In addition there was
little stoichiometry between the potassium, RNA and phosphorus con-
tents under different growth limiting conditions. However, a cor-
relation between the cellular potassium content and the efficiency
of utilisation of both glucose and oxygen was revealed which led to
the suggestion that potassium is intimately involved in either the

generation of energy by oxidative phosphorylation, or in the utilisation of this energy for growth, or in both processes. Support for the first notion was provided by the observation that isolated mitochondria from potassium limited organisms phosphorylated ADP less efficiently than mitochondria from glucose limited organisms (Aiking & Tempest, 1976).

Cellular magnesium, in contrast to potassium, is nearly all cell bound and mainly to the ribosomes (Marquis, Porterfield & Matsumura, 1973). Thus, the molar ratio of RNA to Mg^{2+} constitutes a valid criterion for assessing the involvement of Mg^{2+} in the stabilisation of the ribosomes, under different environmental conditions. Figure 3.11 shows the variation in the RNA/Mg^{2+} molar ratio of Aspergillus nidulans in response to changes in the dilution rate. Over the range 0.02 h^{-1} to 0.175 h^{-1} there was a trebling in value of the RNA/Mg^{2+} molar ratio, a finding which differs from that reported for Candida utilis (Aiking & Tempest, 1976) where the RNA/Mg^{2+} molar ratio shows only a slight variation with growth rate. Coupled with this increase in the RNA/Mg^{2+} molar ratio, there was an increase in the hyphal concentrations of the organic cations, spermine and spermidine (Bushell & Bull, 1974), which when considered with the hyphal Mg^{2+} concentration produced a growth rate independent molar stoichiometry for RNA/cation close to 2:1. While experiments in vitro have demonstrated ionic binding of polyamines to RNA (Raina & Telaranta, 1967; Viotti, Bagni, Sturani & Alberghina, 1971) there appears to be a finite limit to the amounts of spermidine and spermine that can replace Mg^{2+} at the ribosome level. Thus, Viotti and colleagues (Viotti, Bagni, Sturani & Alberghina, 1971) observed maximal values of 59 nmoles of spermidine and 2.2 nmoles of spermine per μmole RNA nucleotide for Neurospora crassa ribosomes. Assuming that the ribosomes of Aspergillus nidulans have a similar capacity for polyamine binding then it seems unlikely that the increased
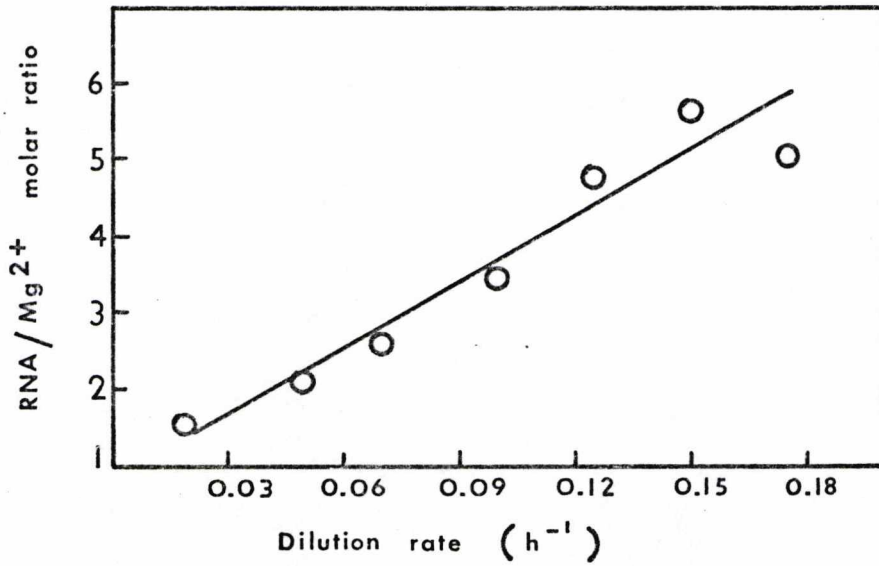
Figure 3.11. Stoichiometry between hyphal RNA and magnesium at different dilution rates. An average molecular weight for RNA (nucleotide) of 340 was assumed.

levels of spermine and spermidine observed within the range 0.07 h^{-1} to 0.175 h^{-1} are primarily concerned with ribosomal stabilisation: values ranged from 11 to 15 nmoles/µmole RNA (total) for spermine and 163 to 338 nmoles/µmole RNA (total) for spermidine (Bushell & Bull, 1974). In a number of systems a stimulatory effect of poly- amines on RNA (Fox & Weiss, 1964; Raina & Cohen, 1966) and protein (Takeda, 1969) synthesis has been observed and it is conceivable that the increased synthesis of polyamines by Aspergillus is associ- ated with the enhanced rates of RNA and protein synthesis incurred at the increased growth rates. It must be pointed out, however, that the results obtained for Neurospora were derived from batch culture studies and therefore may not be strictly comparable with those of chemostat grown Aspergillus.

3.3.5. Wall composition

One of the more significant findings to emerge from observations of the growth of Aspergillus nidulans at different rates relates to the greatly altered levels of wall components in hyphae which had been cultured at a dilution rate of 0.05 h^{-1}. This response indicates a considerable reorganisation of wall metabolism which is probably concerned with the differentiation of hyphal tips at growth rates between 0.05 h^{-1} and 0.02 h^{-1}. In view of Zonneveld's (1972) propo- sal that wall α-1,3 glucan serves as the main carbon and energy source for cleistothecium development during surface growth of Aspergillus nidulans the build up of this polymer at a dilution rate of 0.05 h^{-1} and its subsequent decline below 0.05 h^{-1} (Figure 3.7) is of especial interest. Although mycelial synthesis of wall α glu- can (α glucan content of wall X proportion of wall material pres- ent in mycelium) was not significantly influenced by changes in dilution rate (Table 3.6) susceptibility of the reserve polymer to α-glucanase activity in vivo may depend upon wall structure. Thus,

Table 3.6.   Synthesis of wall α-glucan by the mycelium at
             different dilution rates.

Dilution rate (h^{-1})	Wall α-glucan synthesis (g glucan/100 g mycelium)
0.02	2.07
0.05	1.76
0.07	1.54
0.10	1.43
0.125	1.51
0.15	1.38
0.175	1.43

the reduced wall content at 0.05 h^{-1} (Table 3.2) when considered
with wall thickness measurements (Table 3.1) may be indicative of a
less dense, less well integrated wall structure than normal, which
may facilitate conidiation.

3.3.6.   Cationic binding properties of the hyphal wall

The capacity of Gram-positive bacterial walls to bind Mg^{2+} ions has
been correlated with the levels of wall teichoic and teichuronic
acids (Meers & Tempest, 1970); in the former case Mg^{2+} is bound
through phosphate groups attached to glycerol or ribitol residues
and in the latter case through the carboxyl groups of glucuronic
acid. Thus, it has been suggested that these anionic polymers func-
tion as primary binding sites for Mg^{2+}, and possibly other cations,
prior to assimilation (Meers & Tempest, 1970). The presence of
uronic acids in fungal cell walls (Gancedo, Gancedo & Asensio, 1968;
Bull, 1970) has, therefore, led to speculation concerning their role
in the cation assimilation process. In an attempt to elucidate the
importance of the negatively charged wall components of Aspergillus
nidulans in cation binding and hence, cation assimilation, cation
binding capacities (Table 3.4) were compared with the levels of
individual wall components.

In comparison with the ion binding properties of the walls of _Staphylococcus aureus_, _Aspergillus nidulans_ adsorbed significantly lower quantities of K^+, Na^+ and Mg^{2+}. Cutinelli & Galdiero (1967) obtained values of 0.56, 0.34 and 1.05 µg cation bound/mg cell wall for the K^+, Na^+ and Mg^{2+} binding capacities, respectively, of the bacterial wall at pH 7.2. However, even among bacteria, Mg^{2+} binding capacities may cover a wide range of values: Meers & Tempest (1970) reported values of 13.3 to 19.0 µg Mg^{2+} bound/mg wall for _Bacillus subtilis_ var. _niger_. Since the difference in the Mg^{2+} binding capacities of the microbial walls cannot wholly be correlated with the different levels of cellular Mg^{2+} the inference is that the degree of binding is of relatively minor importance in the cation assimilation process, compared with the rate at which cations are removed from the cell wall and conveyed across the cell membrane. Comparison of the steady state levels of hyphal K^+ and Mg^{2+} with the K^+ and Mg^{2+} binding capacities of the wall and the amount of wall material present in the mycelium suggests that in _Aspergillus nidulans_ this rate of transfer is reduced at increased growth rates.

The maximum amount of Ca^{2+} bound by _Aspergillus nidulans_ and _Staphylococcus aureus_ is similar. The value of 1.72 µg Ca^{2+} bound/mg wall at pH 7.2 for Staphylococcus falls within the range of values displayed by _Aspergillus nidulans_ at different growth rates. A value of 2.4 mg Ca^{2+} bound/g mycelium (measurement made at pH 6.0) for Ca^{2+} binding to the mycelial surface of _Penicillium notatum_ (cf. extreme values of 0.096 and 0.61 mg Ca^{2+} bound/g mycelium, calculated as Ca^{2+} binding capacity X proportion of wall material present in mycelium, for _Aspergillus nidulans_) has been reported by Cuppoletti & Segel (1975) but, it is probable that this value includes Ca^{2+} binding to the membrane, as isolated walls were not used for the determination. Assuming that the individual binding potentials reported in Table 3.4 are realised _in vivo_, the different affinities

of the cations for the hyphal wall may be a reflection of the relative importance of each of these cations in hyphal growth. Thus, the high degree of Ca^{2+} binding may be of particular significance. The role of Ca^{2+} in reducing cell permeability has been noted in several instances. Thus, cells of Rhizobium trifolii grown in defined medium deficient in Ca^{2+} were more permeable than cells grown in media containing excess Ca^{2+} (Humphrey & Vincent, 1965) and this observation correlated with the reduced level of Ca^{2+} in the walls of the "Ca^{2+} deficient rhizobium" (Humphrey & Vincent, 1962). It was concluded that Ca^{2+} played a role in securing wall components in a firm steric arrangement and glucuronic acid was suggested as a possible binding site (Vincent & Humphrey, 1968). Calcium ions have been reported to decrease the loss of the sugar alcohols, mannitol and arabitol and other unidentified compounds from the mycelium of the marine fungus, Dendryphiella salina (Allaway & Jennings, 1970); presumably the "protective Ca^{2+} ions" were localised at the cell surface. The cell walls of higher plants bind Ca^{2+} through the carboxyl groups of the galacturonic acid residues of pectic substances (Kohn & Furda, 1967; Somers, 1973) and this observation taken in conjunction with the proposal put forward for Rhizobium trifolii walls would point to a common role for hexuronic acids in Ca^{2+} adsorption. In Aspergillus nidulans however, the similarity of response of wall phosphorus and protein contents and the Ca^{2+} binding capacity to changes in the growth rate may indicate that protein carboxyl or anionic phosphate groups could also assume or augment this function. It is therefore worth noting that in Achyla Ca^{2+} is bound to the wall via a low molecular weight glycoprotein (Le'John, Cameron, Stevenson & Meuser, 1974).

The substantial difference in the amounts of Ca^{2+} and Mg^{2+} that were adsorbed by the wall was totally unexpected and may indicate specificity of cation binding. The absence of a common trend in the

individual cationic binding capacities with growth rate lends support to this view. To what extent uronic acid residues are involved in Mg^{2+} binding cannot be ascertained from the present data. However, even assuming complete dissociation of uronic acid carboxyl groups, the degree of Mg^{2+} binding was a great deal lower than would be expected if uronic acid was primarily concerned with Mg^{2+} adsorption.

CHAPTER 4. THE INFLUENCE OF GROWTH TEMPERATURE ON THE COMPOSITION AND PHYSIOLOGY OF ASPERGILLUS NIDULANS

4.1. INTRODUCTION

Information relating to the effects of growth temperature on micro-
bial metabolism has been derived predominantly from batch culture
studies. While significant changes in the levels of metabolic
products in batch grown microorganisms can to some extent be attrib-
uted to alterations in temperature, additional factors arising from
inadequate control of environmental parameters may complicate inter-
pretation of the changes observed. In particular, the maximum spec-
ific growth rate of a microorganism varies considerably in response
to temperature, a relationship which, within certain temperature
limits, can be described by the Arrhenius equation (Arrhenius,
1908);

$$k = Ae^{-\mu/RT} \qquad (xxxix)$$

In this equation, k is the reaction velocity constant; A is the
Arrhenius constant; μ, the temperature characteristic of the process;
R, the gas constant; and T, the absolute temperature. This relation-
ship, which adheres at temperatures below the optimum for growth,
does not obtain, however, at superoptimal temperatures, when thermal
denaturation of cell macromolecules and organelles may assume sig-
nificant proportions.

In the chemostat, however, where the culture environment is
rigorously controlled, the unequivocal effects of temperature on
microbial growth can be determined systematically. Various aspects
of bacterial (Tempest & Hunter, 1965; Harder & Veldkamp, 1967) and
yeast (Brown & Rose, 1969; Jones & Hough, 1970) physiology have been
studied in this manner but, to date, only one report pertaining to
the continuous growth of a mould at different environmental temper-
atures has been published and this was concerned chiefly with
secondary metabolism (Rowley & Pirt, 1972). The present report,
which deals mainly with primary metabolism, also presents data on

wall chemistry, an aspect which has not been previously investigated in eukaryotic microorganisms using the continuous culture technique.

4.2. RESULTS

4.2.1. Hyphal growth and morphology

The effect of growth temperature on the physiology of Aspergillus nidulans was studied over the range 22°C to 50°C. Glucose was present in the culture medium at a concentration of 11.7 g/l and the pH was controlled at 6.8. Although the specific growth rate of the mould was controlled at 0.10 h^{-1}, steady state cultures were not obtained at 22°C or 50°C as washout occurred at both temperatures (Figure 4.1a,b).

At all temperatures examined diffuse, filamentous growth was produced, except at 22°C, when 10 to 20% of the culture was in the form of small, hard, elliptical pellets. At higher temperatures the presence of a small number of conidia was observed. These conidia were not produced on typical conidiophores but from phialides borne at the hyphal tips. Conidiation occurred following a shift of temperature from 38°C to 40°C and again when the temperature was shifted up from 42°C and 45°C. At 42°C swollen cells were evident in several of the hyphae. The diameter of these swollen cells did not exceed 1.5 to 2 times the normal hyphal diameter. Mycelial lysis, as judged by light and electron microscopy, was not observed at extreme temperatures.

Hyphal diameter and wall thickness were independent of temperature within the range 25°C to 50°C but increased when the temperature was lowered from 25°C to 22°C (Table 4.1).

During steady state growth glucose was not detected in culture filtrates and the pH remained steady at 6.8, no addition of acid or alkali being necessary to maintain the pH.

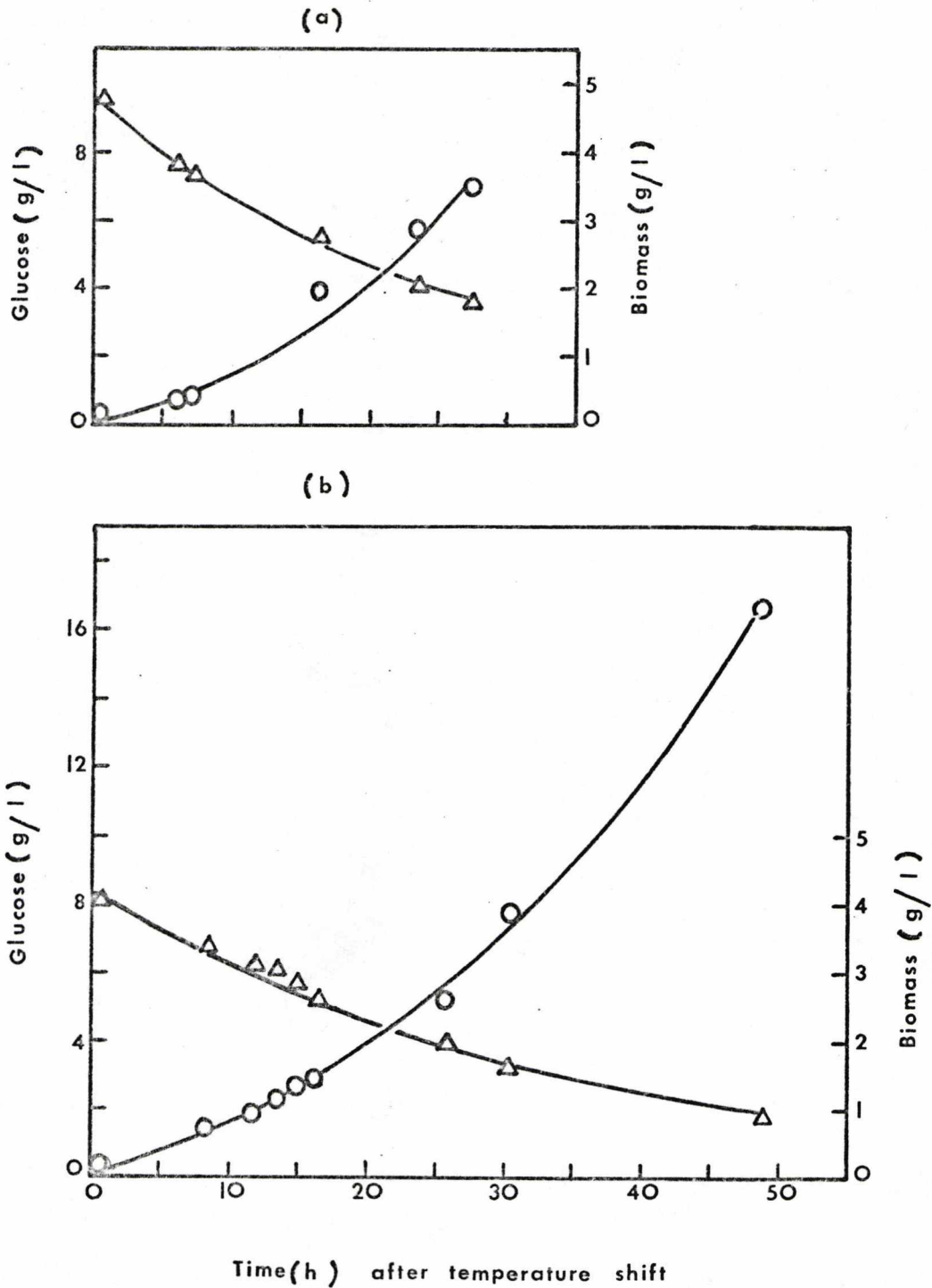The apparent maximum specific growth rates of the mould at 22°C

Figure 4.1. Culture washout after temperature shifts:
(a) 45°C to 50°C; (b) 25°C to 22°C;
biomass, △ ; growth limiting substrate
concentration, O .

Table 4.1. Hyphal diameter and wall thickness at different
temperatures.

Temperature (^{o}C)	Hyphal diameter (μm)	Wall thickness (μm)
22	1.60 ± 0.45[a]	0.091 ± 0.030[a]
25	1.16 ± 0.14	0.075 ± 0.006
30	0.93 ± 0.13	0.064 ± 0.005
35	1.16 ± 0.14	0.073 ± 0.005
38	0.98 ± 0.14	0.071 ± 0.005
40	1.02 ± 0.14	0.071 ± 0.005
42	1.02 ± 0.13	0.074 ± 0.014
45	1.01 ± 0.09	0.071 ± 0.009
50	0.99 ± 0.10	0.075 ± 0.005

Each result is the mean of 10 measurements.
[a]Standard deviation.

and 50°C, as determined from washout data (Figure 4.2a,b), were
0.073 h^{-1} and 0.062 h^{-1}, respectively. The value obtained at 22°C
may have been lower than the "true" value, because the culture
existed in a heterogeneous morphological state.

Temperature related changes in culture biomass and respiratory
activity are shown in Figure 4.3a,b. The optimum temperature for
mycelial production was found to lie between 35°C and 38°C. Between
25°C and 40°C there was an increase in the oxygen uptake rate, but
above 40°C the rate remained constant. An increase in carbon dioxide
output was also observed as the temperature was raised from 25°C to
45°C, but this did not parallel the increase in oxygen uptake, as
demonstrated by the higher RQ values at lower temperatures.

4.2.2. Hyphal composition

The macromolecular composition of the hyphae varied considerably
with temperature (Figure 4.4a). The DNA content showed negligible
dependence upon temperature in the range 22°C to 40°C, but between
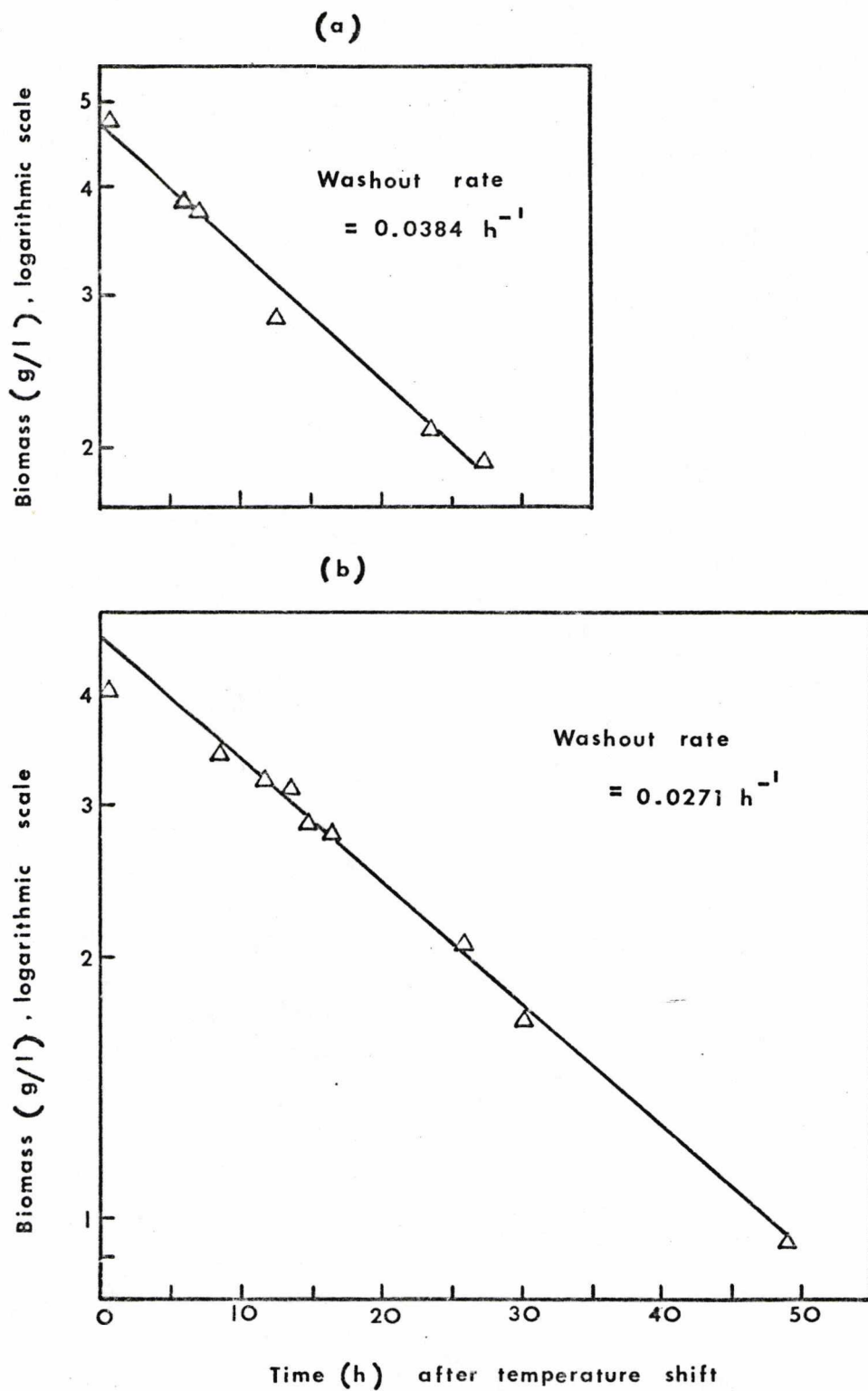
Figure 4.2. Determination of culture washout rates: (a) at 50°C after a shift from 45°C; (b) at 22°C after a shift from 25°C.

Figure 4.3. Effect of temperature on: (a) biomass production, growth yield; (b) Q_{O_2}, O ; Q_{CO_2}, □ ; RQ, △ .

Figure 4.4. Effect of temperature on hyphal contents of:
(a) DNA, O ; RNA, △ ; protein, ▲ ; carbohydrate,
□ ; (b) magnesium, O ; amino acid pool, △ ;
phosphorus, ● ; potassium, ◻ . All values are
quoted as % w/w of mycelium.

40°C and 50°C there was a decrease of 25%. The RNA content showed no significant change within the range 30°C to $50^{\c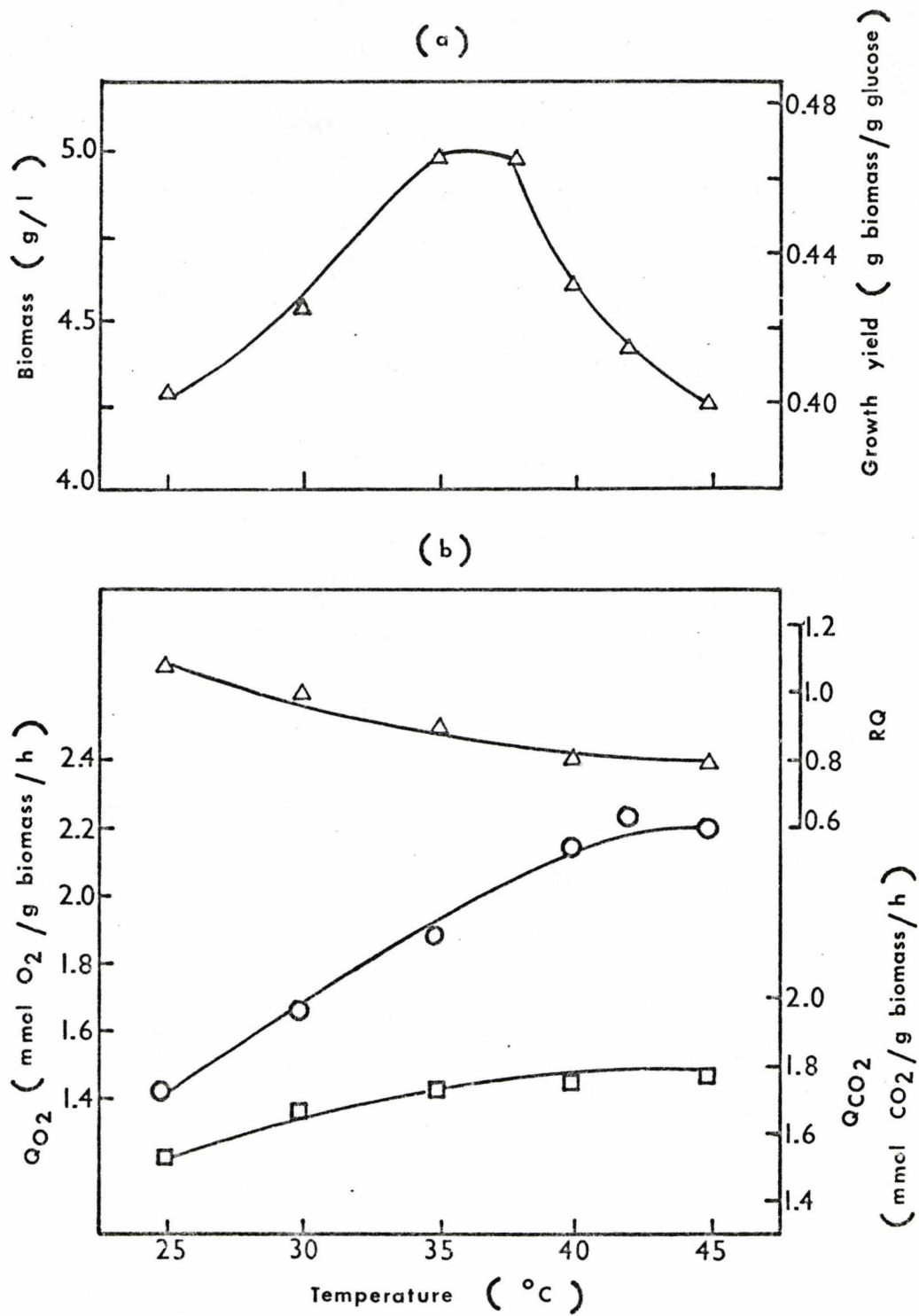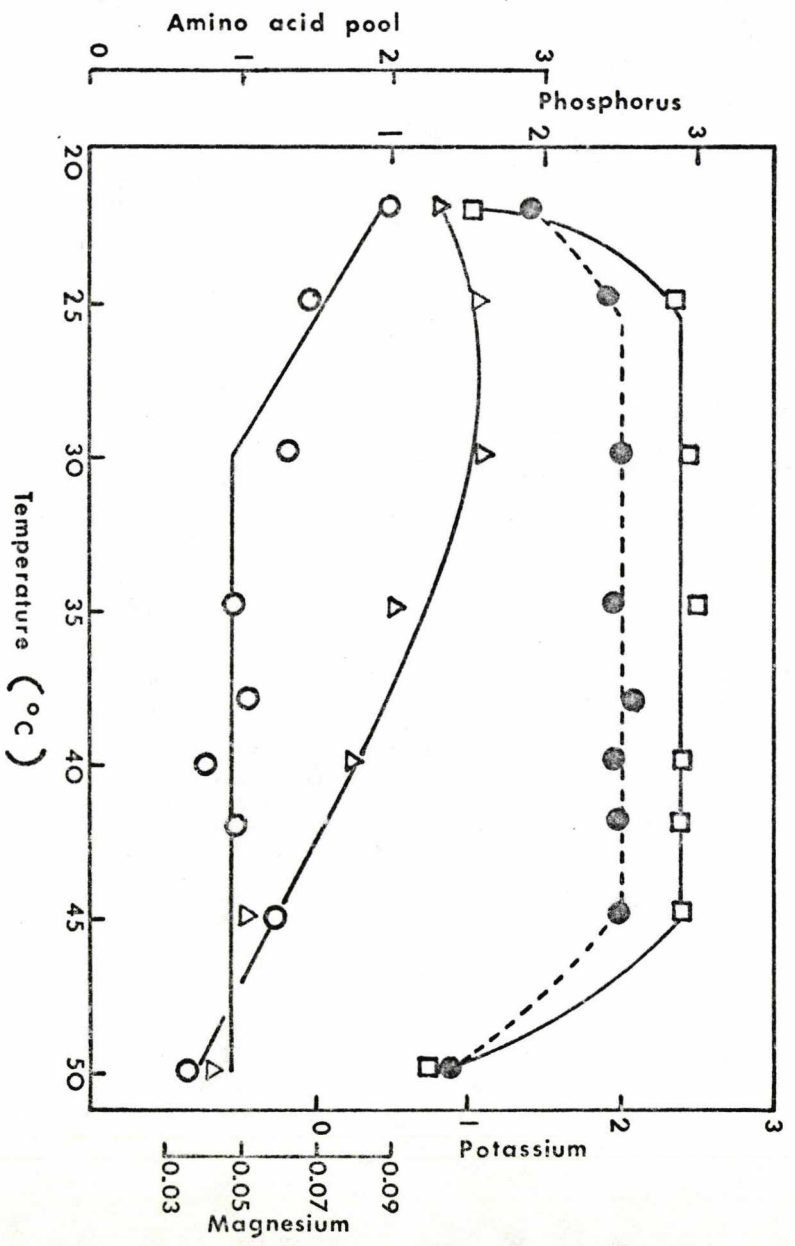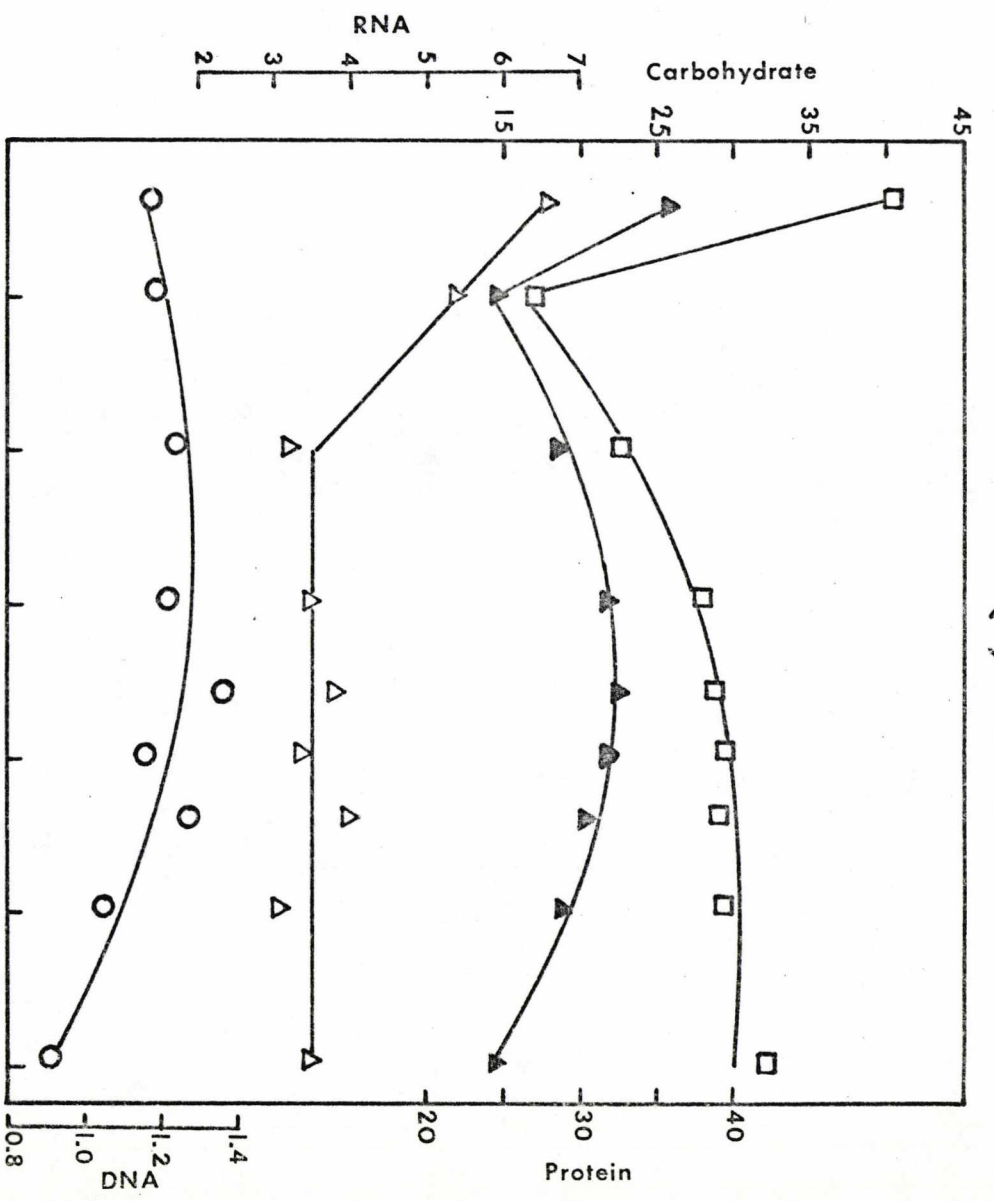irc}$C, but a dramatic rise, representing an increase of 112%, occurred by reducing the temperature from 30°C to 22°C. This increase, however, was insufficient to support steady state growth at 22°C (D, 0.10 h^{-1}) and this probably represents the minimum temperature for growth of Aspergillus nidulans in defined medium in a chemostat. Protein and carbohydrate contents increased above 25°C, protein to a peak value at 38°C and carbohydrate to a constant level at 35°C and above. Between 25°C and 22°C increases in protein and carbohydrate levels were 47% and 139% respectively. Although the levels of the macromolecular components of hyphae grown at 22°C were subject to the additional effects of growth rate ($\mu = 0.073\ h^{-1}$), the increases observed in RNA, protein and carbohydrate contents were purely temperature induced as no comparable differences were reported in hyphae which had been grown at a fixed temperature, but at different rates (Chapter 3).

The size of the internal amino acid pool, which fell markedly above 30°C, maintained a constant level in the range 22°C to 30°C (Figure 4.4b). Variations in intracellular magnesium, potassium and phosphorus are also shown in Figure 4.4b. The levels of potassium and phosphorus remained steady as the growth temperature was altered except under conditions which produced culture washout i.e. 22°C and 50°C when hyphal concentrations of both components decreased. Changes in the magnesium content followed closely the changes in RNA such that the RNA (nucleotide)/Mg^{2+} molar ratio remained constant and independent of temperature (Figure 4.5).

The proportion of hyphal material which was attributable to wall material showed little change between 25°C and 35°C, but rose significantly above 35°C (Table 4.2).
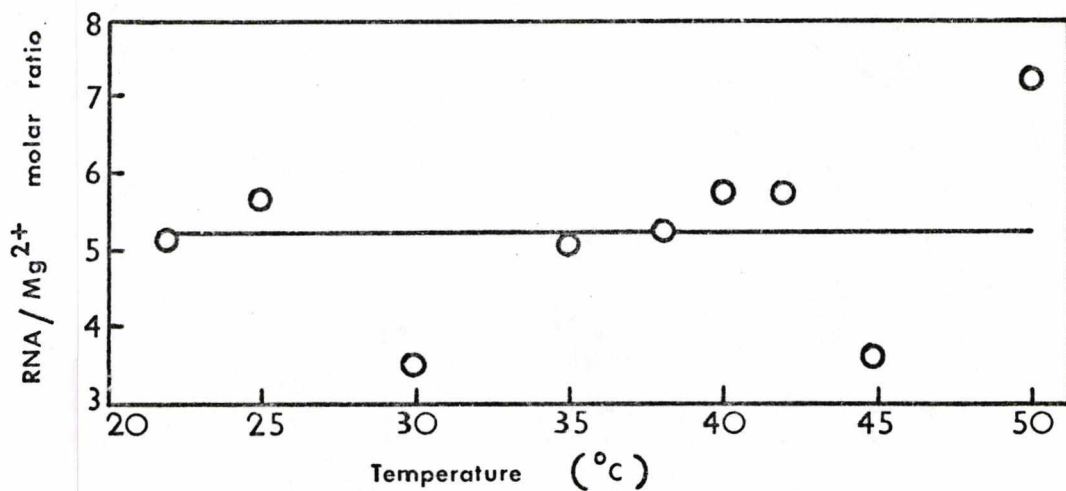
Figure 4.5. Stoichiometry between hyphal RNA and magnesium at different temperatures. An average molecular weight for RNA (nucleotide) of 340 was assumed.

Table 4.2. Effect of temperature on the proportion of
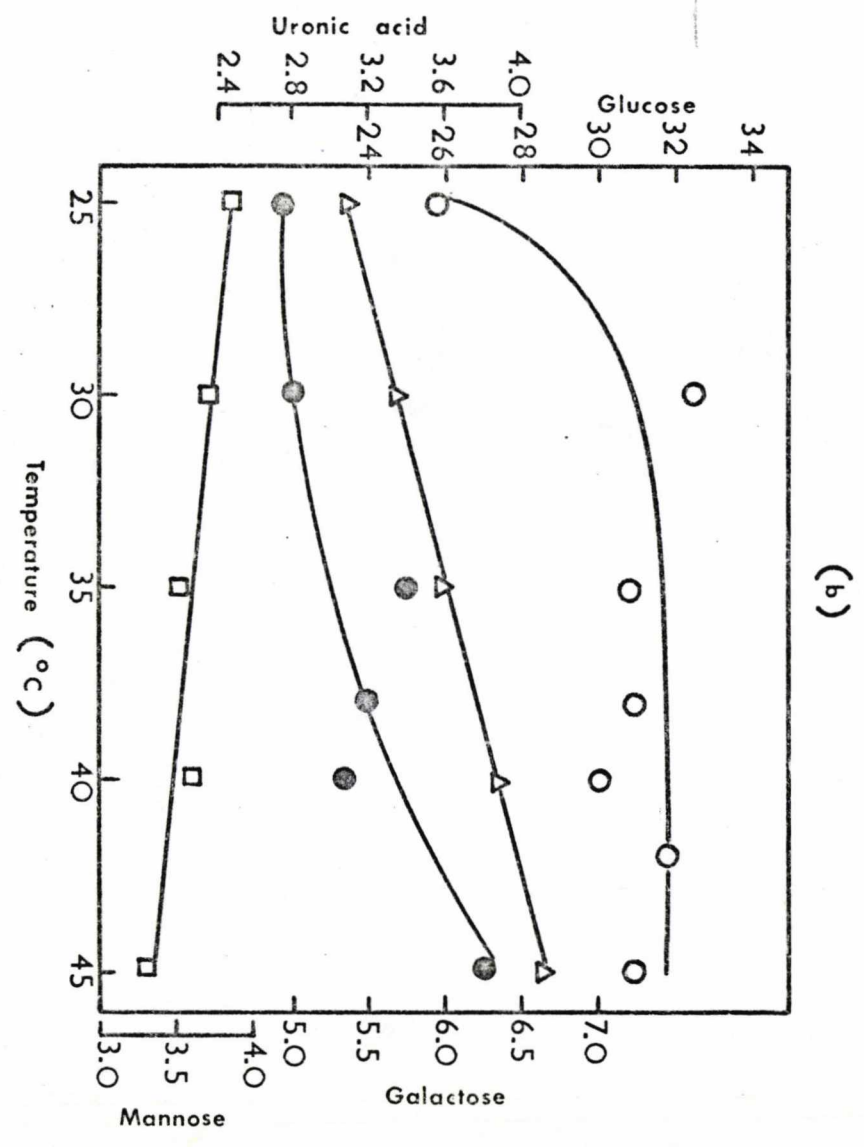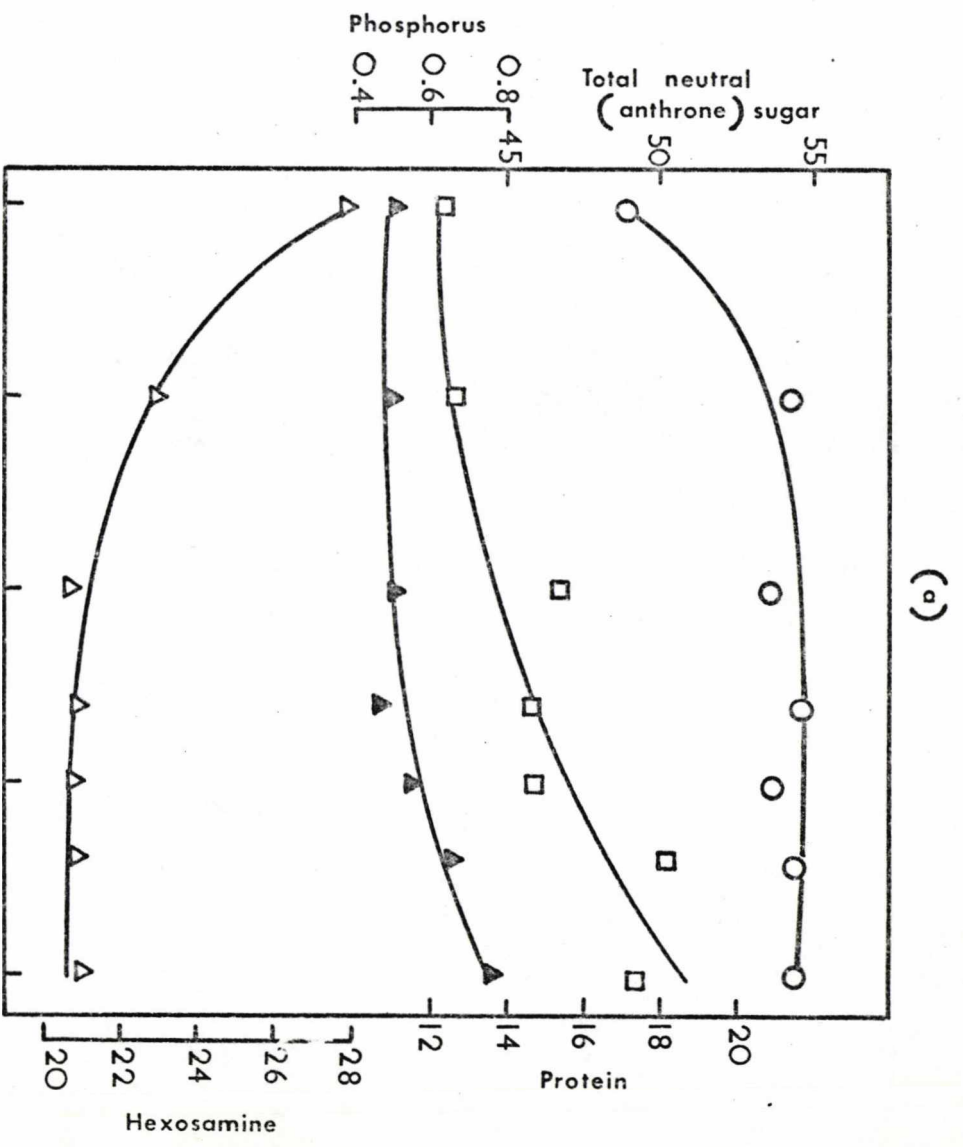wall material present in the mycelium.

Temperature (^{o}C)	Wall content (% w/w of mycelium)
25	21.0
30	18.3
35	22.8
38	29.0
40	26.0
42	31.9
45	19.1

4.2.3. Wall composition

Figure 4.6a,b shows the temperature associated changes in wall composition. Neutral carbohydrate, which accounts for the major portion of the wall by weight, followed a pattern similar to that exhibited by whole mycelia. The glucose content paralleled that of neutral carbohydrate, while mannose and galactose, respectively decreased and increased as the temperature was raised. The hexosamine content did not vary above 35°C but increased synthesis was observed between 35°C and 25°C. Overall, the total carbohydrate content of the wall showed little variation with temperature (Table 4.3). The hyphal wall contents of protein, phosphorus and uronic acid all increased directly with temperature.

A batch culture study by Katz & Rosenberger (1970a) showed that an increase of temperature from 30°C to 41°C elicited a common response in the wall phosphorus, protein and glucose contents of two strains of Aspergillus nidulans; both protein and phosphorus levels were higher at 41°C while the level of glucose was much reduced. The different pattern of glucose variation in batch and chemostat culture once again underlines the importance of the latter system in assessing quantitative aspects of phenotypic variation in micro-

Figure 4.6.   Effect of temperature on hyphal wall contents
              of: (a) hexosamine, Δ ; phosphorus, ▲ ; protein,
              □ ; total neutral (anthrone) sugar, O ;
              (b) mannose, □ ; uronic acid, ● ; galactose, Δ ;
              glucose, O . All values are quoted as % w/w of
              wall.

Temperature (°C)

(b)

Uronic acid
Glucose
Galactose
Mannose

(a)

Phosphorus
Total neutral (anthrone) sugar
Protein
Hexosamine

organisms.

Table 4.3. Total carbohydrate content of the hyphal wall
            at different temperatures.

Temperature (^{o}C)	Total carbohydrate (% w/w of wall)
25	77.1
30	77.5
35	74.6
38	75.8
40	74.6
42	75.5
45	75.5

4.3. DISCUSSION

4.3.1. Morphology

The vegetative morphology of Aspergillus nidulans was little affec-
ted by variations in temperature. This is in contrast to the changes
observed by Anderson & Smith (1972) during growth of Aspergillus
niger over the range 30°C to 44°C. These workers observed an in-
crease in branch formation between 30°C and 41°C, while at 44°C
there was a complete inhibition of apical growth, although spherical
growth of conidia did occur to produce so-called "giant cells". Be-
cause apical growth is dependent upon the translocation of wall pre-
cursors to the extending hyphal tip, such changes may reflect an
interference of this process. The increase in hyphal diameter of
Aspergillus nidulans between 25°C and 22°C (Table 4.1) may indicate
a similar restriction on translocation, possibly brought about by a
reduced rate of cytoplasmic streaming: studies with various phycomy-
cetes have revealed an approximate doubling of the rate of streaming
within the temperature range 19°C to 28°C (Burnett, 1968). An alter-
native explanation for restricted apical growth would be the

activation of chitin synthetase at subapical sites as a result of temperature dependent inactivation or inhibition of synthesis of the proteinaceous chitin synthetase inhibitor (Cabib & Farkas, 1971; McMurrough & Bartnicki-Garcia, 1973).

The hyphal swelling noted at high temperatures concurs with the report of Anderson & Smith (1972) who evidenced the onset of swollen hyphae in Aspergillus niger at 41°C. This observation undoubtedly indicates variations in cell wall structure leading to greater plasticity. Proteinaceous bonds, including disulphide bonds, between polysaccharide fibrils are believed to be important in higher plant morphogenesis (Lamport, 1965, 1969). Moreover, disruption of disulphide bond formation in Saprolegnia by dithiothreitol leads to swelling of the hyphal tip (Heath, 1970). Thus, alterations in the linkages between protein and carbohydrate may occur in Aspergillus at elevated temperatures: Emiliani & Ucha de Davie (1962) have indicated the presence of glycoprotein complexes in Aspergillus phoenicis. In this context, it is of interest to note that the degree of peptide cross bridging in the peptidoglycan of Bacillus coagulans walls is lower in cells grown at 55°C as compared with cells grown at 37°C and there is also a change in morphology (Novitsky, 1973).

4.3.2. Oxygen utilisation and yield of mycelium

The specific rate of oxygen utilisation by Aspergillus nidulans, like that of several microorganisms (McFeters & Ulrich, 1972; Rowley & Pirt, 1972) was enhanced by increasing the temperature of cultivation. This observation may be indicative of an increased maintenance energy requirement at elevated temperatures; increased maintenance requirements have been demonstrated in several bacteria with increasing temperature (Marr, Nilson & Clark, 1963; Topiwala & Sinclair, 1971; Mennett, 1972; Matsché & Andrews, 1973). Although it

has been suggested that a large portion of this maintenance energy may be needed for resynthesis of protein and nucleic acids during turnover (Marr, Nilson & Clark, 1963) it is unlikely that turnover of these macromolecules would assume significant proportions below the optimum growth temperature. However, since the environmental temperature induces similar variations in the lipid composition of all microorganisms i.e. increased saturation of fatty acid residues as the temperature is raised (Kates & Baxter, 1962; Sumner, Morgan & Evans, 1969; Chang & Matson, 1972), it is more probable that the re-organisation of cellular lipids and more specifically, cellular membranes necessitates an increased energy requirement at elevated temperatures.

Although an increased maintenance energy requirement would explain the lowered mycelial yield at temperatures above $38^{\circ}C$, the fall in yield below $38^{\circ}C$ may be a consequence of uncoupled growth. Uncoupled growth has been observed in some bacteria at temperatures on either side of the optimum and here the reduced growth efficiency is the result of energetic uncoupling, i.e. failure to make effective use of the ATP generated by catabolic reactions (Senez, 1962; Forrest, 1967; Ng, 1969). Whether energetic uncoupling or uncoupling of oxidative phosphorylation occurs in <u>Aspergillus nidulans</u> remains to be determined.

4.3.3. <u>Macromolecular composition: Carbohydrate, protein and DNA</u>

Several batch culture studies of microorganisms have revealed that the synthesis of polysaccharides, either capsular or intracellular, is increased at suboptimal temperatures. For example, the production of extracellular dextrans by Leuconostoc is reduced at $37^{\circ}C$ as compared to $25^{\circ}C$, an effect which has been attributed to the inactivation of dextransucrase at temperatures above $30^{\circ}C$ (Neely, 1960). Comparable experiments with a dextran producing Lactobacillus have

indicated the presence of a temperature sensitive dextran synthesis-
ing system (Dunican & Seeley, 1963). The starch content of the col-
ourless alga, Polytoma uvella decreases within the temperature range
22°C to 30°C, probably as a result of the inactivation of starch
synthesising enzymes (Mangat & Badenhuizen, 1971).

Observations on carbon limited chemostat cultures of unicellu-
lar organisms have also shown increased synthesis of carbohydrate at
suboptimal temperatures. In the yeast, Candida utilis, the level of
carbohydrate increases by 24% within the range 30°C to 15°C, when
the growth rate is controlled at a value of 0.10 h^{-1} (Brown & Rose,
1969). Tempest & Hunter (1965) observed an increase of the same
order in Aerobacter aerogenes between 40°C and 30°C but a further
lowering of the temperature from 30°C to 25°C was accompanied by a
147% rise in the carbohydrate content. Aspergillus nidulans differs
from these two organisms in that it synthesises increased amounts of
carbohydrate as the temperature is raised above 25°C; the increase
in carbohydrate, 139%, between 25°C and 22°C, however, is of the
same order as that shown by Aerobacter aerogenes between 30°C and
25°C (Tempest & Hunter, 1965). The data presented in Figure 4.7a,b
show an increase in the levels of both intracellular and extracellu-
lar (wall) carbohydrate in Aspergillus nidulans between 25°C and
45°C; intracellular carbohydrate was calculated by subtracting the
amount of extracellular carbohydrate i.e. wall neutral carbohydrate
material present in the mycelium (neutral carbohydrate content of
wall X proportion of wall material present in mycelium) from the
total carbohydrate content of the mycelium. The results indicate
that the increased level of carbohydrate in mycelia grown within the
range 25°C to 35°C (Figure 4.4a) is largely intracellular. This
finding is therefore similar to that of Brown & Rose (1969) for NH_4^+
limited Candida utilis. This organism contains an increased quantity
of intracellular carbohydrate, i.e. trehalose and glycogen, as the
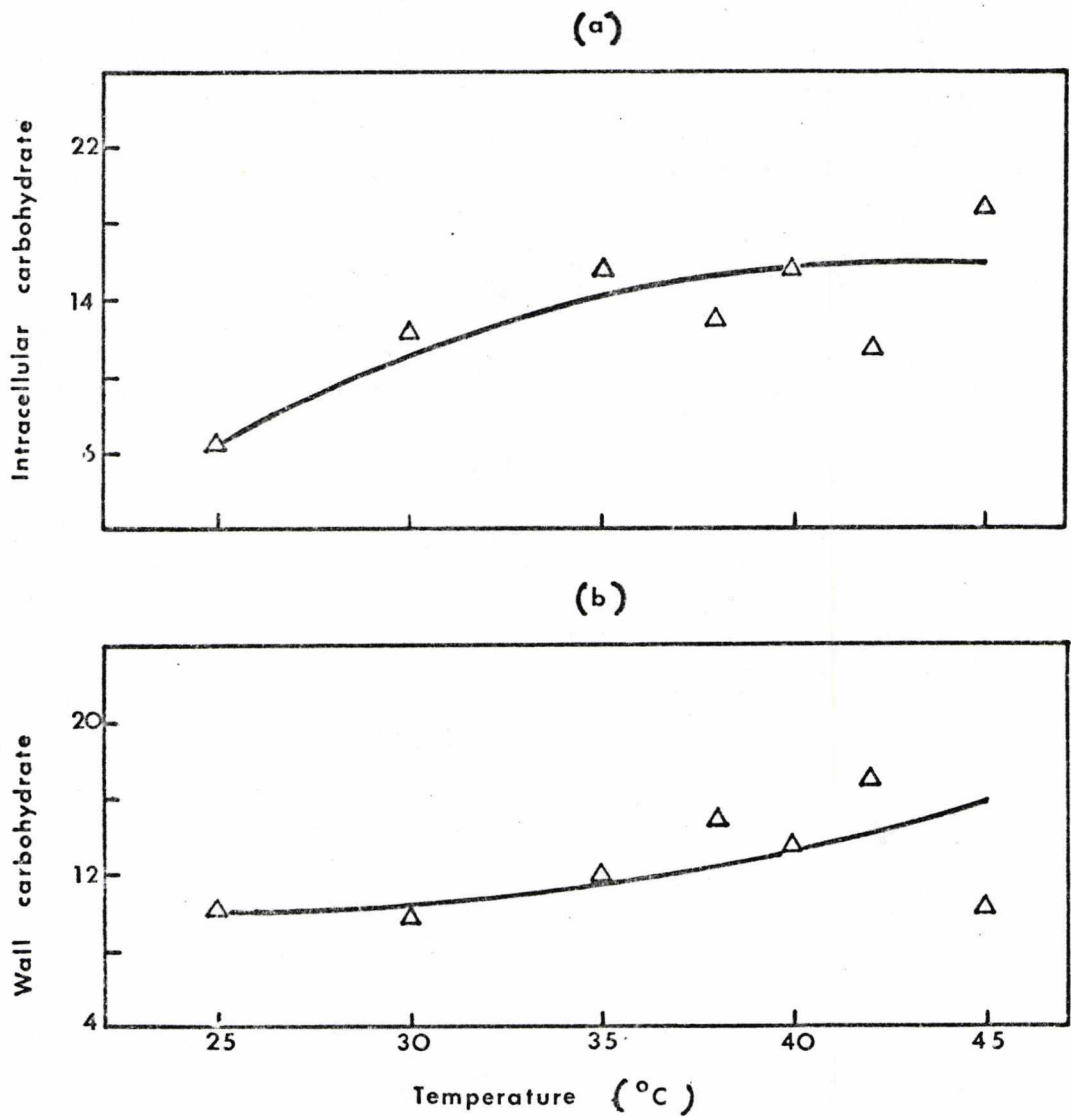
Figure 4.7. Effect of temperature on: (a) intracellular,
(b) extracellular (wall) carbohydrate contents
of mycelium. Values are quoted as % w/w of
mycelium.

temperature is raised from 15°C to 30°C and this increase is manifest, also, in the total cellular carbohydrate content (Brown & Rose, 1969). The enzymic basis for the altered pattern of intracellular carbohydrate synthesis in the yeast has not been elucidated. However, in the case of glycogen, an increase in the ATP pool as a result of low temperature energetic uncoupling (the fall in yield below 30°C may indicate such uncoupling) may provide an explanation since it has been shown that glycogen synthetase activity is inhibited by ATP (Rothman-Denes & Cabib, 1971). Insufficient data were available to determine whether the additional carbohydrate present in Aspergillus nidulans at 25°C was intracellular or extracellular in nature.

Continuous culture investigations of bacteria and yeast have indicated heterogeneity in the patterns of variation of total cellular protein with temperature. Growth of Candida utilis (Brown & Rose, 1969) and a psychrophilic pseudomonad (Harder & Veldkamp, 1967) at suboptimal temperatures evokes increased protein synthesis while the reverse behaviour is shown by glycerol limited cultures of Aerobacter aerogenes (Tempest & Hunter, 1965). At supraoptimal temperatures, also, there is a rise in the protein content of the pseudomonad (Harder & Veldkamp, 1967). However, variations in cellular protein levels of the extent shown by Aspergillus nidulans between 25°C and 22°C, i.e. a 47% increase over a 3°C interval, have not been reported. It is conceivable that this rise represents an increase in enzyme protein possibly associated with increased carbohydrate (polysaccharide) synthesis; additional synthesis of enzymic protein during low temperature adaptation has been proposed for the plankton alga, Skeletonema costatum, which doubles its protein content within the range 20°C to 7°C (Jørgensen, 1968).

The small variation in the DNA content of Aspergillus nidulans in response to changes in temperature is in agreement with observations made on unicellular organisms (Harder & Veldkamp, 1967; Brown &

Rose, 1969).

4.3.4. Efficiency of RNA in protein synthesis

Batch grown bacteria and yeasts cultivated at different temperatures show little variation in their RNA content (Schaechter, Maaløe & Kjeldgaard, 1958; Brown & Rose, 1969). However, during chemostat growth at low temperatures increased synthesis of RNA has been observed (Tempest & Hunter, 1965; Harder & Veldkamp, 1967; Brown & Rose, 1969). Tempest & Hunter (1965) found that the additional RNA synthesised by Aerobacter aerogenes at suboptimal temperatures was located in the ribosomal fraction of the cells, an observation which led them to consider ribosomal activity as a temperature-dependent function. Lowering the environmental temperature was assumed to cause a fall in ribosomal activity, so that in order to maintain a constant rate of protein synthesis (consistent with a fixed growth rate in the chemostat) an increase in the concentration of ribosomes was necessary to compensate the reduced ribosomal efficiency. In batch culture, however, where the cells already contain their maximum content of ribosomes, a lowering of temperature cannot evoke further synthesis of RNA, with the result that the growth rate falls and the RNA content remains unaltered.

Aspergillus nidulans, like the unicellular organisms, Aerobacter aerogenes (Tempest & Hunter, 1965), Candida utilis (Brown & Rose, 1969) and a psychrophilic pseudomonad (Harder & Veldkamp, 1967) synthesises increased amounts of RNA during chemostat culture at low temperatures. The data presented in Figure 4.8 show the temperature dependence of the protein synthesising activity of RNA (protein/RNA X μ) for Aspergillus nidulans. Although this activity remained constant over the range $30^{\circ}C$ to $40^{\circ}C$ there was a sharp fall towards extreme temperatures. Thus, at $25^{\circ}C$ and $50^{\circ}C$ the level of activity was only 50% of the maximum value. It would seem, therefore,
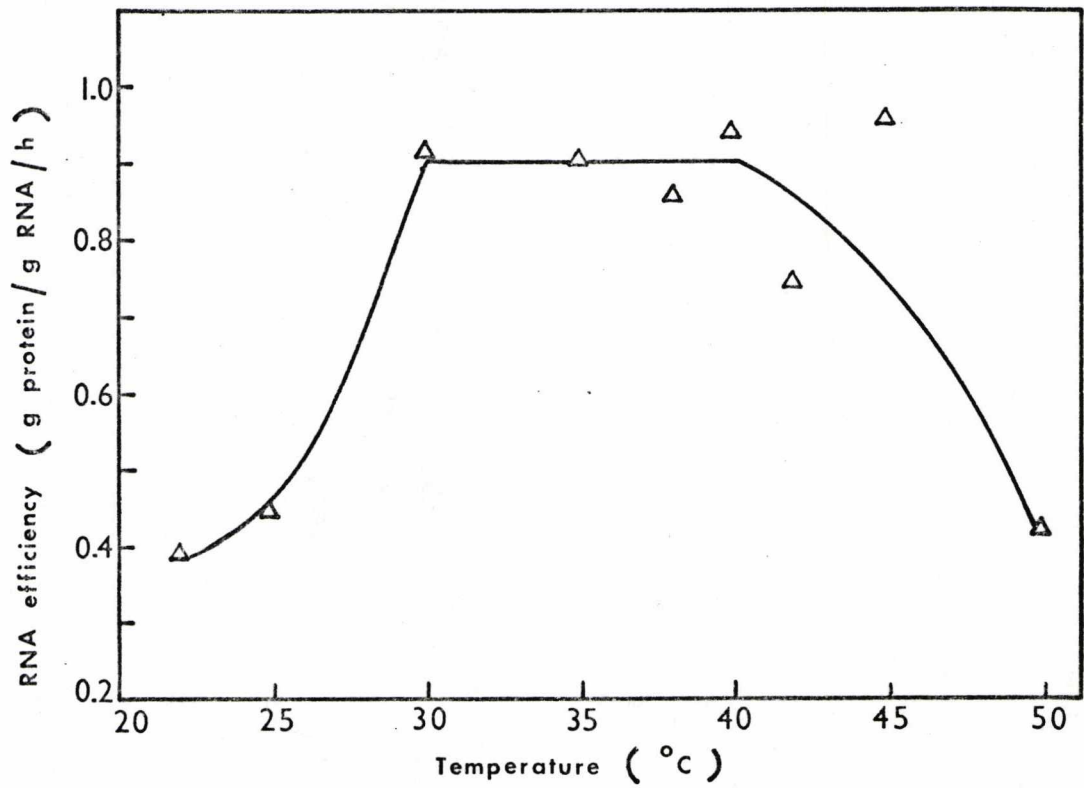
Figure 4.8.   Effect of temperature on the efficiency of RNA
in protein synthesis.

that the doubling of the RNA content between $30^{o}C$ and $22^{o}C$ (Figure 4.4a) adequately compensates the reduced efficiency of RNA in protein synthesis over the same range. However, at temperatures above $40^{o}C$ the reduced efficiency is not accompanied by an increase in RNA synthesis and this may mean that the rate of protein turnover has reached significant proportions.

While it is difficult, from the present data, to evaluate the effect of temperature within the range $30^{o}C$ to $50^{o}C$ on the ribosomal activity and functional polysome concentration of Aspergillus nidulans, in vitro analyses of eukaryotic protein synthesising sys- tems have revealed the existence of temperature sensitive steps in the peptide chain initiation process. Thus, formation of the 40S ribosomal subunit Met-tRNA$_f$ complex in the rabbit reticulocyte lysate system is markedly reduced at $42^{o}C$ (Mizuno, 1975), an effect which has also been observed in L cells (Shochetman & Perry, 1972). In Krebs ascites cells, however, the formation of the 40S ribosomal Met-tRNA$_f$ complex is not affected by temperature within the range $37^{o}C$ to $45^{o}C$, but the binding of this complex to mRNA and a 60S sub- unit to produce an 80S complex is far less efficient at $45^{o}C$ (Austin & Kay, 1975). Thus, the sensitivity of the chain initiation process to high temperatures may be a general feature of eukaryotic cells. If this is indeed the case, then a decrease in the polysome populat- ion would be expected during growth of Aspergillus nidulans in the chemostat at elevated temperatures and a corresponding increase in ribosomal activity so that growth may proceed at a fixed rate. It is interesting to note at this point that analyses of eukaryotic cell- free protein synthesising systems have demonstrated that peptide chain elongation proceeds normally at elevated temperatures (Shochetman & Perry, 1972; Austin & Kay, 1975; Mizuno, 1975). Fur- thermore, Sprechman & Ingram (1972) observed that the rate of trans- lation of "hemoglobin mRNA" by ribosomes of the rabbit reticulocyte

system increased within the temperature range 22°C to 45°C, and by using the thermostable supernatant factors of <u>Bacillus</u> <u>stearother-</u> <u>mophilus</u> and the ribosomes of <u>Escherichia</u> <u>coli</u>, Cox and colleagues (Cox, Pratt, Huvos, Higginson & Hirst, 1973) demonstrated a 5-fold increase in the rate of polyphenylalanine synthesis on raising the temperature from 37°C to 55°C.

Although inhibition of the initiation reaction of protein synthesis has also been observed at low temperatures in bacteria (Szer, 1970; Friedman, Lu & Rich, 1971), low temperature inactivation of the peptidyl transferase of <u>Escherichia</u> <u>coli</u> ribosomes has been reported (Miskin, Zamir & Elson, 1968) and it is possible that a lesion of this type is responsible for the reduced RNA efficiency of <u>Aspergillus</u> <u>nidulans</u> within the temperature range 30°C to 22°C. The high degree of compensation (99%) between RNA content and RNA efficiency over the range 30°C to 22°C suggests that all ribosomes and ribosomal subunits are associated in functional polysomes.

CHAPTER 5.   THE INFLUENCE OF pH VALUE ON THE

COMPOSITION AND PHYSIOLOGY OF

ASPERGILLUS NIDULANS

5.1. INTRODUCTION

Studies of fungal growth kinetics have revealed a diversity of response to concentrations of H^+ and OH^- ions in the culture medium. Penicillium chrysogenum, for example, attains a peak maximum specific growth rate within the range 7.0 to 7.4 (Pirt & Callow, 1960), while the optimum range for growth of Gibberella fujikuroi (Borrow, Brown, Jeffreys, Lessell, Lloyd, Lloyd, Rothwell, Rothwell & Swait, 1964) and Candida utilis (Andreeva, Shul'govskaya & Rabotnova, 1970) traverses several pH units.

The effect of pH on the growth rates of Candida utilis (Andreeva, Shul'govskaya & Rabotnova, 1970) and Propionibacterium shermanii (Ibragimova, Neronova & Rabotnova, 1969) has been treated as a case of non-competitive inhibition by H^+ and OH^- ions, which can be described by Michaelis-Menten type kinetics:

$$\mu = \frac{\mu_o K_p}{K_p + p} \tag{xxxx}$$

In this equation μ is the specific growth rate; μ_o, the value of μ with no inhibitor present; p, the inhibitor concentration; and K_p, a constant which represents the inhibitor concentration at which $\mu = \mu_o/2$. By analogy with the Monod equation, which demonstrates the dependence of growth rate upon the concentration of growth-limiting substrate, the above equation describes inhibition of a single metabolic "bottleneck" reaction for growth. Muzychenko and colleagues (Muzychenko, Kantere & Gurkin, 1973) suggested that such inhibition was likely to be caused by a limitation on the rate of diffusional transfer of the substrate-permease complex due to locally high charge densities within the pores of the membrane. The demonstration of pH-independent yield coefficients, concomitant with a slowing down of the specific growth rate on the acid side of the pH optimum in Penicillium chrysogenum (Pirt & Callow, 1960) and

Trichoderma viride (Brown & Halsted, 1975), is consistent with Muzy-chenko's suggestion that glucose transfer into the cell is restrict-ed by the medium H^+ ion concentration. The foregoing examples serve to illustrate the necessity of controlling the specific growth rate in studies relating the effects of environmental pH on microbial physiology.

The influence of pH value, under chemostat conditions, on bacterial growth has been studied in some detail. Thus, respiratory activity and growth efficiency (Harrison & Loveless, 1971), meta-bolic product (propionic and acetic acids) formation (Ibragimova, Neronova & Rabotnova, 1971), macromolecular composition (Tempest & Hunter, 1965; Ibragimova, Neronova & Rabotnova, 1971; Sakharova & Ibragimova, 1975) and cell wall composition (Ellwood & Tempest, 1972; Archibald, Baddiley & Hepstinall, 1973) have all been invest-igated. Among eukaryotic microorganisms, respiratory metabolism has been studied over a limited pH range (2.43 to 2.77) in Candida utilis, (Lirova, Andreeva & Taptykova, 1973) while variations in melanin biosynthesis (Rowley & Pirt, 1972) and morphology (Pirt & Callow, 1959) with culture pH have been reported in filamentous fungi.

An investigation by Cantrell & Dowler (1971) has shown that pH has little effect on the macromolecular composition of Pythium, but as the batch method for mycelial cultivation was used the data obtained by these workers must be treated with due regard to this factor. The present account relates the different extents to which the cellular and hyphal wall composition of Aspergillus nidulans are affected by culture pH value under steady-state conditions.

5.2. RESULTS

5.2.1. Hyphal growth

Hyphal growth was examined over the pH range 3.1 to 7.9 with

temperature controlled at $30^{\circ}C$, culture dilution rate at 0.10 h^{-1} and glucose at a level of 12.5 g/l. The culture pH was varied by the automatic addition of either 2 M H_2SO_4 or 2 M KOH. Filamentous growth was achieved under all conditions, but at pH 3.1 the culture contained a few small mycelial pellets. Moreover, at pH 3.1 glucose was detected in culture filtrates at a concentration of 0.23 g/l, although steady state growth was obtained. A proportion of the hyphae grown at pH 4.1 and pH 3.1 showed swellings, but the diameter of swollen hyphae did not exceed 1.5 to 2 times the normal hyphal diameter. The data presented in Table 5.1 indicate that pH had little effect on the mean hyphal diameter and wall thickness.

Table 5.1. Hyphal diameter and wall thickness at different pH values.

pH	Hyphal diameter (μm)	Wall thickness (μm)
3.1	1.07 ± 0.11[a]	0.067 ± 0.005[a]
4.1	1.19 ± 0.13	0.070 ± 0.014
5.0	1.18 ± 0.18	0.070 ± 0.014
6.0	0.97 ± 0.10	0.075 ± 0.005
6.9	0.93 ± 0.13	0.064 ± 0.005
7.9	0.92 ± 0.12	0.071 ± 0.009

Each result is the mean of 10 measurements.
[a]Standard deviation.

Biomass synthesis and growth yield in cultures of *Aspergillus nidulans* were greatest at pH 6.9 (Figure 5.1a). Oxygen consumption increased within the range pH 7.9 to 5.0 but fell at pH values below 5.0 (Figure 5.1b). Carbon dioxide output increased towards low pH values and the respiratory quotient (RQ) which showed little variation between pH 4.1 and 7.9 rose significantly below pH 4.1 (Figure 5.1b).
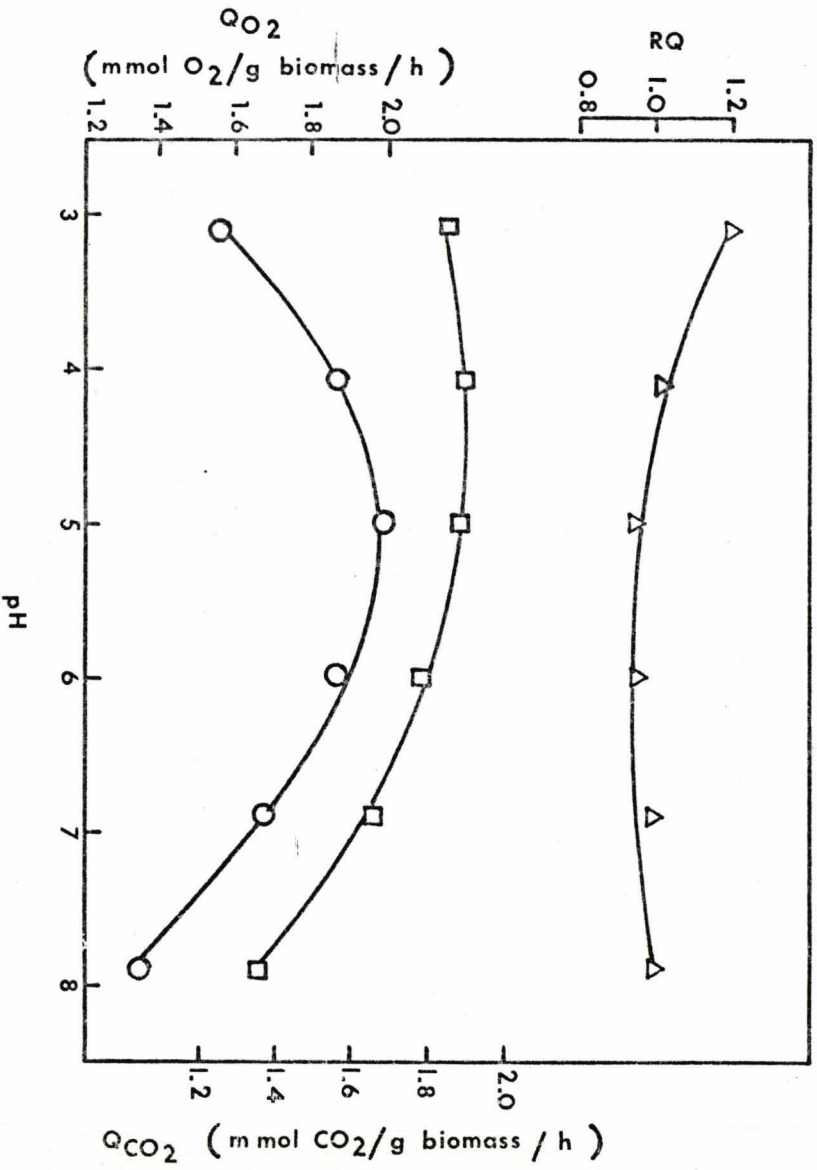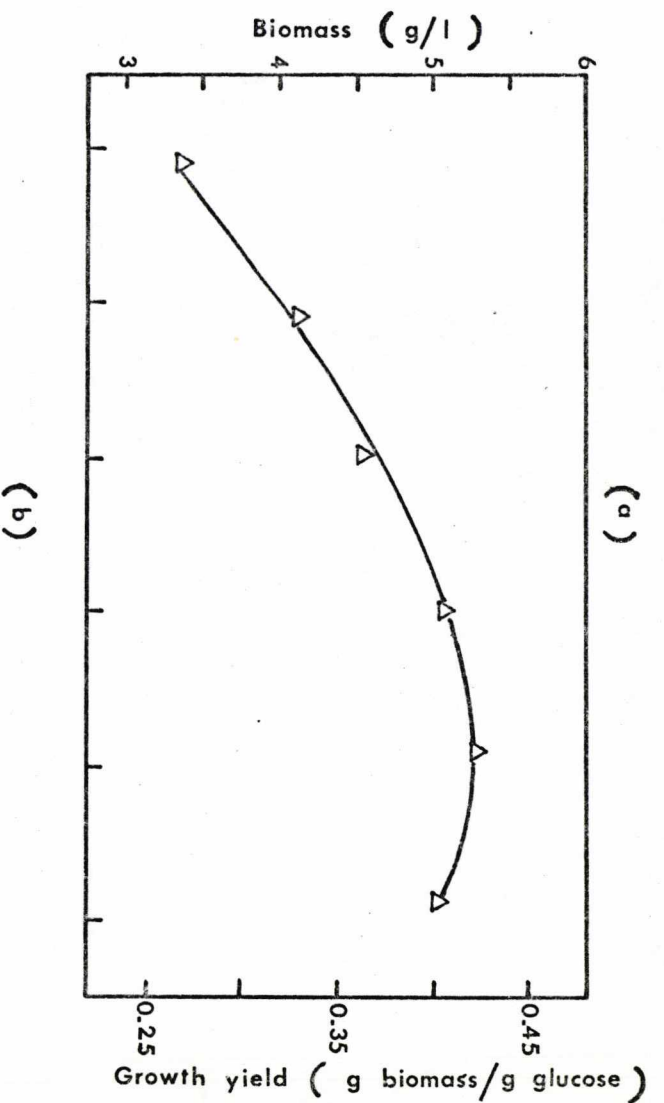
Figure 5.1. Effect of pH on: (a) biomass production, growth yield; (b) Q_{O_2}, O ; Q_{CO_2}, □ ; RQ, △ .

5.2.2. Hyphal composition

Variations in hyphal composition in response to changes in culture pH are shown in Figure 5.2a,b. Protein and carbohydrate both exhibited small decreases as the pH value was increased. The DNA content fell above pH 6.0, while RNA showed a 32% reduction as the pH was raised over the range 3.1 to 7.9. The amino acid pool, which remained invariant over the range pH 3.1 to 6.9, decreased by 38% between pH 6.9 and 7.9. Phosphorus and potassium each constituted a constant proportion of the hyphal dry weight at pH values above 4.1, but the levels of both components fell between pH 4.1 and 3.1. The magnesium content also dropped over the latter range and again above pH 4.1 showed a decline. The molar stoichiometry between RNA and Mg^{2+} showed a marked dependence upon pH value, the major variation occurring below pH 4.1, when the RNA/Mg^{2+} molar ratio almost trebled in value (Figure 5.3).

As the pH was raised a decreased proportion of the hyphal dry weight was accounted for by wall material (Table 5.2).

Table 5.2.   Effect of pH on the proportion of wall material present in the mycelium.

pH	Wall content (% w/w of mycelium)
3.1	29.1
4.1	29.1
5.0	26.7
6.0	23.7
6.9	18.3
7.9	17.5

5.2.3. Wall composition

In contrast to the whole hyphae the wall composition was drastically affected by the culture pH: the results are shown in Figure 5.4a,b.
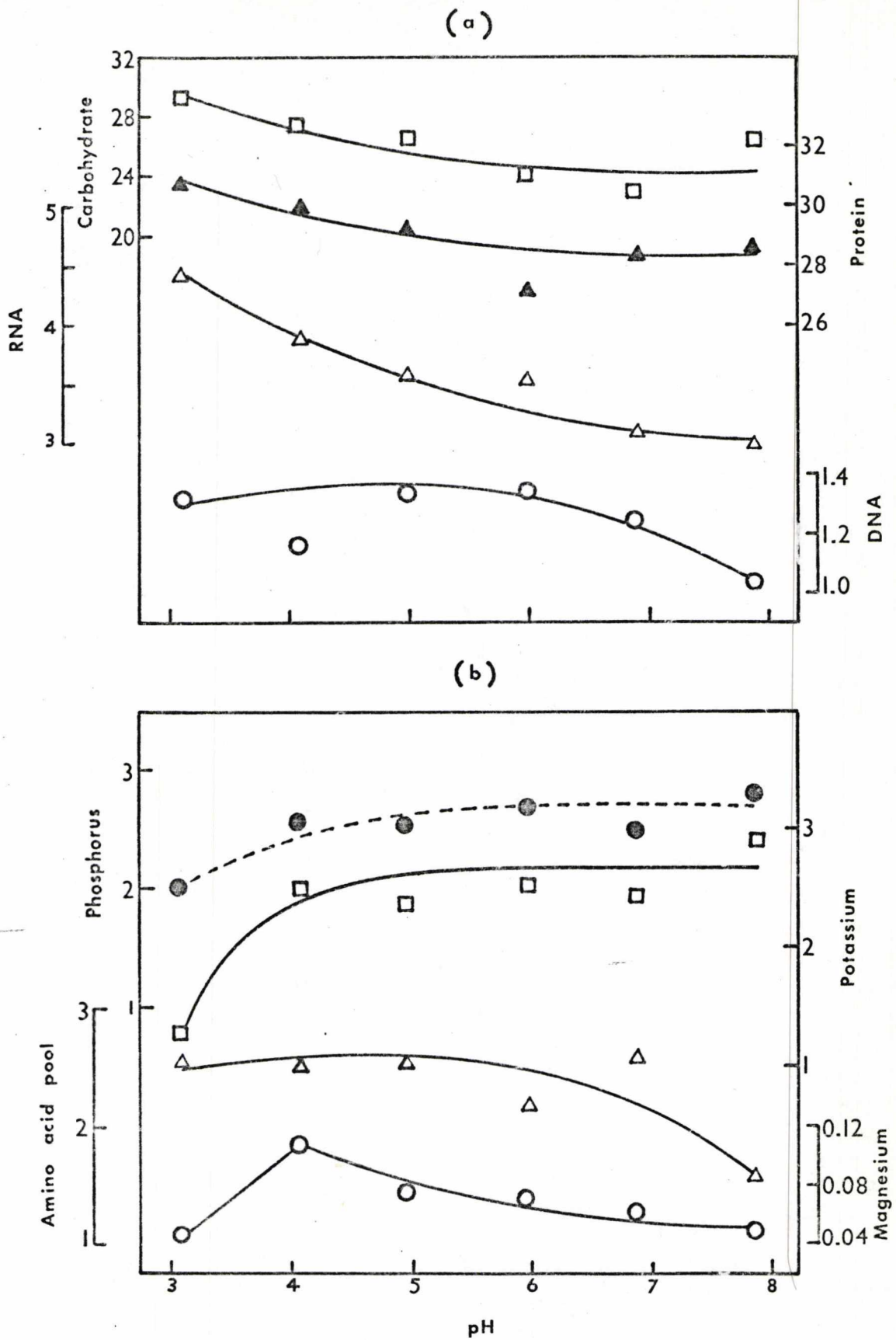
Figure 5.2.   Effect of pH on hyphal contents of: (a) DNA, O ;
RNA, △ ; protein, ▲ ; carbohydrate, □ ; (b) magnesium,
O ; amino acid pool, △ ; potassium, □ ; phosphorus,
● . All values are quoted as % w/w of mycelium.
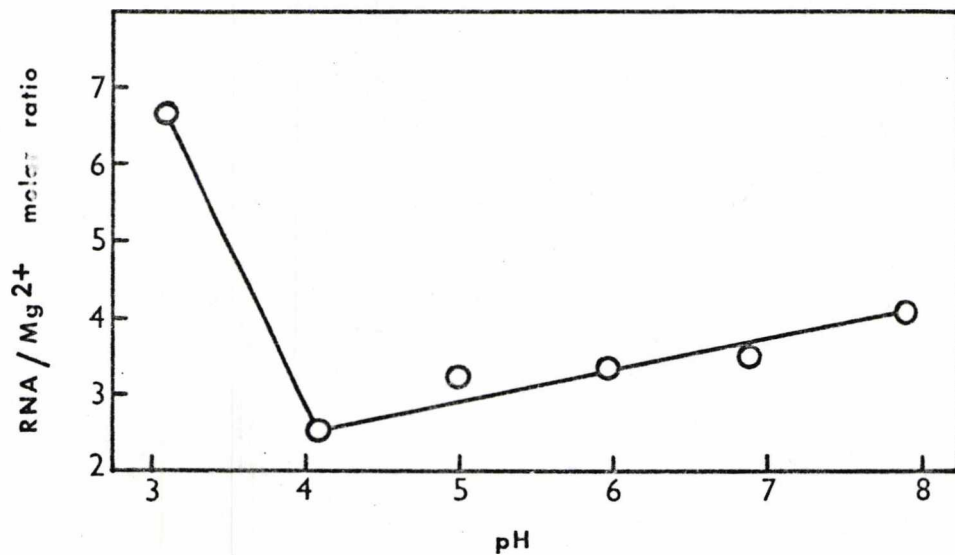
Figure 5.3. Stoichiometry between hyphal RNA and magnesium at different pH values. An average molecular weight for RNA (nucleotide) of 340 was assumed.

Figure 5.4. Effect of pH on hyphal wall contents of:
            (a) phosphorus, ▲ ; protein, ☐ ; hexosamine,
            △ ; total neutral (anthrone) sugar, ○ ;
            (b) uronic acid, ◉ ; mannose, ☐ ; galactose,
            △ ; glucose, ○ . All values are quoted as
            % w/w of wall.
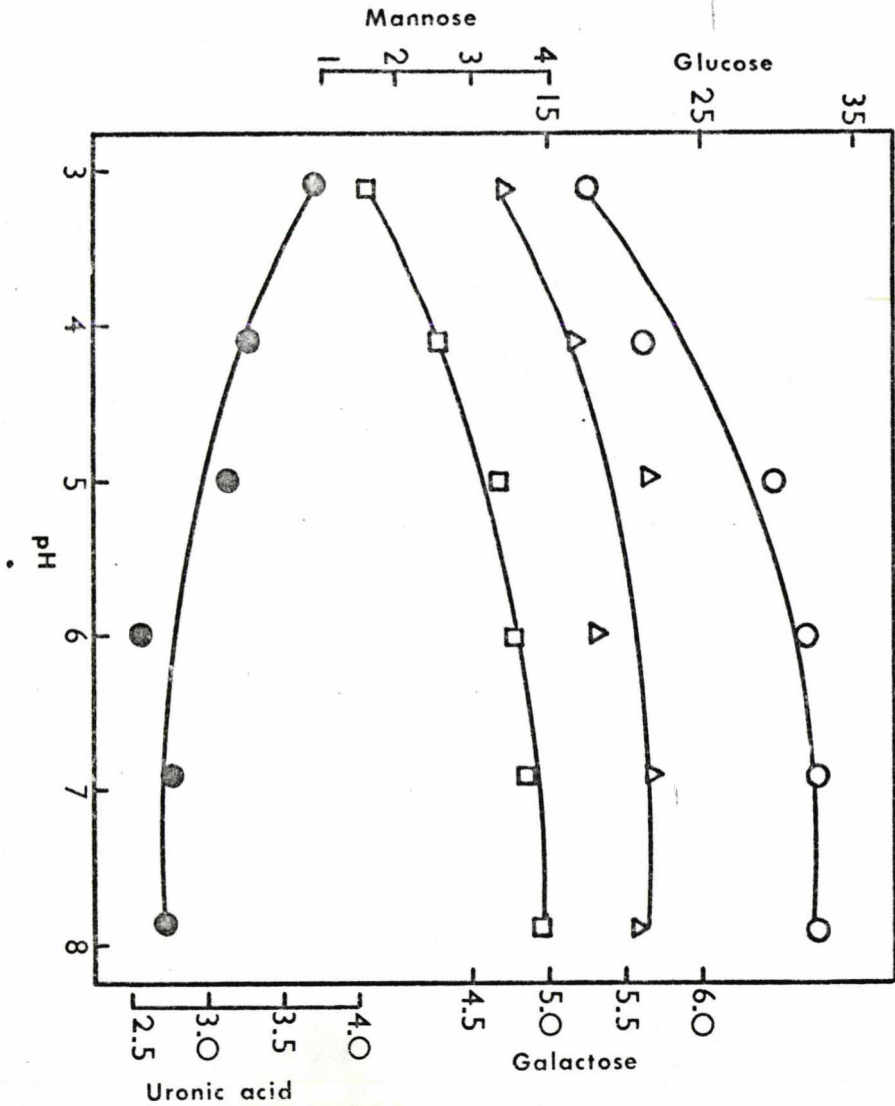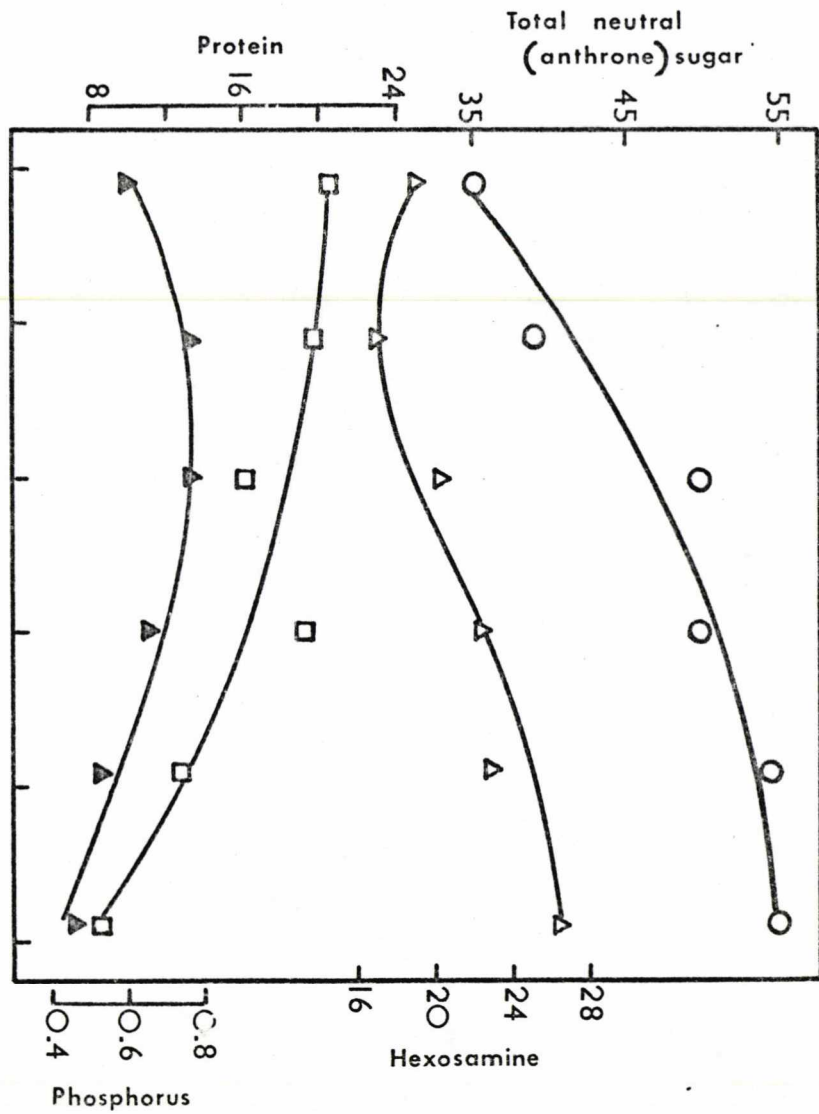
Of the components least affected galactose showed a 19% increase over the range 3.1 to 7.9 and the uronic acid content fell by 27% over the same range. The total neutral sugar, mannose and glucose contents all exhibited substantial increases between pH 3.1 and 7.9 (values rose by 55%, 140% and 93%, respectively), but in all three cases 77% of the total increase occurred between pH 3.1 and 5.0. The mannose: galactose: glucose molar ratio was constant except at the lowest pH values (Table 5.3).

Table 5.3. Effect of pH on the molar ratio of mannose: galactose: glucose in the hyphal wall.

pH	Molar ratio		
	mannose	galactose	glucose
3.1	2	6	21
4.1	2	4	17
5.0	2	3	18
6.0	2	3	18
6.9	2	3	18
7.9	2	3	17

There was a 40% increase in the level of hexosamine and overall, the total carbohydrate content of the wall rose by 50% over the pH range examined (Table 5.4).

Table 5.4. Total carbohydrate content of the hyphal wall at different pH values.

pH	Total carbohydrate (% w/w of wall)
3.1	54.3
4.1	56.4
5.0	70.3
6.0	72.5
6.9	77.5
7.9	81.3

A 50% reduction in the protein content was observed over the range 3.1 to 7.9 and the level of phosphorus also dropped significantly between the two pH extremes.

5.3. DISCUSSION

5.3.1. Morphology

The morphological changes observed during growth of Aspergillus nidulans at different pH values were not as extensive as those previously reported for mycelial fungi. Abundant conidial production has been demonstrated in batch grown Penicillium griseofulvum and Trichoderma viride at pH values of 4.35 and 3.15, respectively, while large globose cells (sporophore initials) terminal to the main or lateral mycelial branches were noted at still lower pH values (Bent & Morton, 1963). In chemostat grown Penicillium chrysogenum, short, much-branched, swollen and distorted hyphae have been observed at a pH value of 7.4 (Pirt & Callow, 1959). In the latter case the thickness of the swollen hyphae reached values up to six times the normal hyphal thickness, a range three times greater than that observed for Aspergillus at pH 3.1 and 4.1. Pirt & Callow (1959) suggested that the aberrant hyphal morphology might be the result of changes in wall structure or composition, a view which is supported by the observations made with Aspergillus nidulans (Figure 5.4).

Also, in agreement with the observations of Pirt & Callow (1959) pellet formation in Aspergillus nidulans occurred in association with an increase in the branching frequency of the mycelium.

5.3.2. Oxygen utilisation and yield of mycelium

An increase in the maintenance energy requirement of Aspergillus nidulans within the pH range 6.9 to 5.0 is suggested by the simultaneous increase and decrease in the rate of oxygen utilisation and yield of mycelium, respectively: Harrison & Loveless (1971) also

reported an increase in the Q_{O_2} of chemostat grown _Escherichia coli_ as the pH was reduced from 6.6 to 5.4. This increased maintenance requirement is probably concerned with the active extrusion of H^+ ions from the cytoplasm by means of H^+ ion pumps situated in the plasmalemma. Below pH 4.1, however, the fall in Q_{O_2} may be an indication that _Aspergillus nidulans_ can no longer maintain a pH differential, possible interference of normal respiratory metabolism also being involved; in _Candida utilis_ damage to the electron transport chain in the region of the cytochromes and dehydrogenases occurs below pH 2.77 (Lirova, Andreeva & Taptykova, 1973). The concurrent drop in the levels of mycelial K^+ and Mg^{2+} furthers the view that appreciable uptake of H^+ occurs. It is interesting to note in this context that in yeast H^+ ions competitively inhibit the transport of all cations below pH 4.0 (Suomalainen & Oura, 1971) and that this is accompanied by H^+ uptake (Armstrong & Rothstein, 1967).

5.3.3. Efficiency of RNA in protein synthesis

In common with many continuously cultivated unicellular prokaryotes (Tempest & Hunter, 1965; Ibragimova, Neronova & Rabotnova, 1971; Sakharova & Ibragimova, 1975) the mycelial contents of DNA, protein and carbohydrate of _Aspergillus nidulans_ did not alter significantly in response to variations in environmental pH. However, a significant decrease in the level of RNA occurred at high pH values, a tendency which has also been observed in populations of _Bacillus megaterium_ (Sakharova & Ibragimova, 1975).

Figure 5.5 presents data on the change in the protein synthesising activity of RNA (protein/RNA X D) of _Aspergillus nidulans_ as a function of various pH values. Lowering the pH from above neutrality produced a 27% drop in the efficiency of RNA in protein synthesis. Chemostat grown cultures of bacteria and yeast also show variations in the efficiency of the protein synthesising system as the
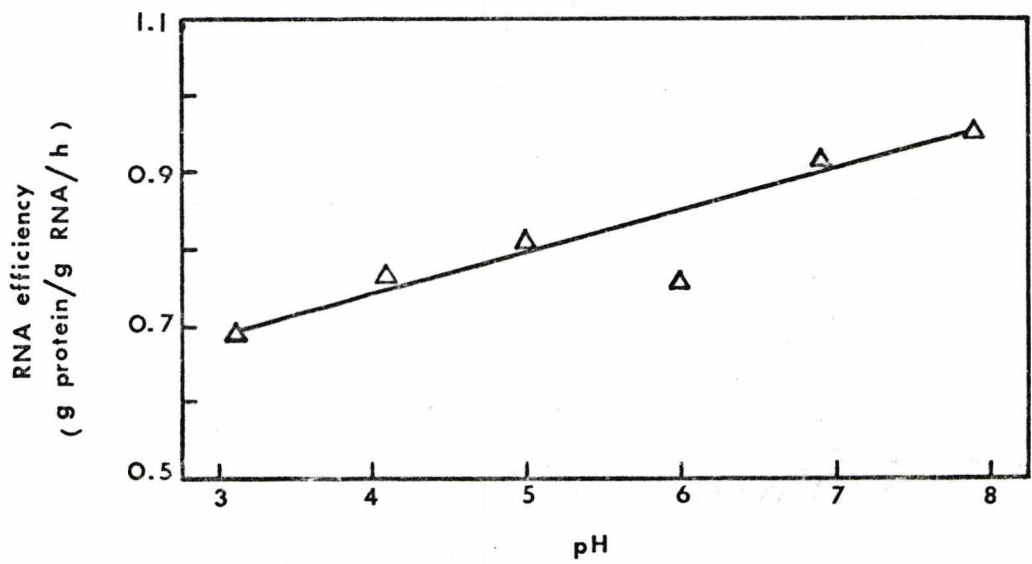
Figure 5.5.   Effect of pH on the efficiency of RNA in protein
            synthesis.

environmental pH is altered (Tempest & Hunter, 1965; Alroy & Tannen-baum, 1973; Sakharova & Ibragimova, 1975). An investigation of the influence of different environmental parameters (μ, pH, temperature) on the protein/nucleic acid ratio of <u>Candida</u> <u>utilis</u> led Alroy & Tannenbaum (1973) to propose that the pH-induced changes in the protein synthesising efficiency of the yeast were solely manifestations of the changes in the reduced dilution rate, D/D_c (where D is the dilution rate and D_c is its critical value) which accompanied alterations in the culture pH value; thus, nucleic acid efficiency varied in exactly the same way as D_c. A reassessment of the protein and nucleic acid data obtained by Tempest & Hunter (1965) for <u>Aerobacter</u> <u>aerogenes</u> grown at a fixed rate but in media of varying pH has shown that this relation also holds true for the bacterium (Alroy & Tannenbaum, 1973). Extending these observations to <u>Aspergillus</u> <u>nidulans</u> a drop in μ_{max} (D_c) is indicated as the pH is lowered from 7.9.

CHAPTER 6.   EFFECTS OF SODIUM CHLORIDE ON THE

PHYSIOLOGY OF ASPERGILLUS NIDULANS

6.1. INTRODUCTION

A large number of terrestrial fungi are capable of growth in varying dilutions of sea water (approximate NaCl concentration 3.5%) (Jones & Byrne, 1976). As growth of terrestrial fungi, in saline environments, is accompanied by a reduction in the maximum specific growth rate (Watson, 1970; Charlang & Horowitz, 1971), studies relating the specific effects of sodium chloride on cell physiology must, of necessity, be performed under chemostat conditions.

Variations in the cellular (Tempest & Meers, 1968; Tempest, Meers & Brown, 1970) and wall (Meers & Tempest, 1970; Ellwood & Tempest, 1972) composition of bacteria, in response to changes in the culture sodium chloride concentration, have been noted under steady state conditions, while in Candida utilis metabolic pathways are influenced by the presence of sodium chloride (Watson, 1970). A survey of the sodium chloride tolerance of terrestrial fungi (Tresner & Hayes, 1971) showed that, of the organisms tested, members of the genus Aspergillus were among the most tolerant. It was therefore of interest to develop further this observation. Hence, in the present work an attempt is made to determine the way in which the physiology of Aspergillus nidulans changes to adapt to different sodium chloride concentrations.

6.2. RESULTS

6.2.1. Hyphal growth

Preliminary experiments were carried out in order to determine the range of NaCl concentrations which Aspergillus nidulans would tolerate. A defined medium agar, incorporating 0, 5, 10, 15, 20 or 25% (w/v) NaCl, was inoculated with vegetative hyphae and growth examined at intervals during incubation at 30°C. Growth was completely prevented at concentrations of NaCl exceeding 10% (w/v). Growth in the presence of increasing NaCl concentrations was accompanied by a

reduction in the colony radial growth rate (Figure 6.1) and 10% (w/v) NaCl markedly reduced conidiation.

For chemostat experiments the concentration of NaCl in the medium was increased progressively from 0 to 8% (w/v) NaCl. The growth rate, pH and temperature were controlled at 0.10 h^{-1}, pH 6.8 and $30^{o}C$ respectively, and glucose at a level of 12.5 g/l. The addition of NaCl to a culture, previously grown in NaCl-free medium evoked an initial response, characterised by the appearance of conidia at hyphal tips. This condition, however, merely represented a transition phase prior to the establishment of a steady state. Media containing 8% (w/v) NaCl caused a drop in the mycelial dry weight as time progressed and empty hyphal compartments were evident throughout the exposure period. Although analytical data for mycelium grown in the presence of 8% (w/v) NaCl are included in this report, it is improbable that the culture attained a steady state at this concentration.

Culture dry weight and mycelial yield fell as the percentage of NaCl was raised, while non-metabolised glucose was detected in culture filtrates at NaCl concentrations of 4% (w/v) and above (Figure 6.2a).

The respiration rate dropped when the NaCl concentration was raised above 2% (w/v) and there was a corresponding but less marked fall in carbon dioxide production (Figure 6.2b); as a result the respiratory quotient (RQ) exhibited a slight increase as the NaCl concentration was raised (Figure 6.2b).

Hyphal size and wall thickness were largely unaffected by the presence of NaCl (Table 6.1).

Additional alkali was required to maintain a steady culture pH value as the NaCl concentration was increased (Table 6.2).
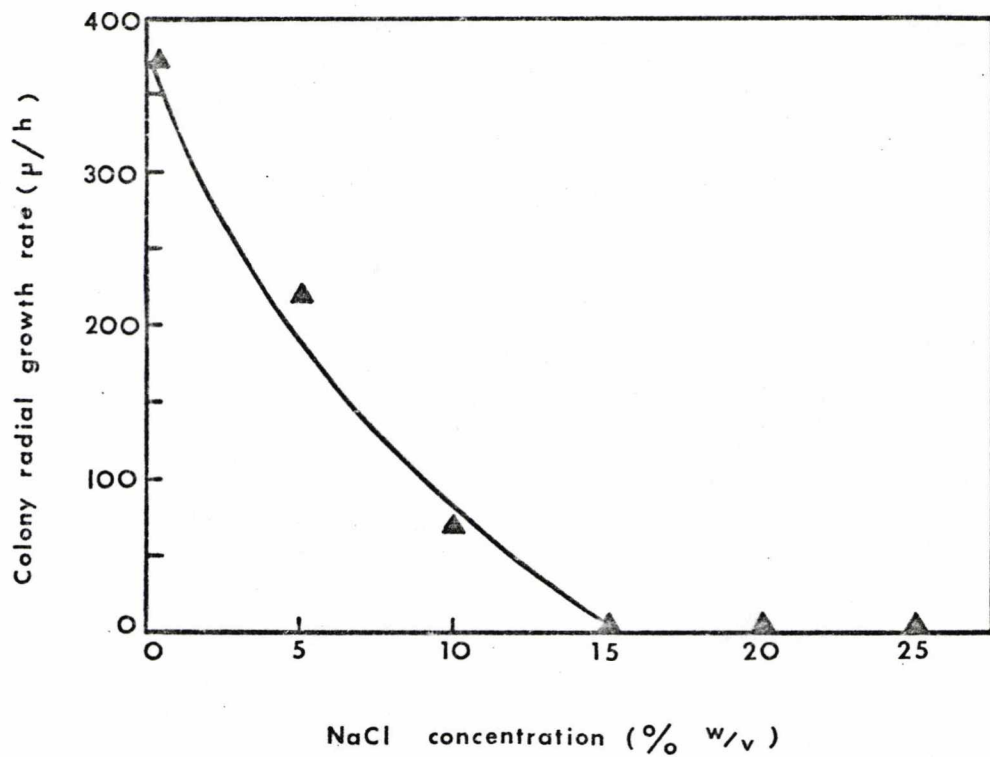
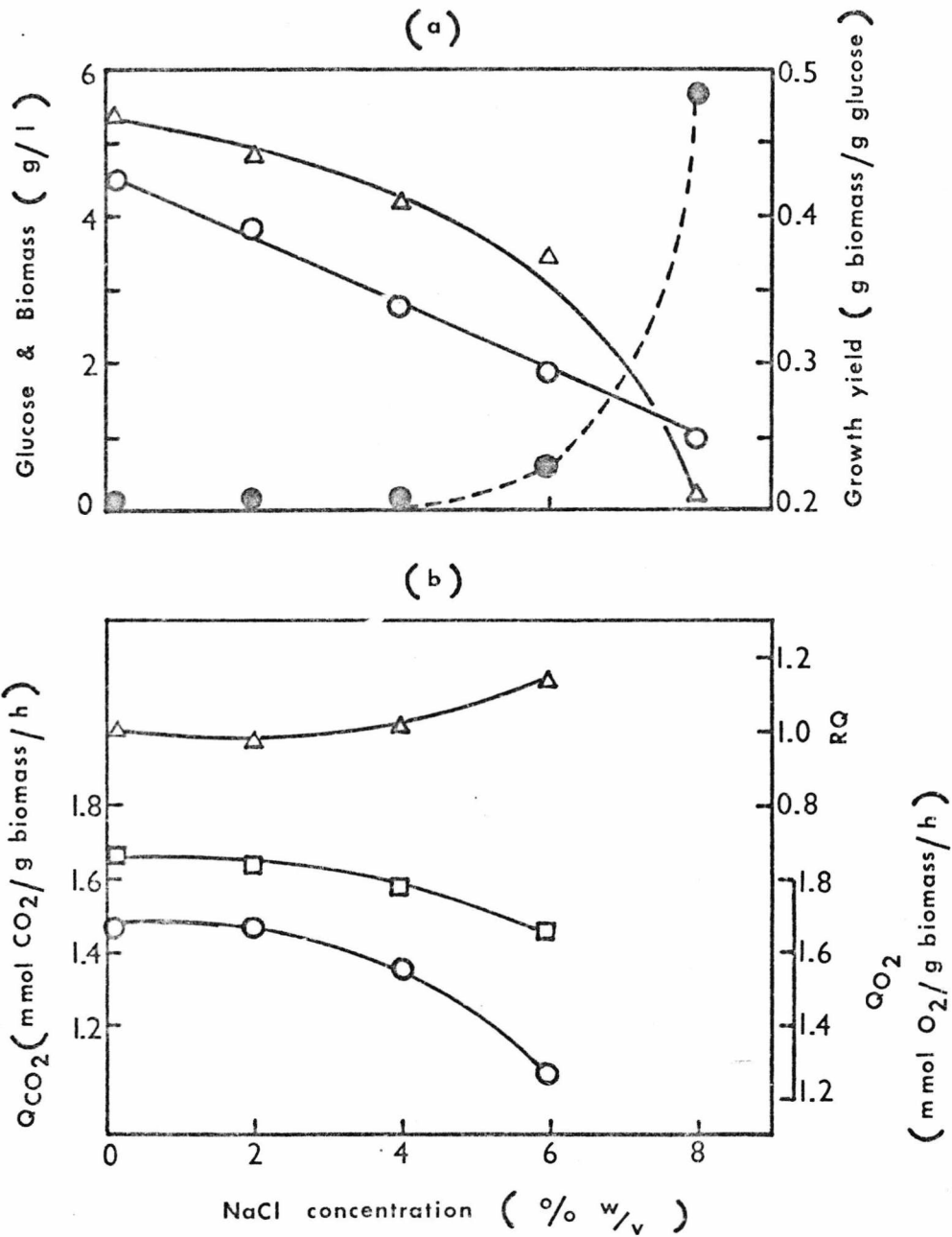Figure 6.1. Effect of NaCl concentration on colony radial growth rate.

Figure 6.2. Effect of NaCl concentration on: (a) biomass
production, △ ; growth limiting substrate
concentration, ● ; growth yield, ○ ; (b) Q_{O_2}, ○ ;
Q_{CO_2}, □ ; RQ, △ .

Table 6.1. Hyphal diameter and wall thickness at different
NaCl concentrations.

NaCl concentration (% w/v)	Hyphal diameter (μm)	Wall thickness (μm)
0	0.93 ± 0.13[a]	0.064 ± 0.005[a]
2	1.00 ± 0.15	0.063 ± 0.003
4	0.99 ± 0.19	0.065 ± 0.005
6	1.10 ± 0.20	0.077 ± 0.003
8	0.93 ± 0.03	0.068 ± 0.008

Each result is the mean of 10 measurements.
[a]Standard deviation.

Table 6.2. Effect of NaCl concentration on alkali consumption.

NaCl concentration (% w/v)	Alkali (2 M KOH) required for pH control (ml/g biomass/h)
0	0
2	0.57×10^{-3}
4	0.78×10^{-3}
6	1.24×10^{-3}

6.2.2. Hyphal composition

Changes in hyphal composition are shown in Figure 6.3a,b. The
protein, RNA and DNA contents exhibited small increases as the NaCl
concentration was raised. The level of carbohydrate increased by 74%
over the entire range of concentrations studied. The internal amino
acid pool, phosphorus and potassium levels all fell in a similar
manner. The magnesium content decreased less markedly than either
potassium or phosphorus and reached a constant value when the NaCl
concentration was 4% (w/v). This value, 0.04%, probably represents
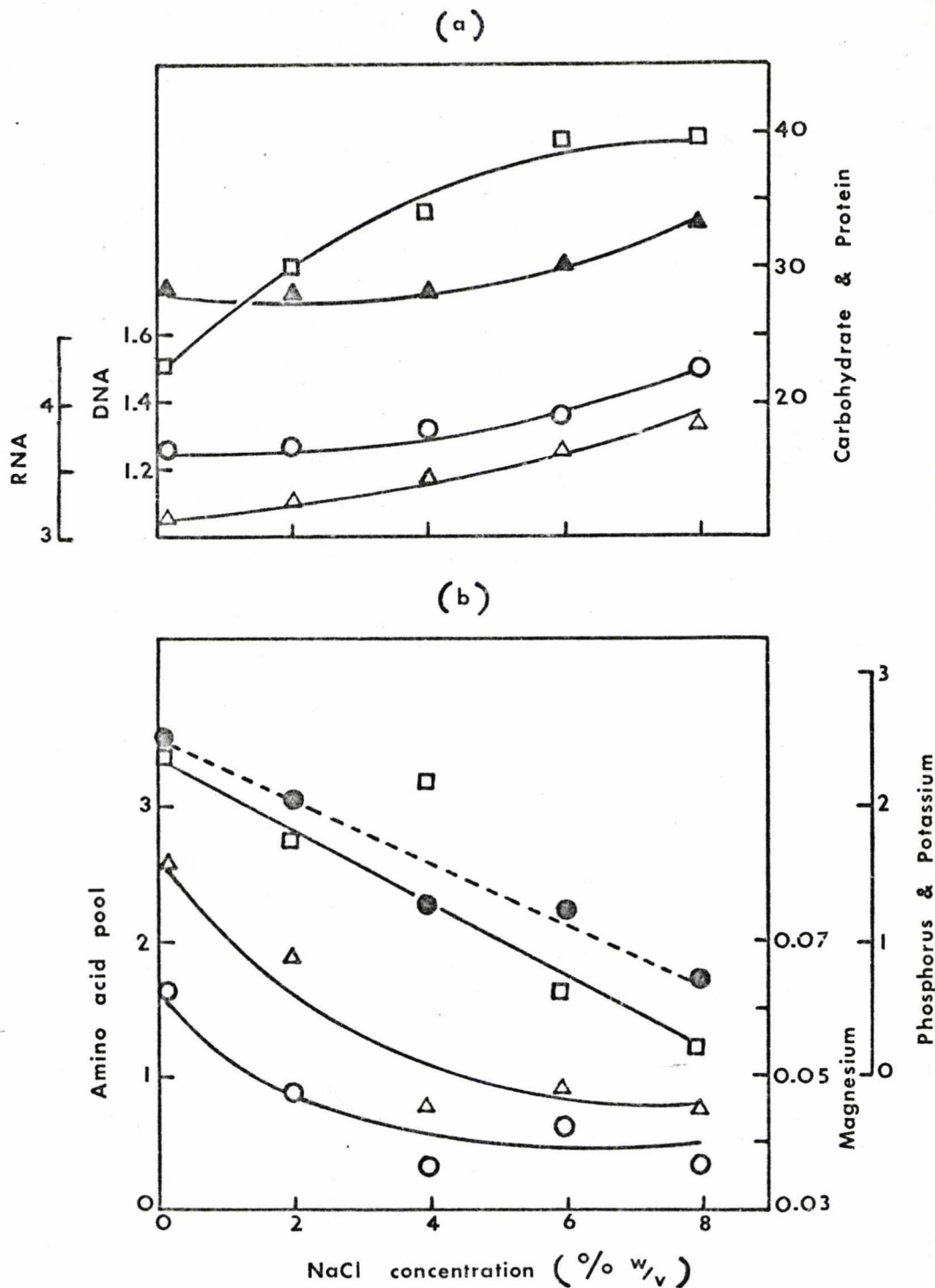
Figure 6.3. Effect of NaCl concentration on hyphal contents
of: (a) RNA, △ ; DNA, O ; protein, ▲ ; carbohydrate,
□ ; (b) magnesium, O ; amino acid pool, △ ;
potassium, □ ; phosphorus, ● . All values are
quoted as % w/w of mycelium.

the basic, minimal magnesium content of the mycelium. The molar ratio of RNA to Mg^{2+} increased progressively with increasing NaCl concentration (Figure 6.4).

There was some variation in the quantity of wall material present in the mycelium as the culture salinity was altered (Table 6.3).

Table 6.3. Effect of NaCl concentration on the proportion of wall material present in the mycelium.

NaCl concentration (% w/v)	Wall content (% w/w of mycelium)
0	18.3
2	16.6
4	14.0
6	28.7
8	21.2

6.2.3. Wall composition

The influence of changes in culture salinity on hyphal wall composition is shown in Figure 6.5a,b. As the NaCl concentration was increased there was a small increase in the level of neutral carbohydrate counterbalanced by a reduction in the hexosamine content. Thus, a constant proportion of carbohydrate was maintained in the wall (Table 6.4).

Table 6.4. Total carbohydrate content of the hyphal wall at different NaCl concentrations.

NaCl concentration (% w/v)	Total carbohydrate (% w/w of wall)
0	77.5
2	78.0
4	77.2
6	77.1
8	80.6

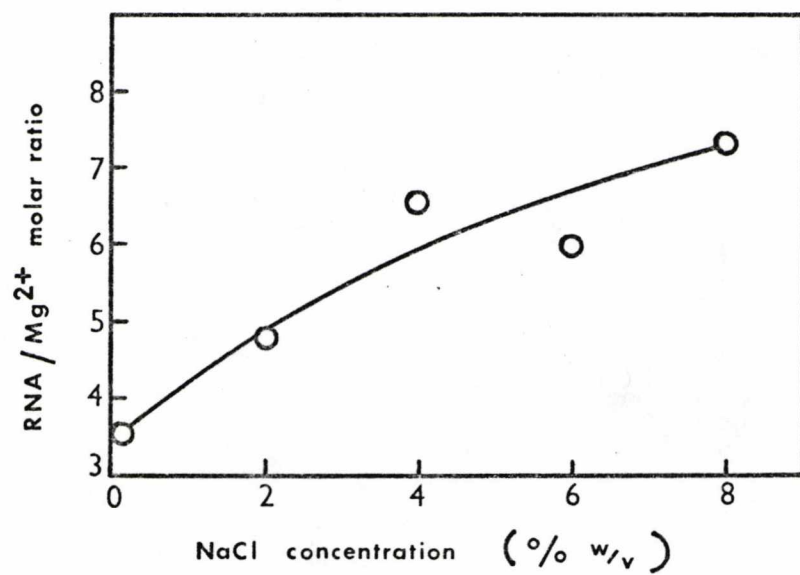Figure 6.4.   Stoichiometry between hyphal RNA and magnesium at
different NaCl concentrations. An average molecular
weight for RNA (nucleotide) of 340 was assumed.

Figure 6.5.   Effect of NaCl concentration on hyphal wall
contents of: (a) phosphorus, ▲ ; hexosamine, △ ;
protein, □ ; total neutral (anthrone) sugar, ○ ;
(b) uronic acid, ● ; mannose, □ ; galactose, △ ;
glucose, ○ . All values are quoted as % w/w of wall.
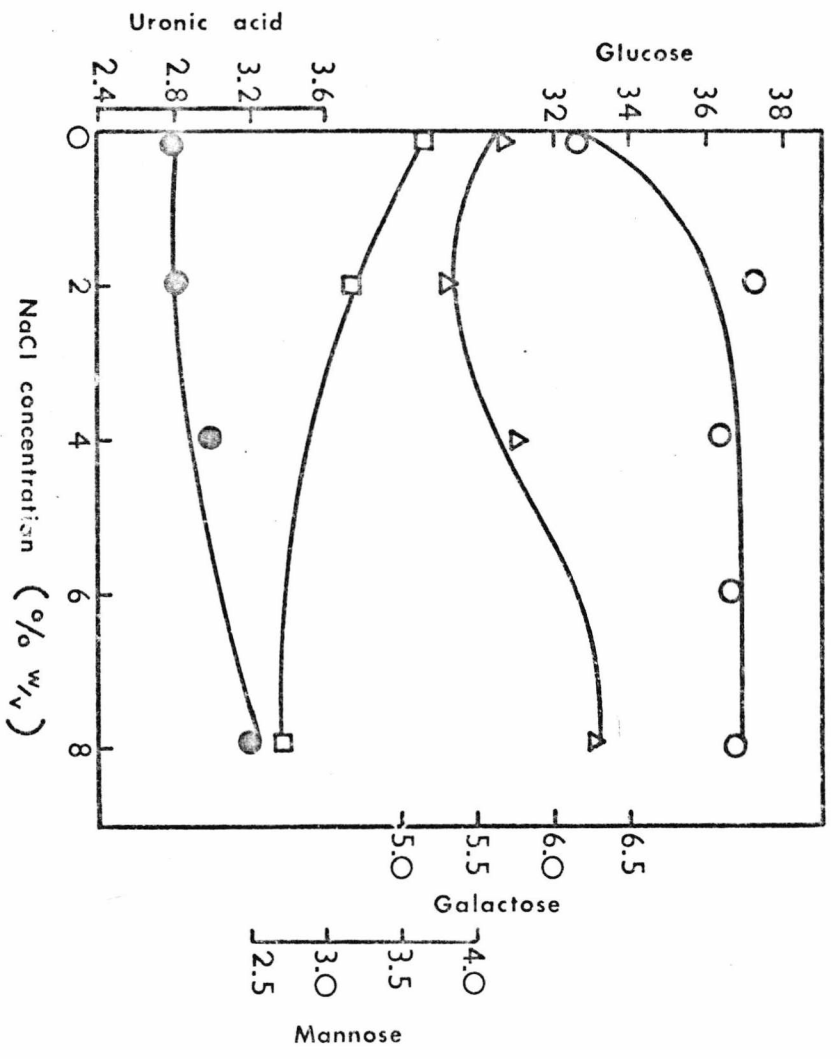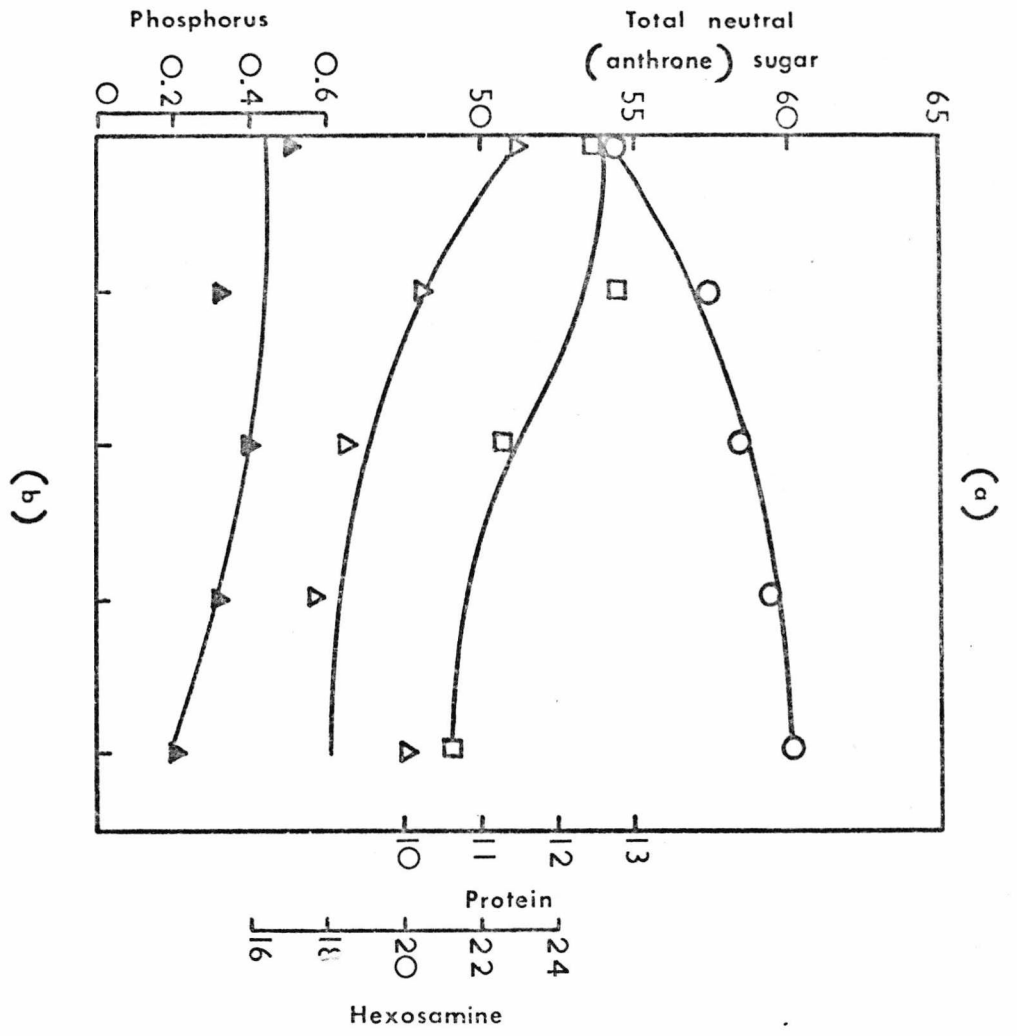
The glucose content followed a pattern similar to total neutral sugar, galactose and uronic acid increased slightly, while mannose showed a 27% reduction in content between 0 and 8% (w/v) NaCl. The level of protein fell by 16.5% and the 60% drop in phosphorus content was of the same order as that shown by the whole mycelium, 71%.

6.3. DISCUSSION

6.3.1. Sodium chloride tolerance: Osmoregulation

The degree of NaCl tolerance shown by Aspergillus nidulans 13 mel is similar to that reported by Tresner & Hayes (1971) for several other strains of Aspergillus nidulans. These workers found that all the strains tested could withstand a NaCl concentration of 10% (w/v) and some were even capable of growth in the presence of 25% (w/v) NaCl. The marked fall in conidial production observed at 10% (w/v) NaCl may be a reflection of a gradual drop in conidial production over the entire NaCl concentration range, a response which may be governed by the hyphal Mg^{2+} content; chemostat grown cells contain less Mg^{2+} as the NaCl concentration is raised (Figure 6.3b) and conidiation in submerged culture at low dilution rates is preceded by a substantial rise in the level of Mg^{2+} (Chapter 3). With respect to the decrease in the colony growth rate in the presence of NaCl (Figure 6.1), it is interesting to note that the repressive effect which NaCl has on the growth of Neurospora crassa in submerged culture can be alleviated by increasing the external Mg^{2+} concentration (Charlang & Horowitz, 1971).

The halotolerant nature of Aspergillus nidulans undoubtedly resides in its ability to prevent intracellular accumulation of Na^+ ions, a property which may reflect the possession by the organism of a highly efficient Na^+ extrusion mechanism, or a cytoplasmic membrane which is relatively impermeable to Na^+ ions. While there is no direct evidence to support either view, in non-halophilic yeasts the

activity of the Na^+ extrusion system correlates well with the degree of NaCl tolerance (Norkrans & Kylin, 1969). Thus, it is reasonable to postulate that NaCl influences hyphal growth by influencing reactions which take place at the cell surface. The initial stages in cation assimilation involve binding of the cations to the surface, so that any reduction in the intracellular concentration of cations, in this case Mg^{2+} and K^+, can be expected to reflect an interference in the uptake mechanism. Thus, in yeast Na^+ ions inhibit K^+ transport by competing for either of two K^+ binding sites (Armstrong & Rothstein, 1967). The reduced level of phosphorus in Aspergillus nidulans mycelia observed at high salt concentrations (Figure 6.3b) may be a consequence of a K^+ requirement for phosphate uptake, as has been demonstrated for yeast (Schmidt, Hecht & Thannhauser, 1949) and Escherichia coli (Weiden, Epstein & Schultz, 1967). In this context the potassium ion exercises its influence by increasing the buffering capacity of the cell and this augments phosphate uptake. Cells low in potassium can absorb only a few phosphate ions (Rothstein, 1961). Magnesium uptake also may be indirectly influenced by the presence of NaCl. In baker's yeast the synthesis of a carrier for the transport of bivalent cations involves a phosphorylation step which is closely coupled with reactions involved in the absorption of phosphate (Jennings, Hooper & Rothstein, 1958). These alterations in the pattern of mineral uptake, induced by NaCl, probably contribute to the increased acidity of the culture fluid at high salt concentrations (Table 6.2).

The respiratory data (Figure 6.2b) suggest a decreased level of energy production at concentrations of NaCl exceeding 2% (w/v). However, because the rate of glucose uptake increased with increasing NaCl concentration (Table 6.5) it seems that catabolic and anabolic reactions are less effectively coupled in the presence of NaCl. In the marine fungus, Dendryphiella salina, the rate of glucose

catabolism is reduced in the presence of NaCl and this coincides with a reduced mycelial K^+ content (Allaway & Jennings, 1970).

Table 6.5.   Effect of NaCl concentration on the rates of glucose uptake and hyphal carbohydrate accumulation.

NaCl concentration (% w/v)	Glucose uptake rate (A) (g glucose/ g biomass/h)	Hyphal carbohydrate accumulation rate (B) (g carbohydrate/ g biomass/h)	Ratio B/A (g carbohydrate/ g glucose)
0	0.234	0.0227	0.097
2	0.254	0.0299	0.118
4	0.293	0.0339	0.116
6	0.340	0.0395	0.116
8	0.398	0.0395	0.099

Allaway & Jennings (1970) suggested that K^+ ions are required to activate some step in glucose catabolism. In the glycolytic sequence there are three possible steps which have been shown to have a specific requirement for K^+: these are the reactions catalysed by phosphofructokinase (Paetkau & Lardy, 1967), aldolase (Rutter, 1961) and pyruvate kinase (Rose & Rose, 1969). A similar K^+ cofactor requirement may exist in Aspergillus nidulans, and since many of the glycolytic enzymes also require Mg^{2+} for activation, the lowered hyphal cationic content could limit glucose catabolism. On the basis of the above considerations, it is suggested that the substantial increase in the mycelial carbohydrate content (Figure 6.3a) may represent an accumulation of free glucose or other soluble carbohydrates, such as sugar alcohols; in Dendryphiella mannitol and arabitol accumulate as a result of a decrease in glucose degradation (Allaway & Jennings, 1970). Since an increase in the soluble carbohydrate content would produce a corresponding rise in the internal

osmotic pressure, this response would enable the hyphae to maintain a steady osmotic balance at all salt concentrations, a function which is a prerequisite for halotolerance. The net osmotic disturbance suffered by the hyphae was obviously small, as the hyphal size showed little variation (Table 6.1) and plasmolysis was not evident. In several algal and plant cells there is an accumulation of soluble carbohydrate in the protoplasm in response to high external osmotic pressures (Kauss, 1974b; Ben-Amotz, 1974); continuously cultivated Chlorella vulgaris doubles its non-cellulosic carbohydrate content over the NaCl range 0 to 2% (w/v) (Lisovskii & Sypnevskaya, 1969). The data presented in Table 6.5 show that there is a constant relationship between the rate of glucose uptake and the rate of hyphal carbohydrate accumulation (hyphal carbohydrate content X D) and this supports the view that the increased glucose requirement is primarily concerned with providing a source of osmotically active material. A reduced level of acetyl-CoA, the major end product of glycolysis, brought about by the decrease in glucose catabolism, may be responsible for the enhanced glucose uptake. This metabolite has been shown to regulate glucose uptake in Aspergillus nidulans (Romano & Kornberg, 1969).

Thus, in Aspergillus nidulans osmotically active carbohydrate appears to accumulate in response to the restricted entry of cationic species, a process which may enable a considerable portion of cellular energy to be conserved. There exists, however, a finite limit to the quantity of carbohydrate that can be accumulated, as demonstrated by the plateau attained at a NaCl concentration of 8% (w/v). At this value the hyphae can no longer maintain an osmotic balance, with the result that the plasmalemma experiences tensions not normally encountered and, hence membrane damage occurs.

6.3.2. Efficiency of RNA in protein synthesis

The protein synthesising activity of RNA (protein/RNA X D) dropped to a slight extent in the presence of NaCl, but the lowest level of activity was only 11% below that observed in NaCl-free medium (Figure 6.6). This reduced efficiency may be connected with the lowered hyphal K^+ and Mg^{2+} levels since both cations are essential for various stages in protein synthesis (Sachs, 1957; Lubin, 1963). It is clear, however, that the hyphal K^+ and Mg^{2+} contents would need to be reduced to an extremely low level before the activity is seriously hindered. Hence, the small variation in RNA efficiency may conceal a supplementary stimulation of ribosomal activity by organic cations (polyamines). It is of interest to note that increased synthesis of spermine and spermidine has been observed in response to decreases in the cellular levels of K^+ (Smith, 1970) and Mg^{2+} (Viotti, Bagni, Sturani & Alberghina, 1971; Bushell & Bull, 1974) in higher plants and filamentous fungi, respectively.

6.3.3. Wall metabolism

Although growth of Aspergillus nidulans in media of varying salinity was accompanied by alterations in the levels of wall components, these changes were not as extensive as those observed in response to changes in environmental temperature (Chapter 4) or pH (Chapter 5). The overall carbohydrate content remained steady over the range 0 to 8% (w/v) NaCl (Table 6.4) which indicates that the availability of hexose phosphate intermediates for wall polysaccharide synthesis is not restricted in the presence of NaCl. The variations in the levels of individual carbohydrate components therefore, probably reflect alterations in the availability of specific nucleoside phosphates. Since a continuous supply of free amino acids is essential for nucleoside phosphate biosynthesis, the state of the internal amino acid pool may prove to be of considerable importance in the
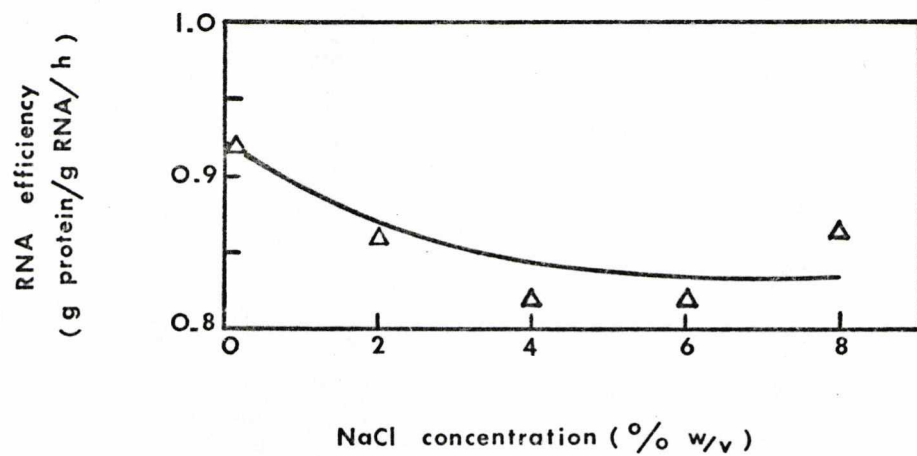
Figure 6.6.   Effect of NaCl concentration on the efficiency of
RNA in protein synthesis.

regulation of cell wall biosynthesis. This view is substantiated by observations on the Gram-positive bacterium, <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u>. Phosphate limited organisms grown in the presence of varying concentrations of NaCl display qualitative changes in the amino acid pool which do not relate to the overall protein composition (Tempest, Meers & Brown, 1970). However, the anionic polymer content of the wall undergoes considerable variation (Ellwood & Tempest, 1972) and this undoubtedly reflects the changed patterns of nucleoside phosphate synthesis imposed by the altered amino acid pool content. Although a detailed analysis of the amino acid pool content of <u>Aspergillus</u> <u>nidulans</u> was not undertaken, the reduced level of wall hexosamine, which was observed at high salt concentrations (Figure 6.5a), would indicate that the pool content of glutamine is lower.

Growth of non-halophilic organisms in saline media appears to be influenced to a large extent by cell wall metabolism. Thus, phosphate limited cells of <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u>, which normally lack wall teichoic acid, synthesise increased amounts of this component in the presence of NaCl, a response which enables increased quantities of Mg^{2+} to be adsorbed by the wall prior to assimilation (Ellwood & Tempest, 1972). This type of response is essential if growth is to continue since it has been demonstrated that Na^+ ions competitively inhibit the binding of Mg^{2+} to bacterial walls (Strange & Shon, 1964). Furthermore, the ability of <u>Aspergillus</u> <u>nidulans</u> to resist the increased internal osmotic pressures brought about by increases in culture salinity, reflects the capacity of the organism not only to maintain a strict balance between wall lysis and wall synthesis during growth in saline media, but also to retain an optimal wall structure, a view which is supported by the relatively small variation in wall composition which occurred over the NaCl range 0 to 8% (w/v).

CHAPTER 7. GENERAL DISCUSSION

7.1. The inter-relationship between magnesium and ribonucleic acid

In a detailed chemostat study of Aerobacter aerogenes, Tempest and colleagues (Dicks & Tempest, 1966; Tempest & Meers, 1968) demonstrated that the molar stoichiometry between magnesium and RNA (nucleotide) was not influenced significantly by variations in growth rate, temperature or NaCl concentration. That this is not the case for Aspergillus nidulans is shown by the data presented in the foregoing chapters; only in face of changing environmental temperature did the RNA/Mg^{2+} molar ratio maintain a steady value. Such fluctuations can reasonably be interpreted in one of two ways: under conditions which provoke a rise in the RNA/Mg^{2+} molar ratio, (1) there may be considerable involvement of other cations e.g. K^+ and polyamines in the maintenance of a functional ribosomal configuration; (2) the need for Mg^{2+} and/or other cations may be reduced as a result of increased internal neutralisation between phosphate groups and positively charged bases, i.e. purines, pyrimidines and basic amino acids, on the ribosome. In relation to the latter point, work by Tempest, Hunter & Sykes (1965) has shown that the compositional nature of the ribosome is subject to environmental influence. Thus, under conditions of glycerol and Mg^{2+} limitation the proportion of protein present in the ribosomes of Aerobacter aerogenes was found to decrease as the growth rate was raised. The ratio of ribosomal protein to RNA was unaffected by temperature, however, during carbon limited growth (Tempest & Hunter, 1965) and this observation may be relevant to the existence of a temperature-independent constant molar stoichiometry between RNA and magnesium in Aspergillus nidulans.

7.2. Potassium and the efficiency of cell synthesis

Of the many physiological roles in microorganisms that have been ascribed to potassium (e.g. maintenance of ribosomal activity,

osmoregulation, stimulation of respiration) that of facilitating the utilisation of glucose is considered of major importance among those eukaryotes so far examined in detail (see for example, Allaway & Jennings, 1970; Aiking & Tempest, 1976). In a carefully devised chemostat experiment, Aiking & Tempest (1976) varied the intracellular K^+ content of Candida utilis, by progressively shifting the specific growth limitation of the culture from potassium to glucose and compared the K^+ levels with $Y_{glucose}$ (biomass yield with respect to glucose) and Y_{O_2} (biomass yield with respect to oxygen) values. The results obtained revealed a clear correlation between intracellular K^+ and the efficiency of utilisation of both glucose and oxygen. The comparable data presented in Figure 7.1 for Aspergillus nidulans (obtained over a more extensive range of environmental conditions than was studied by Aiking & Tempest, 1976) are in basic agreement with those reported for Candida utilis, although a K^+-independent component of the $Y_{glucose}$ value is indicated by the failure of the extrapolated curve (to a theoretical zero mycelial K^+ content) to meet the origin. However, Aspergillus nidulans differs from Candida utilis in that the efficiency with which oxygen is utilised appears to be restricted by high intracellular K^+ concentrations (Figure 7.2).

7.3. Calcium and the integrity of the cell membrane

Numerous reports exist which indicate the importance of divalent cations i.e. Mg^{2+} and Ca^{2+} in maintaining the structural and functional integrity of the plasma membrane (see for example, Tempest & Strange, 1966; Indge, 1968; Hagler & Lewis, 1974; Ledebo, 1976). The metal ions are considered to act as bridges between adjacent negatively charged groups on the phospholipids or proteins of the membrane. On the basis of wall binding studies it was intimated that Ca^{2+} was a likely candidate for this role in Aspergillus nidulans

Figure 7.1. Relationship between mycelial potassium content
and $Y_{glucose}$ value. Individual points represent
collective data obtained from experiments on:
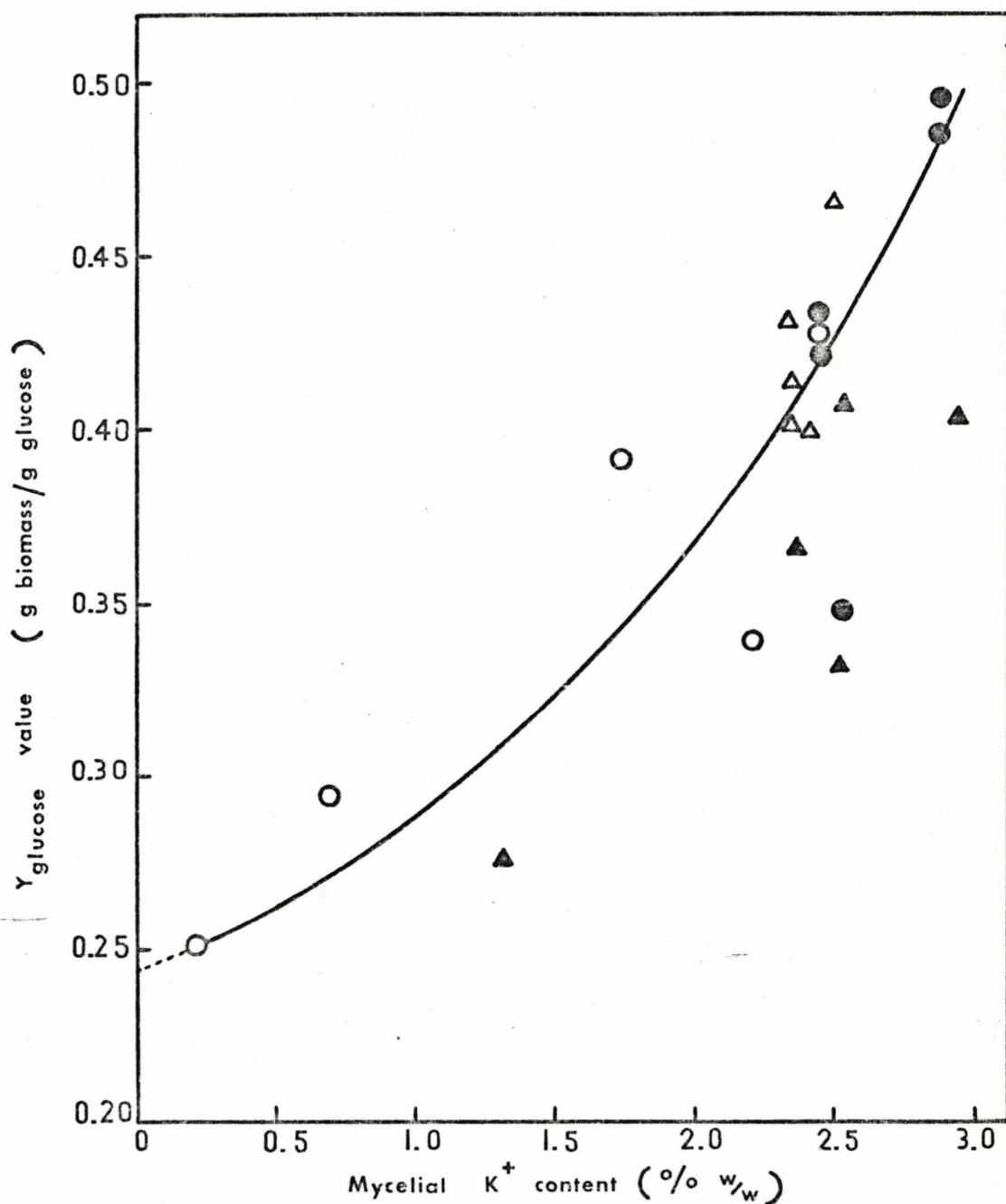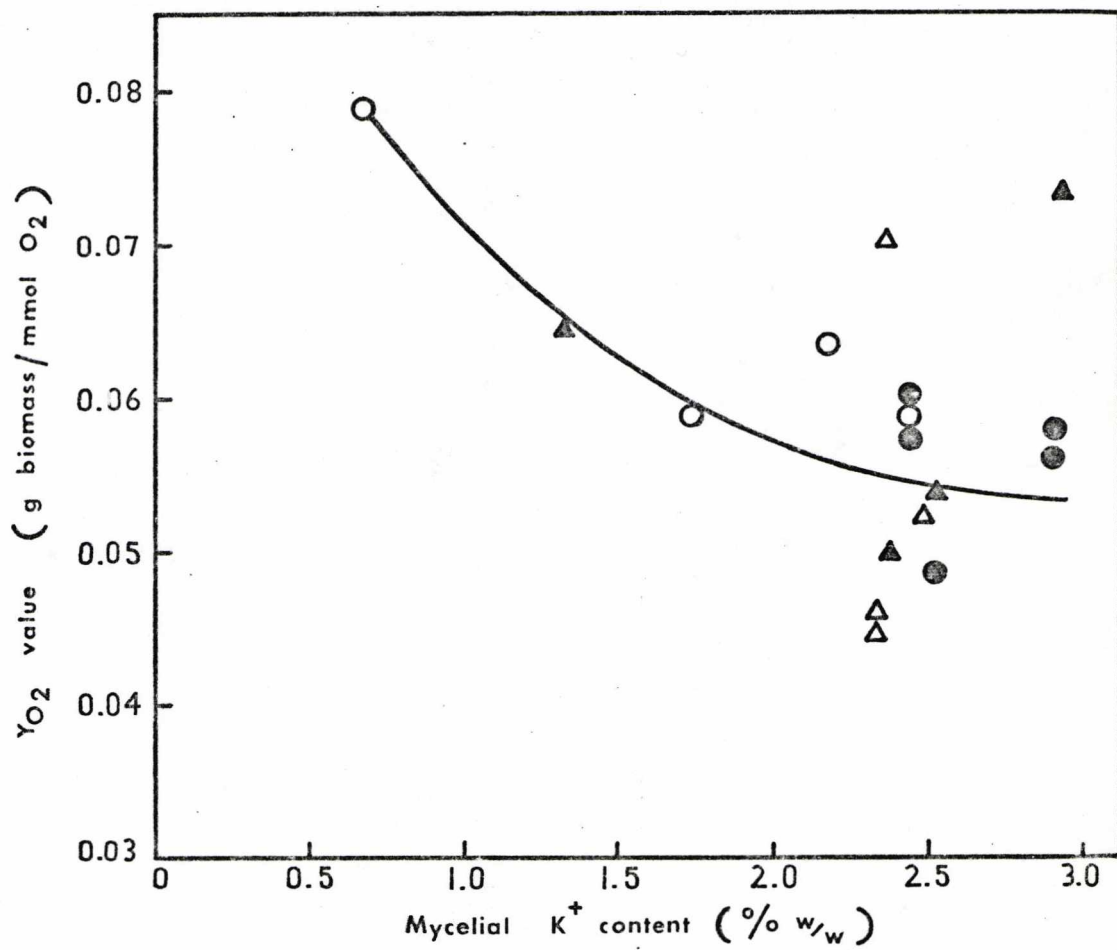growth rate, ● ; temperature, △ ; pH, ▲ ; NaCl, ○ .

Figure 7.2. Relationship between mycelial potassium content
and Y_{O2} value. Individual points represent
collective data obtained from experiments on:
growth rate, ● ; temperature, △ ; pH, ▲ ; NaCl, ○ .

(Chapter 3), prior adsorption of the cation to wall phosphate and/or protein possibly facilitating this function. Hence, the significant rise in the wall phosphorus and protein levels of Aspergillus nidulans in response to increased temperatures and decreased pH values may reflect an additional requirement for Ca^{2+} ions to stabilise the membrane structure. Diamond & Rose (1970) also observed that the protective effect of Ca^{2+} (against osmotic lysis) for sphaeroplasts of Saccharomyces cerevisiae was dependent upon the temperature at which the yeast had been cultured, the effect increasing within the range $15^{\circ}C$ to $30^{\circ}C$.

7.4. Wall composition

Table 7.1 summarises the degree of variability in wall composition of Aspergillus nidulans for a limited range of environmental parameters. The ratio B/A is included solely to provide a means of comparing the changes observed in individual wall components. Thus, of the components examined protein, glucose, mannose and phosphorus were the most sensitive to environmental changes.

Table 7.1. Extent of variation in hyphal wall composition.

Wall component	Extreme values observed[a] (% w/w of wall)	Ratio B/A
Protein	8.5^A - 20.5^B	2.4
Hexosamine	17.2 - 28.0	1.6
Total neutral (anthrone) sugar	35.1 - 61.8	1.8
Total carbohydrate	54.3 - 82.7	1.5
Glucose	17.4 - 43.6	2.5
Mannose	1.6 - 4.0	2.5
Galactose	4.7 - 6.7	1.4
Uronic acid	2.3 - 4.4	1.9
Phosphorus	0.15 - 0.78	5.2

[a] Values represent collective data derived from experiments on growth rate, temperature, pH and NaCl concentration.

Overall, there appears to be a considerable flexibility in wall composition not associated with gross morphological change. Several reports which have dealt with the interconversions of different morphological types (see for example, Marks, Keller & Guarino, 1971) tend to single out specific components as key factors. However, the complex polymeric nature of the wall plus the failure to correlate induction of similar morphological forms with similar changes in wall composition must question the former ideology. It is conceivable that the quantitative changes observed in wall content are bound up with variations in the nature and degree of intramural cross-linkages which are instrumental in the integration of wall structure. To date, information regarding such linkages in fungi is scanty. However, a few reports have indicated the existence of protein-polysaccharide linkages (Emiliani & Ucha De Davie, 1962; Le'John, Cameron, Stevenson & Meuser, 1974) and one can envisage the latter being affected by alterations in the levels of either glycan or protein components with possible morphological manifestations.

Total wall carbohydrate content was little affected by variations in environmental conditions, except in mycelia grown in the presence of differing H^+ ion concentration. When one considers that the majority of carbohydrate is confined within the fibrillar element of the wall in the form of glucans and chitin, and that biosynthesis of the latter components is believed to occur extracellularly (see Chapter 1) the inference is that pH exerts a considerable influence on the polymerisation steps involved in wall biosynthesis. Taken in conjunction with observations made with strain BWB 224 of Aspergillus nidulans, it would appear that extracellular polymerisation reactions, in general, in this organism are restricted at low pH values: in strain BWB 224 production of melanin, a microfibrillar coating on the outer surface of the wall, increases with pH in the range 3.0 to 7.9 (Rowley & Pirt, 1972).

One interesting fact to emerge from the cell wall study of
Aspergillus nidulans is the similarity of response of both the
protein and phosphorus contents to different environmental param-
eters, an observation which may be taken as presumptive evidence for
the existence of a phosphoprotein component in the wall. However,
the level of variation of each of these components was not the same
and hence an association between either phosphorus or protein and
other wall constituents is also indicated. Chemical fractionation
studies of the wall by Bull (1970) tend to corroborate the above
views. Thus, more than 50% of the total wall protein and phosphorus
was found in the hot acid-soluble fraction together with neutral and
amino sugars while the initial cold alkali-soluble fraction also
contained phosphorus along with glucose, galactose and galactosamine.

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