Growth-Associated aspects of mould physiology

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ABSTRACT.

The technique of continuous-flow culture was used to study the growth kinetics of and the effects of, growth rate on biochemical composition and anaplerotic metabolism on the mould, *Aspergillus nidulans*.

The validity of classical microbial growth equations, which have been well tested for unicells, was established for mould growth. Continuous cultures of *A.nidulans* followed Monod kinetics when growing by simple hyphal elongation but exhibited anomalous growth kinetics when growing by branch formation. The kinetics of growth in batch culture involving the production of an extracellular metabolite followed the "logistic" model more closely than the Monod model. The effect of growth rate upon respiratory parameters, glucose uptake and energy yield, and the kinetics of growth in the transient state were also studied.

An inter-relationship between hyphal magnesium, polyamine and RNA concentrations was established, which held for vegetative cultures at all growth rates. A relationship between the ratio of magnesium to polyamine and ribosomal efficiency was also established. Ribosomal efficiency was itself proportional to growth rate at all values of μ below 65% μmax, reaching a maximum value which was evident in the growth rate range, μmax-65% μmax. Zero ribosomal efficiency was observed at the minimum specific growth rate (μmin) under which conditions differentiation into conidia took place. The response of ribosomal efficiency to abrupt changes in growth rate in transient states

was immediate and positive. The effect of dilution rate upon mycelial wall content, DNA concentration and on the concentrations and energy required for synthesis of carbon-rich "storage-polymers" is also discussed.

This work describes an investigation of anaplerotic metabolism, particularly that involving CO_2 fixation. The inhibitory effect of high aeration conditions upon spore germination appeared to reflect a critical requirement for bicarbonate in the growth medium. Further investigation revealed a significant phosphoenol pyruvate carboxylase (PEPC) activity in batch culture. Pyruvate carboxylase (PC) activity was also detected but at lower levels. Glucose-limited chemostat cultures showed a marked increase in both carboxylase activities. Maximum PEPC levels increased from 1.1×10^{-2} µmol protein^{-1} h^{-1} to 1.0µmol mg^{-1} h^{-1} in chemostat culture and PC activity from 1.5×10^{-3} µmol mg^{-1} h^{-1} to 2.5×10^{-2} µmol mg^{-1}h^{-1}. Both enzyme activities were growth rate dependent. Measurements of bicarbonate fixation by intact mycelium indicated that virtually all the assimilated bicarbonate was fixed by PEPC and PC. The feasibility of making more efficient use of the carbon source with respect to biomass production by maximising carboxylase activities was investigated by (a) manipulating chemostat culture conditions to provide optimum bicarbonate concentration, (b) producing step-down and step-up transitions states and (c) constructing a two-stage continuous culture system.

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SECTION I.

1. INTRODUCTION

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SECTION I.

The application of continuous-flow culture techniques to the study of fungal biochemistry and physiology.

1. INTRODUCTION.

Statements describing the metabolic status of a micro-organism should, ideally, contain an explicit definition of the rate and morphological mode of growth of that organism as well as a description of the environmental conditions, capable of effecting the metabolic response described. This is necessary so that others may achieve the repetition and consequent duplication of reported effects. The validity of this assertion is readily illustrated with reference to the filamentous micro-fungi, which exhibit a range of morphological states whose diversity may be effected by small changes in culture environment, resulting in corresponding changes in cell physiology. The growth kinetics and metabolism of dispersed filamentous, stromatic or pellet cultures are markedly different from each other as well as from differentiating cultures, e.g. those undergoing conidiation, and each of these states results from the imposition of a specific set of environmental conditions. Similarly, the culture morphology may influence its own micro-environment, thus, the somewhat thixotropic broth produced by a mycelial culture results in a situation where conventional gas transfer dynamics may be inoperable.

In order to define and achieve a physiological state capable of study, the open culture system, as defined by Herbert (1961a) is the method of choice. With such a system

both the input of substrates and the output of biomass and growth products occur, resulting in the possibility that biomass growth and output will balance, giving rise to a steady state in which, theoretically, constant conditions may be maintained indefinitely. This contrasts with the closed or batch culture system which provides constantly changing cell physiology and environmental conditions and, consequently, will only provide material for the study of transient changes. Substrate-limited continuous cultures with balanced growth characteristics may be achieved using the chemostat (Novick & Szilard, 1950) which has several advantages over other culture systems. Some of these have been enumerated by Pirt (1972) as follows:

(i) growth rate may be controlled and varied;

(ii) physical and nutritional conditions may be varied while maintaining a constant growth rate;

(iii) substrate-limited growth may be achieved;

(iv) culture biomass may be maintained at a constant concentration, independantly of growth rate;

(v) rapid and large scale conversion of substrate into biomass takes place.

When the technique of continuous-flow culture is applied to filamentous moulds a number of technical difficulties may be encountered. However, recent developments in equipment and techniques (see Rowley & Bull, 1973) have lead to a situation where continuous-flow culture is becoming increasingly the method of choice for research into fungal physiology. In the present study, the application of classical chemostat theory (Monod, 1950; Herbert, Elsworth & Tellings, 1956) to the behaviour of continuous-flow cultures of fungi is tested and the system, thus evaluated, is applied to the study of growth-

associated aspects of fungal biochemistry and metabolism.

2. MATERIALS & METHODS.

(a) Organisms.

The majority of experiments were carried out with a prototrophic, hyaline mutant, 13mel, of *Aspergillus nidulans* (Eidam) Wint. (Bull & Faulkner, 1965). This mutant was chosen because of its inability to produce melanin which, in the wild-type interferes with many spectrophotometrically-based procedures. Some growth experiments were duplicated using a yellow sporing prototrophic recombinant strain, BWB224, of *Aspergillus nidulans*.

(b) Culture Media.

A chemically defined glucose-salts minimal medium (ph 6.4) was used routinely for preparing batch and continuous-flow cultures. Slopes and plates of solid medium were prepared by adding 3% New Zealand agar to the glucose-salts solution.

The detailed composition of the medium which is based on that described by Carter and Bull (1969) was as follows:

(i) D-glucose as the sole carbon and energy source at a concentration of $15gl^{-1}$;

(ii) sodium nitrate ($6gl^{-1}$);

(iii) mineral salts mixture (gl^{-1}), EDTA $Na_2.2H_2O$ 0.6; $MgSO_4.7H_2O$ 0.25; $CaCl_2$ 0.05; $ZnSO_4.7H_2O$ 0.02; $MnSO_4.4H_2O$ 0.02; $CuSO_4.5H_2O$ 0.005; $FeSO_4.7H_2O$ 0.1; Na_2SO_4 0.5; $Na_2MoO_4.2H_2O$ 0.005;

(iv) KH_2PO_4 - Na_2HPO_4 buffer of pH 6.4 to produce a final concentration of 0.1M for liquid medium and 0.025M for solid medium.

For convenience, constituents (ii), (iii) and (iv) were prepared and stored in bulk (20 l.) as 20 times concentrated solutions. Subsequently they were mixed and diluted to final concentration prior to autoclaving, in volumes of 20 l., at 15 lb. in^{-2}.

40 min. In continuous-flow fermentations, antifoam (polypropyleneglycol P2006; Shell Chemical Co., Ltd., London, S.E.1) was added automatically to the culture at a predetermined rate.

Using the medium described, it was found that the pH was; maintained at a constant value in continuous-flow cultures, without the necessity for acid or alkali addition.

(c) Inoculation procedure.

The sequence of events, used routinely for the inoculation of *Aspergillus nidulans* in the initiation of continuously stirred tank reactor (CSTR) cultures is best represented by the flow diagram in Figure 1. The transfer of culture from stage V to stage VI was effected 24 hours after the initial inoculation from stage IV.

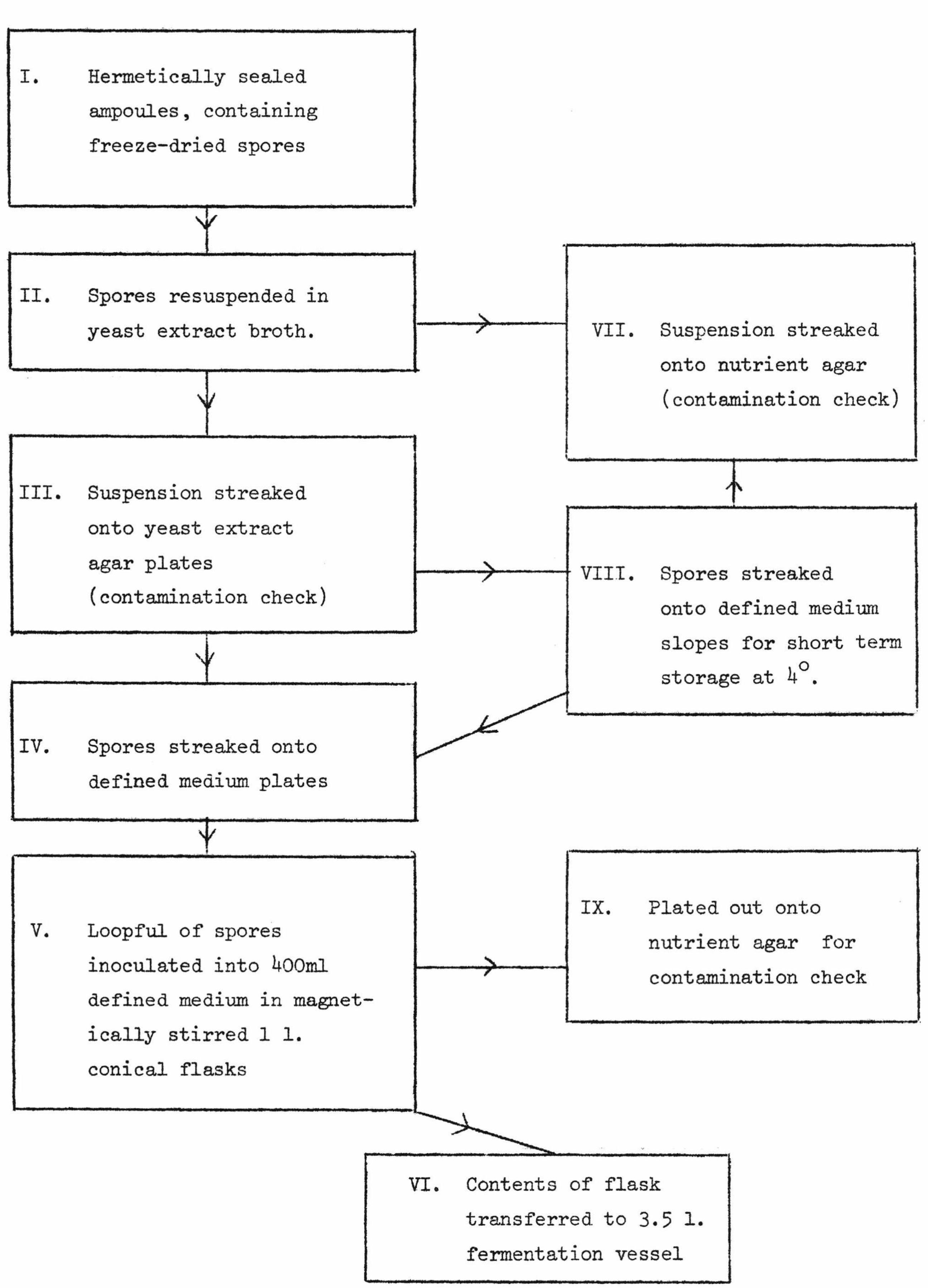
(d) Flask cultures.

Two methods of producing submerged liquid cultures of *Aspergillus nidulans* in conical flasks were investigated.

In the first method, a 1 litre conical flask containing 400ml of chemically defined medium was used as a culture vessel. Agitation was effected by means of an orbital shaker. (L.H. Engineering, Ltd., Stoke Poges, Bucks) operating at 90rpm. To increase aeration in the culture, the flask was fitted with a stainless steel coil which ran around the base of the flask and which caused turbulence in the culture when the flask was rotated. Using a spore inoculum harvested from a three-day old plate culture, a liquid culture consisting of pellets, some 3mm in diameter was produced after 24h incubation at 30°C.

An alternative procedure was also used in which a 1 litre flask, containing 400ml of medium, was inoculated in the same

Figure 1. Diagram Describing Inoculation Procedure.



way as in the previous method. In this case, the wire coil was omitted from the flask and the culture was agitated by means of a Teflon-coated magnetic stirrer bar, driven by an electric stirrer unit (A. Gallenkamp & Co.,Ltd., London, E.C.2). operating at half the maximum speed. At a temperature of 30°C a well-dispersed, homogeneously, filamentous culture was produced after 24h. incubation.

(e) Fermentation Equipment.

(i) Introduction.

Adaptation of the CSTR for the cultivation of moulds has been described by Solomons (1972) and Rowley & Bull (1973); the equipment in this work embodies many of the design features discussed and described in these two publications. Various types of continuous-flow culture equipment have been advocated for mould growth; they include the compartmentalised "tower" fermenter (Ross & Wilkin, 1968), the cyclone column chemostat (Dawson, 1963), the horizontal tubular vessel described by Means, Savage, Reusser & Koepsell (1962) and the vertical stirred and plug flow tubular reactors developed by Greenshields (see Greenshields and Smith, 1974). However, the "Porton" type of CSTR used in the present study provides great flexibility in operation and enabled batch as well as continuous-flow cultures to be established.

(ii) Assembly and Operation.

The apparatus used in the present study is illustrated in Plate 7. The basic unit consists of a stainless steel and glass fermenter vessel (Model 1/1000; 3.2 1.) manufactured by L.H. Engineering, Ltd. The vessel (N) comprises a section of

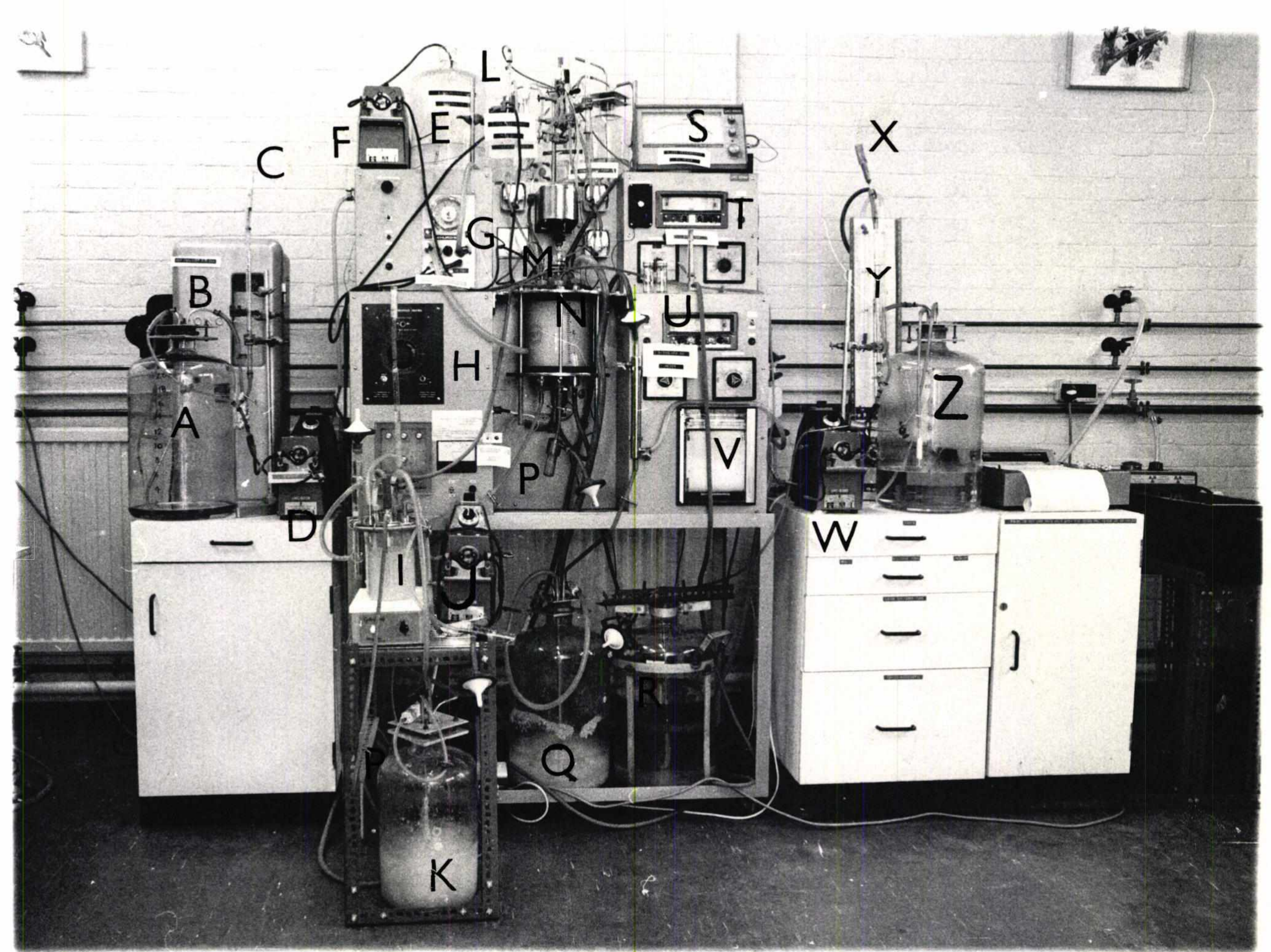
Pyrex glass pipe (Q.V.F. Ltd., Stoke-on-Trent), fitted with stainless steel top- and base-plates. The top-plate is fitted with a ball-race gland, to carry the stirrer shaft which is driven through a universal coupling (M); agitation is provided by an eight-bladed paddle-type impeller. The pyrex pipe is separated from the base-plates by means of two circular neoprene rubber gaskets (Edwards High Vacuum Ltd., Crawley, Sussex). Feed, effluent and control lines are introduced into the fermenter by means of stainless steel or Teflon tubing set into silicone-rubber bungs (Esco (Rubber) Ltd., London, E.C.3.) which are secured in the top- and base-plates by knurled port locks.

All the internal surfaces of the culture vessel consist, where possible, of highly polished stainless steel, glass or Teflon in order to prevent accreted mycelial growth adhering to the vessel and its contents. Surface growth reduces the effective culture volume, produces a non-homogenous culture, causes depletion of nutrients and may introduce errors into analyses. The accreted fungal growth described here, however, is probably distinct from the "wall-growth" described by Topiwala and Hamer (1971) which consists of monolayers of micro-organiams, attracted electrostatically to culture vessel surfaces. To further discourage accreted growth, the culture volume is adjusted so that the vessel is almost full, thereby ensuring that all surfaces are constantly washed with the culture.

Growth in the medium feed lines is prevented by supplying the carbon source separately from the other medium constituents. Glucose solution, at a concentration of $400gl^{-1}$, is pumped from a 20 1. glass bottle (A) by means of a flow inducer (Watson-Marlow Ltd., Menlow, Bucks), (D). The nitrogen source

PLATE 1. Apparatus for continuous mould cultivation.

A glucose reservoir
B CO_2 analyser
C glucose feed meter
D glucose feed pump
E HCO_3 probe: electrolyte reservoir
F HCO_3 probe: electrolyte pump
G Churchill pump
H stirring speed control
I stage II, 1.0 *l* culture vessel
J stage I - stage II transfer pump
K stage II overflow reservoir
L HCO_3 probe : flow cell
M universal drive coupling
N stage I and single stage 3.5 *l* culture vessel
P sampling port
Q stage I overflow reservoir
R spare medium reservoir
S HCO_3 probe : pH meter
T pH meter
U dissolved oxygen meter
V multipoint chart recorder
W medium feed pump
X medium feed meter
Y air inlet meter
Z medium holding tank



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mineral salts and buffer constituents are pumped from a holding vessel (Z) by means of the flow inducer (W); the supply being replenished during the course of the fermentation from the portable 20 l. vessel (R). The pressure of the culture air supply was monitored and controlled by a device supplied by DeVilblis, Ltd., Bournemouth, Hants. Gas flow rates were monitored using 'Rotameters' (G.E.C. Elliot Automation Ltd., Croydon, Surrey) and controlled by a 'Flowstat' (G.A. Platon, Ltd., Basingstoke, Hants.). To minimise possible depletion of culture volume due to evaporation losses, the exhaust gas outlet pipe was fitted with a stainless steel water-cooled condensor (L.H. Engineering) through which air was conducted to a glass-fibre bacteriological filter (Microflow Ltd., Fleet, Hants.). As a safety precaution against the build-up of excessive air pressure in the culture vessel or any of the air lines due to possible blockage by mycelium, pressure was monitored in the air inlet and outlet pipes with mercury-filled manometers.

For continuous-flow cultures, an overflow pipe consisting of a 1" diameter 'Teflon' tube (Polypenco Ltd., Welwyn Garden City, Herts) is set into the top plate of the vessel. Overflowing culture is conducted, by means of a length of 1" diameter silicone rubber tubing (Jencons (Scientific) Ltd., Hemel Hempstead, Herts) to a 20 l. receiving vessel (Q), which is emptied periodically. To avoid blockage of the overflow pipe by mycelium, the exhaust air was conducted out through the same tube. Alternative procedures for preventing mycelial blockage in the overflow pipe (Righelato & Pirt, 1967) and the take-off pipe in the culture vessel (Brunner & Röhr, 1972) have been

described. The 'pinch valve' system described by Righelato & Pirt, periodically closes the overflow pipe allowing air pressure to build up inside the vessel which, when released, has the effect of blowing any accreted mycelium out of the tube. Brunner & Röhr's system provided a means of intermittently increasing the impeller speed for 30 seconds, thereby causing the shape of the culture broth vortex to change so that the take-off pipe was washed free of surface growth. By increasing the air pressure and the stirring speed, respectively, both these methods result in momentary increases in the culture oxygen transfer rate. Periodic increases in dilution rate due to culture volume depletion also result in each case and it was consequently felt that the procedure described in the present study was preferable in causing less variation in culture environmental parameters.

The illustration (Plate 1) also shows a continuously operating carbon dioxide gas analyser (B); some of the constituents of a system for measuring dissolved CO_2 (E,F,L and S); and a second stage CSTR which was used during the study of anaplerotic metabolism. These components will be described more fully in later Sections.

(iii) Environmental parameters.

The environmental parameters which were maintained routinely throughout continuous-flow cultivation experiments are summarised in Table 1.

The values of pH (6.4) and temperature (30°C) were chosen to provide comparability of results obtained in the present study with those reported for *Aspergillus nidulans*, strain BWB224 (Carter & Bull, 1969 ; 1971; Carter et al, 1971). A minimum

TABLE 1

Continuous flow culture conditions

a	pH	6.4
b	temperature	$30^{o}C$
c	dissolved oxygen tension	$\geqslant$ 40% saturation
d	stirring rate	1000 r.p.m.
e	oxygen transfer rate*	0.55 $mmol.l^{-1}.h^{-1}.mmHg^{-1}$
f	medium glucose concentration (SR)	15 gl^{-1}
g	CO_2 concentration effluent gas stream.	$\leqslant$ 4% /v/v)

* determined by the sulphite oxidation method of Cooper, Fernstrom & Muller (1944) at an air inflow rate of 1 lh^{-1}.

dissolved oxygen tension of 40% saturation was chosen to ensure that there was little likelyhood of the culture reaching the "critical dissolved oxygen tension" reported by Carter & Bull, (1971); dissolved oxygen tensions between this value and air saturation have little effect on the respiratory metabolism of *Aspergillus nidulans*. A stirring rate of 1000 rpm was the minimum which gave complete mixing at the culture biomass concentration used.

Culture pH was monitored by means of an autoclavable glass electrode model C0050/LHE (Electronic Instruments, Ltd., Richmond, Surrey). A reference calomel electrode model B0070/LHE was connected by a liquid salt bridge, carried in a length of silicone rubber tubing. Facilities existed for automatic pH control by means of acid or alkali addition; however, using the buffer system described, external control was unnecessary for maintaining a constant culture pH. The pH value of the broth was also periodically determined from samples using an external pH meter, calibrated with standard buffer solutions, in order to check the readings given by the autoclavable probe.

(f) Gas Monitoring Systems.

This section describes the procedures and equipment used in the estimation of dissolved CO_2 tension, (pCO_2); dissolved oxygen tension (DOT); partial pressure of oxygen in the effluent gas stream from the fermenter and partial pressure of CO_2 in the effluent gas.

(i) Partial pressure of gaseous oxygen.

A branch was taken from the culture effluent gas stream to give an airflow rate of $100ml.min^{-1}$. The proportion of

oxygen in the dried gas mixture was measured with a paramagnetic oxygen analyser Type OA150 (Servomax Controls, Ltd., Crowborough, Sussex) modified for mains operation. For continuous recording, the analyser was connected to a potentiometric chart recorder (model 27000, Bryans Southern Instruments, Ltd., Mitcham, Surrey).

(ii) Partial pressure of gaseous carbon dioxide.

The proportion of carbon dioxide in the dried effluent gas stream was estimated using two volumetric methods, both of which estimate the volume of CO_2 absorbed from the sample by a soda-lime column. A continuous estimate of CO_2 in the effluent stream was made with a "Mono" automatic recording gas analyser (Smail, Sons & Co., Ltd., Glasgow). Values obtained using this method were checked with a manually operated 'Orsat' analyser (A. Gallenkamp & Co., Ltd.).

(iii) Calculation of Respiratory Quotients.

Specific rate of culture oxygen uptake (Q_{O_2})

Volume of oxygen consumed per hour $= 60\ (x-z)$ ml (1)

where

x = rate of oxygen flow into the culture (ml min^{-1})

z = rate of oxygen out flow (ml min^{-1})

(z) may be evaluated using experimental data using the expression:

$$z = \frac{ny}{1 - (c + y)} \qquad (2)$$

where n = throughput (ml min^{-1}) of inert gases and nitrogen

y = fractional O_2 content of the effluent gas

c = fractional CO_2 content of the effluent gas

Q_{O_2} (mmol g biomass $^{1}h^{-1}$)

$$= \frac{60\ (x-z)}{22.4 \times \text{total biomass}} \qquad (3)$$

Specific rate of carbon dioxide evolution (Q_{CO_2})

$$\text{Volume of } CO_2 \text{ produced per hour} = \frac{60\ nc}{1 - (c + y)}\ \text{ml} \qquad (4)$$

Q_{CO_2} (mmol g biomass^{-1}h^{-1})

$$= \frac{60\ nc}{\{1 - (c + y) \times 22.4 \times \text{total biomass}\}} \qquad (5)$$

Respiratory quotient (RQ)

Combining (5) and (3)

$$= \frac{Q_{CO_2}}{Q_{O_2}} \qquad (6)$$

(iv) Dissolved oxygen tension.

DOT was measured using a simple steam-sterilizable galvanic oxygen electrode, generously supplied by Pfizer, Ltd., Sandwich, Kent. The construction details of the probe have been outlined by Johnson, Borkowski & Engblom (1964).

The gas tension in the culture medium was varied by changing the air inflow rate.

(v) Dissolved CO_2 tension

Electrodes for measuring dissolved CO_2 tension have been described by Stow, Baer & Randall (1957), and by Severinghaus & Bradley (1958) and an analogue converter for quantifying their output has been introduced by Nicholls, Shepherd and Garland (1967). However, these systems are costly and involve the use of a probe containing a thin and delicate membrane which could be unsuitable for use in a CSTR. A continuous-flow system for measuring carbon dioxide in gaseous mixtures has been developed

(R.W. Knight, 1973) which measures CO_2-mediated pH changes in the electrolyte solution devised by Severinghaus & Bradley (1958). A similar system, for measuring pCO_2 values, was subsequently developed by the present author. The principle of operation is illustrated in Figure 2. Electrolyte (100mM.NaCl; 100mM $NaHCO_3$ is pumped (Watson Marlow flow inducer, model MHRE2) through a filter flow cell, constructed from a membrane filter holder FD300, (A. Gallenkamp & Co., Ltd., London, E.C.2) containing a 47mm membrane filter (Oxoid, London, S.E.1). From the filter, electrolyte passes through the probe, which dips below the surface of the culture. The probe consists of a 1m length of silicone tubing, 2.5mm in diameter, with walls 1mm thick (Jencons Ltd., Hemel Hempstead, Herts) wound around a glass rod in a double helical fashion. Similar lengths of tubing made from 'Teflon' and 'Polypropylene' were substituted in the probe, however, silicone tubing was found to give a faster response to changing culture conditions. The electrolyte passes into the probe in the outer coil of the helix and out through the inner coil when it passes to a stainless steel flow cell, designed by R.W. Knight (Pfizer Ltd., Sandwich, Kent) and constructed in the University of Kent workshops. In the flow cell, the electrolyte washes over a pH electrode and is subsequently pumped into a waste reservoir.

Using solutions of $NaHCO_3$ to calibrate the probe, a pump setting to give an electrolyte throughput rate of $5ml.min^{-1}$ was chosen. At this flow-rate, a constant reading on the pH meter could be obtained 6 to 7 minutes after the bicarbonate concentration in the test solution had been changed. Teflon and polypropylene tubing was also tried in the probe winding but

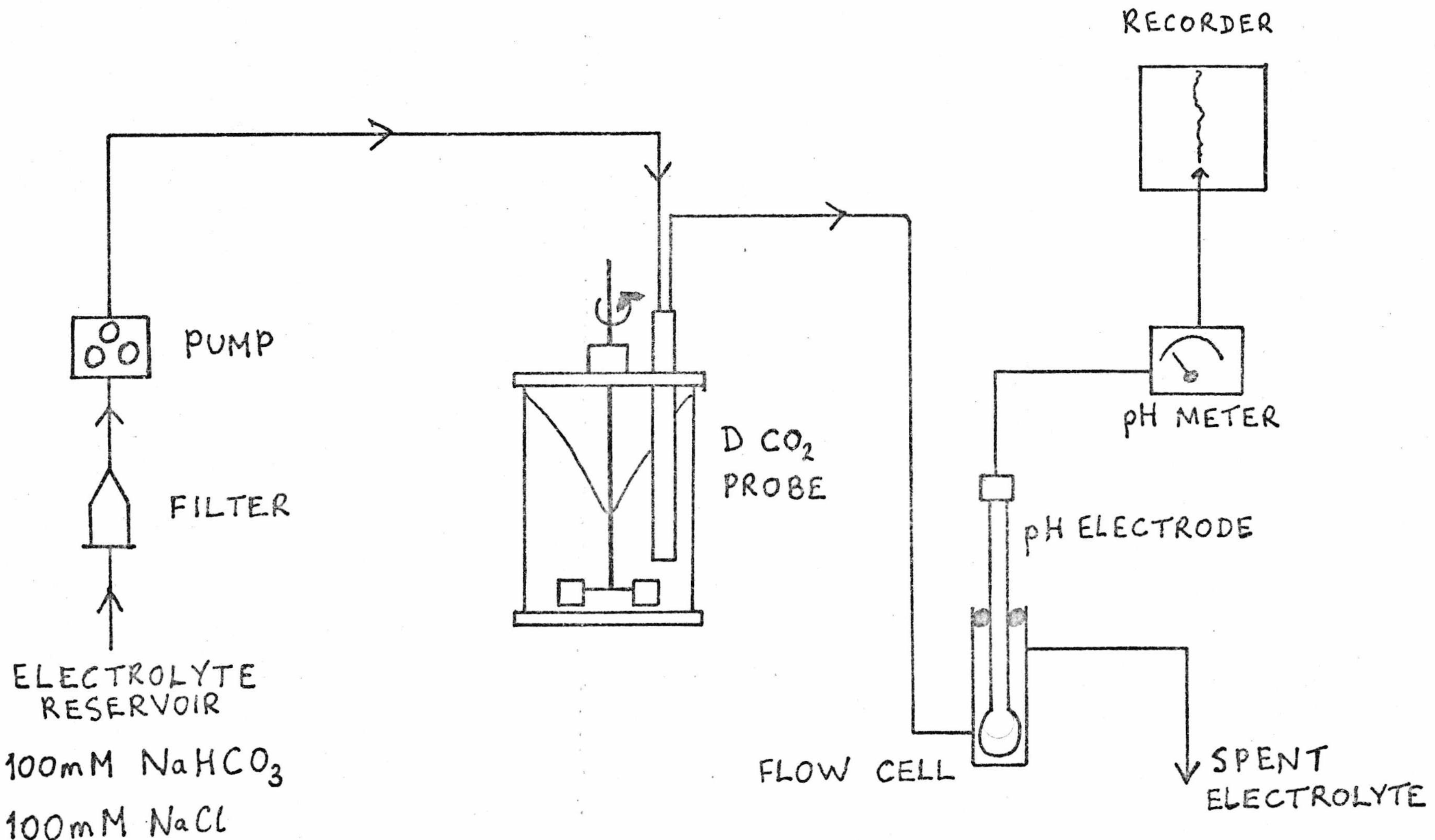


FIGURE 2

only silicone tubing provided a small enough response time for the system to be of practical value. The double helical winding configuration was found to produce the most stable reading.

Dissolved CO_2 values were calculated using the Henderson-Hasselbalch equation (1) as follows:-

$$\log \{HCO_3^-\} = pH - pK' + \log \{CO_2\} \quad (7)$$

where $pK' = -\log (\{H^+\}\{HCO_3^-\}/\{CO_2\})$.

Since $pCO_2 = -\log \{CO_2\}$,

equation (1) may be rearranged to give:

$$pCO_2 = pH - \log \{HCO_3^-\} - pK'$$

Using specified values of pK' (from Umbriet, Burris & Stauffer, 1964) and experimental values of pH and $\{HCO_3^-\}$ the meter reading may be calibrated in terms of pCO_2.

(g) Computer methods.

All calculations involving the use of the computer were made with the University of Kent 1CL4130. Original programmes were written using the machine-oriented language, BASIC (Kemeny & Kurtz, 1971), which was available on the University of Kent time-sharing system. A number of library programmes written in the problem-oriented language ALGOL (Naur, 1963) were also used, in particular the curve-fitting programme PLOFIT (British Aluminium Co. Ltd).

(h) Culture stability and homogeneity checks; sampling methods.

Cultures were sampled from the CSTR by means of a knurled sampling port as described by Evans, Herbert & Tempest (1970), designed to accept a 1 oz. Universal glass container. When

sampling, the first 10ml to emerge from the culture vessel was discarded to avoid the possibility of unrepresentative culture being obtained from the 'dead space' in the sampling line. Wet mounts of culture suspension were examined by phase contrast microscopy for contamination and changes in hyphal morphology. In particular, changes in the following features were sought: branching, vacuolation, autolysis, presence of conidia or conidophores, formation of swollen cells and fragmentation.

Samples were also streaked onto yeast extract agar so that strain stability could be monitored with respect to the selection of morphological mutants. Culture from the samples was plated on nutrient agar as a further check on bacterial contamination.

Continuous culture runs of up to 6 months were achieved with *Aspergillus nidulans*, 13 mel, during which time no detectable changes in strain characteristics were observed.

SECTION II

THE KINETICS OF FUNGAL GROWTH.

1. INTRODUCTION

(a) Exponential growth.

(i) Basic kinetics

(ii) The Monod equation.

(iii) The logistic equation.

(iv) Maximum specific growth rate.

(v) The concept of a "hyphal growth unit".

(b) Stromatic growth.

(c) Continuous-flow cultures.

(i) Introduction.

(ii) Basic growth kinetics.

(iii) Pellet vs. Filamentous growth.

(iv) The maximum specific growth rate of moulds.

(v) Maintained cultures.

(vi) Minimum growth rate.

(vii) The energy yield coefficient, Y^{ATP}.

(viii) Transition states.

SECTION II.

The Kinetics of Fungal Growth.

1. INTRODUCTION.

(a) Exponential growth.

(i) Basic kinetics.

Algebraic descriptions of bacterial growth are well established and have been substantiated by copious experimental evidence. Relatively little attention has been paid to the filamentous fungi, however, an apparent neglect which may be primarily attributed to the inherent practical difficulties encountered in growing fungi (see Section I).

When describing the multiphasic bacterial "growth cycle", Monod (1942,1950) observed that microorganisms in an environment containing an excess of all substrates necessary for growth, are capable of autocatalytic growth. This results in an exponential increase in the rate of culture biomass production during the so-called "logarithmic phase" of the growth cycle. In unicells, exponential growth occurs as a result of binary fission, culture biomass doubling at a constant rate. The kinetics of microbial growth may be summarised as follows:

If one considers an increase in culture biomass from x to x_t gl^{-1}, which takes place during the time period t_0 to t_1 h, then, in incremental notation, the rate of biomass production will be:

$$(x_t - x)/(t_1 - t_0) \quad gl^{-1}h^{-1}$$

or $$\frac{\delta x}{\delta t} \quad gl^{-1}h^{-1}$$

The limit of this quantity as δt approaches zero may be written as a differential function,

$$\frac{dx}{dt} \quad gl^{-1}h^{-1}$$

which is the instantaneous rate of biomass increase at time t. As the organism concentration produced after d t depends on the initial concentration x, dx/dt and x may be related by a suitable constant,

$$\frac{dx}{dt} = \mu x \qquad (8)$$

where μ is the specific growth rate constant (units h^{-1}) as defined by Fencl (1963).

Solving equation (8) an equation describing a straight line whose slope is equal to μ may be obtained:

$$x_t = \int \mu x . dt$$
$$= x_o e^{\mu t} \qquad (9)$$

if growth is exponential

$$\text{and} \qquad \ln x_t = \mu t + x_o \qquad (10)$$

(which is of the form, y = mx + c).

Growth of filamentous moulds, however, is not concerned with binary fission of unicells but with the proliferation of multicellular or coencytic units of variable size. Unrestricted growth in fungi appears, nevertheless, to take place in an exponential manner (Zalokar, 1959; Pirt & Callow, 1960; Trinci, 1969; Griffin, Timberlake & Cheney, 1974).

If the concentration of one of the constituents of a culture growth medium is reduced until its availability limits the growth rate of the organism, the culture is said to be substrate-limited.

The growth rate can then be altered by changing the growth-limiting substrate concentration.

(ii) The Monod equation.

A mathematical model describing substrate-limited microbial growth was proposed by Monod (1942). It is based on the Michaelis-Menten equation for substrate-limited enzyme activity.

$$\mu = \mu_{max} \frac{s}{Ks + s} \qquad (11)$$

where μ_{max} is the maximum specific growth rate under specified physical and nutrient conditions; K_s is the saturation constant and is the growth-limiting substrate concentration at which $\mu = \frac{1}{2} \mu_{max}$ (units gl^{-1}) and s is the culture growth-limiting substrate concentration (gl^{-1}).

(iii) The logistic equation.

The logistic equation (Hockenhull & Mackenzie, 1968) has also been proposed to simulate the microbial growth curve.

Batch curve data has been shown to fit a first order reaction rate expression where growth alone is concerned:

$$\text{i.e.} \quad dx/_{dt} = k_1 x$$

(where k_1 is a constant)

The above equation is identical with equation (8)

$$\text{i.e.} \quad dx/_{dt} = \mu x \quad \text{when} \quad \mu = \mu_{max}$$

$$\therefore \quad dx/_{dt} = \mu_{max} x$$

in conditions of substrate excess.

In addition to the growth term, the logistic equation includes an expression for the rate of decrease of a batch culture population when a state of substrate exhaustion is approached. This has been found to be a second order rate term:

$$-dx/_{dt} = k_2 x^2$$

(where k_2 is a constant, units $g^{-1}h^{-1}$)

Overall:

$$dx/_{dt} = \mu_{max} . x - k_2 x^2$$

Since μ and k_2 are both constants, the equation can be resolved thus:

$$dx/_{dt} = \mu_{max} . x \ (1 - cx) \qquad (12)$$

where $k_2 = \mu c$ and c has units g^{-1}. In this expression, c is known as the "carrying factor".

Maynard-Smith (1971) expressed the equation in the following form:

$$dx/_{dt} = \mu_{max} c \ (1/_c - x) x$$

$1/_c$ has units g biomass and is an expression of the maximum population density of the culture. In Maynard-Smith's terminology, the equation becomes:

$$dx/_{dt} = C . (X_E - x) . x \qquad (13)$$

where x is the initial population concentration (units $g\ l^{-1}$); X_E is the maximum population concentration (units $g\ l^{-1}$) and C is a constant whose units, by analysis, are $g^{-1}\ h^{-1}$.

Computer simulations of the Monod and logistic models are presented in the Results and Discussion, (II,3).

(iv) <u>Maximum specific growth rate</u>.

There is a paucity of published values for μ_{max} in mycology. The data available suggests that maximum specific growth rates are mostly in the order of 0.2 - 0.3 h^{-1} for the filamentous

fungi although a value of 0.8 h^{-1} for *Achlya bisexualis* has been reported. (Griffen, Timberlake & Cheyney, 1974).

(v) The concept of a "hyphal growth unit"

Growth of filamentous fungi is generally brought about by an increase in overall hyphal length; either by the formation of branches or by the elongation of existing hyphae. The contribution of the various parts of the hyphal length to growth is uncertain. Macromolecules are detected in an even distribution throughout the mycelial length (Nishi, Yanagita & Maruyama, 1968; Fencl, Machek, Novak & Seichert, 1969). These observations are supported by electron microscopy (Righelato, Trinci, Pirt & Peat, 1968) which shows that hyphae have an even distribution of mitochondria, ribosomes and nuclei. Katz & Rosenberger (1971), however, have presented evidence that new wall synthesis occurs only at hyphal tips and it would, therefore, appear that growth could become limited by the rate of translocation of macromolecules or their monomers, synthesised further back along the hyphae, if the hyphal length became sufficiently great. Experiments by Trinci (1969) provide further evidence for this assumption. In a study of the growth rates of germ tubes emerging from *Aspergillus nidulans* spores, Trinci observed that hyphae grew exponentially until the germ tube length became 120 μm long. When this critical length was exceeded the hyphal extension rate became linear and exponential growth ensued by means of the formation of new branches at an exponential rate. These observations suggested that hyphae of length less than 120 μm were able to translocate materials from the spore to the growing tip at a rate which allowed exponential growth to occur.

To test whether hyphae growing from a spore inoculum are capable of growth rates differing from those arising from a vegetative inoculum, STR cultures of *Aspergillus nidulans* were prepared from inocula consisting of a spore suspension and from vegetative inocula consisting of exponentially growing mycelia, and the growth rates from the resulting cultures compared (see Materials & Methods, II,2).

Interpreting some observations of shake flask cultures of *Aspergillus nidulans*, Katz, Goldstein & Rosenberger (1972) described a model for exponential growth based upon the frequency of branch initiation. They concluded that there exists a critical hyphal length, below which hyphae can grow at an exponential rate by simple extension. Hyphae above the critical length would form new biomass by means of branch initiation, the rate of new branch formation determining the growth rate. Katz et al further concluded that the overall specific growth rate of a culture is a result of a distribution of several differing component growth rates of its constituent hyphae.

Caldwell & Trinci (1973) reported that the dry weight, total hyphal length and number of hyphal tips of a *Geotrichium candidium* culture increased exponentially at approximately the same specific rate. These results suggested the existence of a unit of hyphal growth consisting of a hyphal tip associated with a constant mean length of hypha. The authors suggested that this 'hyphal growth unit' was duplicated during exponential growth and that it could be defined as follows:

$$\text{Hyphal growth unit } (h, \mu m) = \frac{\text{total hyphal length } (l, \mu m)}{\text{number of hyphal tips}}$$

When the specific growth rate of batch cultures was varied in different ways, the hyphal growth unit of *G. candidium* was shown to vary in length in an analogous manner to the variation of bacterial cell mass under the same conditions (Trinci, 1973). This effect was not observed when the growth rate of *Penicillium chrysogenum* was varied in a chemostat (R.C. Righelato, 1974, personal communication). In this case the hyphal growth unit appears to decrease as dilution rate is increased whereas bacterial cell mass appears to decrease under these conditions (Ierusalinsky, 1962).

(b) Stromatic Growth.

Several authors have reported fungal growth which does not grow exponentially, according to equation 8, (Emerson, 1950; Marshall & Alexander, 1960). In each case, growth appeared to proceed according to a cube root law:

$$x_t^{\frac{1}{3}} = x^{\frac{1}{3}} + kt \qquad (14)$$

where t, x_t and x have the values stated previously and k is a zero order growth rate term. The cultures described, differed from exponentially growing cultures in that they consisted of discrete aggregate or pellets of mycelium. Pirt (1966) stated that the mode of growth of the pellet or stromatic form would grow according to equation (14) if the pellet diameter exceeded a critical value. He suggested that the hyphae in filamentous cultures are all exposed to the medium and are thus capable of contributing to growth. Pellet growth would be limited by oxygen starvation occurring inside the aggregate. Calculations suggested that if oxygen could only enter by simple diffusion,

the growing region of the pellet would be restricted to a peripheral zone, 0.077mm wide. Morphological evidence reported by Camici , Sermonti & Chain (1952) was used to support this theory. Pellet structure may vary between species, however, ranging from densely packed mycelium, where oxygen supply occurs by molecular diffusion, to loosely structured pellets into which the penetration of eddy currents by turbulence in the medium would result in a more efficient supply of oxygen to the interior, (Choudhary & Pirt, 1965). The results of Trinci (1970) differ somewhat from values predicted by Pirt's theory. In an experiment in which the growth of pellets growing singly (i.e. one per flask) was measured; Trinci found that pellet radius increased linearly with time, the dry weight increased at an exponential rate and the cube root of the dry weight increased linearly with time. Increasing the number of pellets in each flask resulted in a decrease in the final pellet diameter and the duration of the initial radial growth rate. Trinci's results lead him to conclude that the growth zone of a hypothetical pellet, 9 .0 mm in diameter was 2.5 mm wide; attributing the discrepancy with Pirt's results to loose packing of the hyphae. In a theoretical and experimental study of the specific respiration rates of *Aspergillus niger* pellets, Kobayashi, VanDedem & Moo-Young(1973) found that respiration rate decreased as pellet size was increased. Three theoretical models were tested to explain the observed phenomena:

Model A; assuming a uniform respiratory activity throughout the pellet.

Model B; assuming that differences in respiratory activity occur as a direct result of age distribution of mycelium within the pellet.

Model C; assuming that each value of specific respiratory activity within the pellet occurs as a direct result of adaptation to the local oxygen concentration.

The authors' conclusion that model C most adequately described the experimental situation is supported by experiments of Huang & Bungay (1973) in which measurements of dissolved oxygen concentrations in *Aspergillus niger* pellets were made using a microprobe. A gradient of dissolved oxygen tension appears to exist within the pellet; beginning, in pellets surrounded by oxygen saturated medium, at a distance 200 μm above the pellet surface. No oxygen was detected at depths greater than 135 μm within the pellet. However, because these experiments were made with pellets suspended in a non-agitated culture medium, Huang & Bungay's results should be interpreted with great care when STRs are being considered, particularly as the oxygen tension gradient within the pellet is continuous with a gradient in the medium. A very useful experiment could be carried out if the dissolved oxygen tension determinations were repeated with the pellet suspended in a well agitated broth.

The initiation of pelleting has been shown to be affected by a number of environmental factors (Whitaker & Long, 1973). Formation of pellets may take place by:

(a) the agglutination of spores at an early stage of germination, e.g. during swelling, or

(b) the aggregation of small clumps of germinated spores.

Evidence for (b) exists in Penicillium (Pirt & Callow, 1959). Galbraith & Smith (1969), however, have postulated that charges in the cell wall may account for pellet formation by mechanism (a), which is then followed by a stabilisation of pellet structure brought about by entanglement of germinating hyphae.

A consequence of pellet growth is its effect upon culture broth rheology. Broths with non-Newtonian flow characteristics result when fungi are grown in the filamentous form, having the effect of promoting the coalescence of gas bubbles (Steel, 1969). This phenomenon results in the inhibition of gas transfer to and from the culture. Cultures consisting of pellets appear to approximate closely to Newtonian fluids and exhibit much faster gas transfer characteristics between the gas phase and the broth (Taguchi, 1971). Moreover, Ross & Wilkin (1968) stated that the pellet growth form was essential for efficient aeration of fungal cultures in tower fermenters. However, the observations of Huang & Bungay (1973) that severe dissolved oxygen tension gradients exist within the pellet suggest that higher oxygen concentrations would be required in the broth to adequately aerate pellet cultures.

(c) Continuous flow cultures.

(i) Introduction.

If, during exponential growth, fresh medium is added to a culture of a rate sufficient to maintain the organism population at a level below maximum density, then growth should be prevented from slowing down (as is the case after three to six generations in a batch culture) and, theoretically, it can continue indefinitely. Moreover, the rate growth will be

limited by the rate of supply of one or more (but usually one) of the nutrients supplied in the fresh medium, thus providing a means of controlling growth rate by specifying the rate of flow of medium into the culture. The concentration of growth-limiting substrate will, with all other essential nutrients in excess, determine the concentration of organisms in the culture produced.

The above proposals form the basis of the principle of continuous culture. The use of this technique for the study of microbial metabolism has been advocated in the critique in Section I.1. Continuous culture plays an important role in many areas of current microbiological and biochemical research. (Tempest, 1970) and the technique has wide applications in industrial processes (Righelato & Elsworth, 1970).

Some of the important theoretical considerations of substrate limited continuous flow systems, i.e. chemostat cultures, may be summarised mathematically and an algebraic treatment of continuous-flow principles is presented below.

(ii) Basic growth kinetics.

Monod's suggestion (Monod, 1949) that the final concentration of organisms achieved in a closed culture when growth-limiting substrate was exhausted, was directly proportional to the initial concentration of substrate may be expressed as follows:-

$$x \propto s_o$$

Thus: $$x = Ys_o \qquad (15)$$

where s is the initial concentration of growth limiting substrate (units, g l^{-1}) and Y is the growth yield factor, defined as that quantity of organisms produced from a given quantity of

growth-limiting substrate (units, g dry weight of organism g dry weight of substrate used^{-1}).

Thus:

$$Y = -dx/_{ds} \tag{16}$$

Substituting (16) in (8):

$$-Y\ ds/_{dt} = \mu x$$

$$ds/_{dt} = -\mu x/_{Y} \tag{17}$$

or:

$$ds/_{dt} = \mu max . \frac{s}{Ks + s} . \frac{x}{Y} \tag{18}$$

where $ds/_{dt}$ is the rate of utilisation of growth limiting substrate by the growing organism.

In continuous cultures, where organisms are simultaneously growing in and being washed out from the fermentation vessel, the net change in concentration of the organisms with time will be determined by the relative rates of each process.

Thus:

The rate of change of organism concentration in the culture vessel	=	Rate of growth of organism	-	Rate of removal of organism

i.e.

$$dx/_{dt} = \mu x - Dx \tag{19}$$

where D is the dilution rate, (units, h^{-1}), an expression relating to the rate of supply of medium to the fermenter and is defined as follows:

$$D = F/_{V}$$

where F is the flow rate of medium entering the fermenter (and leaving it in the situation described by equation 19), with units 1 h^{-1}, and V is the volume of the vessel (units, 1).

Extracting x from (19) and substituting (11):

$$dx/_{dt} = x\ \frac{\mu max\ s}{Ks + s} - D \tag{20}$$

Under conditions of continuous-flow, three situations are possible:

(1) $\mu > D$; where growth is not limited and all essential substrates are present in excess. Such a situation could take place when continuous flow is first applied to a batch culture, before any of the substrates have become growth limiting or in a "step-down transition state" when cultures growing under the regime of a particular dilution rate have another lower dilution rate applied to them (to be discussed later in sub-section vii);

(2) $\mu = D$; in this case, the rate of supply of growth-limiting substrate determines the growth rate of the organism and the culture is in a steady state;

(3) $\mu < D$; where culture is washing out of the fermenter and the organism concentration is progressively decreasing. Such a situation occurs if the dilution rate is greater than μ_{max} or in a "step-up" transition state where a higher dilution rate is applied to a continuous culture in a steady state.

In situation (2), where $dx/_{dt} = 0$, i.e. no net change in biomass concentration occurs, an examination of equation (19) confirms that μ is numerically equal to D.

The kinetics of growth-limiting substrate utilisation may be expressed as follows:

The rate of change of substrate concentration in the culture vessel	=	The rate of input of fresh substrate	-	The rate output of unused substrate	-	The rate of growth limiting substrate use by the organism

i.e.
$$ds/_{dt} = DS_R - Ds - \frac{\mu x}{Y}$$

where s is the concentration of growth limiting substrate present in the culture and S_R is the concentration of growth limiting substrate in medium supplied to the fermenter (units $g\ l^{-1}$ in each case).

$$\therefore \quad ds/_{dt} = D(S_R - s) - \frac{\mu x}{Y} \qquad (21)$$

Substituting equation (11), (21) becomes:

$$ds/_{dt} = D(S_R - s) - \frac{\mu max.s.x}{Y\ (Ks + s)} \qquad (22)$$

By inspection, it can be seen that:

$$x/_{Y} = (S_R - s) = \text{amount of growth limiting substrate utilised by the organism for growth.} \qquad (23)$$

In a steady state, when $dx/_{dt} = 0$ and $\mu = D$; equation 23 may be re-written as:

$$0 = \bar{x} \left(\frac{\mu max\ \bar{s}}{Ks + \bar{s}} - D \right)$$

where $\bar{x}$ and $\bar{s}$ are the steady state concentrations of organism and growth limiting substrate respectively, and are unique for given values of D and S_R.

Thus:

$$D = \frac{\mu max\ \bar{s}}{Ks + \bar{s}}$$

and extracting $\bar{s}$:

$$\bar{s} = \frac{D\ Ks}{\mu max - D} \qquad (24)$$

Similarly, from equation (21):

$$0 = D(S_R - \bar{s}) - \frac{\mu \bar{x}}{Y}$$

thus:

$$\frac{\mu \bar{x}}{Y} = D(S_R - \bar{s})$$

Since D = μ in a steady state,

$$\frac{\bar{x}}{Y} = (S_R - \bar{s}) \tag{25}$$

which is identical to equation (23).

Combining (24) and (25):

$$\bar{x} = Y\left[S_R - \frac{D\ Ks}{\mu max - D} \right] \tag{26}$$

(iii) Pellet vs. Filamentous growth.

The above kinetic treatment of chemostat theory applies only to moulds which grow filamentously. Recalling equation (14) which describes pellet growth:

$$x_t^{\frac{1}{3}} = x_o^{\frac{1}{3}} + k_t$$

differentiating for time

$$x_t \to x_o \quad (=x):$$

$$dt/_{dx} = d/_{dt} \left(x^{\frac{1}{3}}/_k \right)$$

$$= \frac{x^{-\frac{2}{3}}}{3k}$$

$$\therefore \quad dx/_{dt} = 3kx^{\frac{2}{3}}$$

Chemostasis will only occur when

$$\text{Rate of change of culture biomass} = \text{rate of growth of organism} - \text{rate of organism removed} = 0$$

$$dx/_{dt} = 3k\bar{x}^{\frac{2}{3}} - DY(S_R - \bar{s}) = 0$$

Thus:

$$3k\bar{x}^{\frac{2}{3}} = DY(S_R - \bar{s})$$

and

$$k = \tfrac{1}{3}\ DY\ (S_R - \bar{s})\ \bar{x}^{-\frac{2}{3}} \tag{27}$$

Applying the data of Trinci (1970); *Aspergillus nidulans* (BWB224) will grow as pellets in continuous culture at all values of D when:

$$D = \frac{0.0705.\bar{x}^{\frac{2}{3}}}{Y\ (S_R - \bar{s})}\ h^{-1}$$

(iv) The maximum specific growth rate of moulds.

The exponential growth of filamentous fungal batch cultures has been quantified by many authors. When fungi are grown in continuous cultures, however, it has been found that the specific growth rate observed in continuous culture may not be duplicated in the chemostat,(Carter & Bull, 1969; Novak & Fencl, 1973; Solomons, 1972). Using cultures of *Aspergillus nidulans*, *Aspergillus niger* and *Fusarium graminearium*, respectively, these authors were not able to apply dilution rates greater than half the value of the specific growth rate observed in batch cultures. Any attempt to increase the dilution rate to a greater value resulted in the progressive diminution of culture biomass concentration, effected by the rate of culture washout, $D\bar{x}$, having become greater than the rate of growth of the organism $\mu\bar{x}$. Solomons & Scammell (1974) have partially resolved this phenomenon in *Fusarium gravinearium*. A growth rate-dependent requirement for biotin (up to μ = 0.22 h^{-1})and choline chloride (μ = 0.22 to 0.27 h^{-1}) has been demonstrated. Solomons (1972) had already concluded that the attainment of higher growth rates in batch cultures without any addition to the medium could be explained if the required co-factors are present in the spores comprising the inoculum. Novak & Fencl (1973) favour an explanation for this phenomenon based upon the existence of an "intermetabolite" accumulating in the cells and acting as a growth-limiting factor. In this Section a discussion will be presented which attempts to provide an explanation for this

effect in the light of the author's own observations.

(v) Maintained cultures.

The term "maintenance energy" of microbial cultures refers to the energy consumed for functions other than the production of new cell material (Pirt, 1965). This implies that the energy substrate supplied to a microbial culture is used both in the production of new organisms and the maintenance of the existing population:

Thus:

Overall rate of energy substrate utilisation	=	rate of energy substrate used to maintain existing populations	+	rate of energy substrate used for growth	
ds	=	$mx\,dt$	+	Adx	(28)

where A is the amount of energy substrate consumed per unit amount of organism formed and m is the amount of energy substrate consumed per unit time to maintain the organisms in a healty state.

Equation (28) becomes:

$$ds/_{dt} = -(mx + A\,dx/_{dt})$$

substituting (17) and (8):

$$\frac{\mu x}{Y} = mx + A\mu x$$

$$\therefore\ 1/_{Y} = m/_{\mu} + A$$

Because A is a $\delta s/_{\delta x}$ term, it can be seen that it is a reciprocal yield term. Introducing the term Y_g (the "true growth yield", obtained when only the growth limiting substrate utilised for growth is considered; units g.g biomass^{-1}):

$$1/_{Y} = m/_{\mu} + 1/_{Yg} \qquad (29)$$

This is sometimes written as:

$$q = \mu/_{Yg} + m \qquad (30$$

where q is the specific rate of uptake of energy substrate (units g.g biomass^{-1} h^{-1}) and is equal to $\mu/_{Y}$.

Substituting equation (29) in (26) we obtain:

$$\bar{x} = \frac{D\ Yg}{mYg + D} \quad \frac{S_R - D\ K_s}{\mu_{max} - D} \tag{31}$$

when $\mu \rightarrow D$.

Equation (30) is of the form, y = mx + c. Thus, if q is plotted against μ; a straight line is obtained with a slope of $1/_{Yg}$ and an intercept of m. It was found that the value of m obtained with *Penicillium chrysogenum* cultures was equal to the requirement for glucose and oxygen to maintain a glucose-limited culture without growth (Righelato, Trinci, Pirt & Peat, 1968). These authors concluded that the required glucose ration was utilised for the replacement of lysed cells, intracellular turnover of proteins and nucleic acids, the maintenance of osmotic barriers and other aspects of endogenous metabolism. Carter, Bull, Pirt & Rowley (1971) have reported oxygen and glucose maintenance values for *Aspergillus nidulans* (BWB224).

(vi) Minimum Growth Rate.

In his introduction to the 5th Continuous Culture Symposium, Pirt (Pirt, 1972) reviewed a number of reports which suggested that the growth rate of micro-organisms can only be decreased to a finite limit below which all or part of the population ceases to grow. This finite limit was referred to as the minimum growth rate (μ_{min}). Evidence for the existence of a minimum growth rate has been obtained for bacteria(Tempest, Herbert & Phipps, 1967) as well as for fungi.

Pirt & Righelato (1967) observed a marked decay in the rate of penicillin production when the dilution rate of glucose-limited *Penicillium chrysogenum* was set below $0.014h^{-1}$. Concurrently, considerable turnover of both RNA and DNA with a consequent alteration of the ratio of nucleic acids to protein takes place under these conditions (Righelato, Trinci, Pirt & Peat, 1968) while the formation of conidia occurred after about 20 hours. The data of Bainbridge, Bull, Pirt, Rowley & Trinci, (1971) indicated that *Aspergillus nidulans* (BWB224) would not grow if supplied with glucose at 1.5 x the maintenance ration. This would suggest a μmin value of $0.007h^{-1}$. Bainbridge et al observed changes in morphology and chemical composition at this dilution rate but did not observe conidiation.

Pirt (1972) concluded that μmin was approximately equal to 5% of μmax.

Experimental results will be presented in this Section and in Section III which will be offered as evidence for the existence of a minimum growth rate in *Aspergillus nidlans* (13 mel).

(vii) The energy yield coefficient, Y^{ATP}.

In section (v) it was demonstrated that the overall yield coefficient could be resolved into two componants; Y_g, the true growth yield, and a parameter describing the yield of maintenance energy obtained by the organism from the growth limiting substrate. A further variation has been described by Bauchop & Elsden (1960); the concept of Y^{ATP} which describes the weight (g) of organisms produced per mol of ATP utilized. Y^{ATP} values of about 10.5 have been obtained for both anaerobic and aerobic

bacteria (Bauchop & Elsden, 1960; Stouthamer, 1962; Chen, 1964).

An expression for the experimental determination of the number of mols of ATP produced per mol of oxygen utilized by a micro-organism has been derived using the concept of Y^{ATP} (D.E.F. Harrison, 1972, personal communication). Making the following assumptions for a microbial fermentation:

(1) All energy is obtained aerobically.

(2) ATP is the sole intermediary of energy conservation.

(3) The concept of Y^{ATP} applies.

(4) The system is energy-limited for growth, and letting:

Q_{O_2} = the specific rate of oxygen uptake (units mols $O_2.g^{-1}h^{-1}$)

Y_{O_2} = the biomass yield for oxygen (g.cells mols O_2^{-1}).

M_{O_2} = oxygen uptake rate required for maintenance (mols O_2 $h^{-1}g^{-1}$),

then by analogy with equation (30)

$$Q_{O_2} = \mu / Y_{O_2} = M_{O_2}$$

If N is the number of mols ATP produced per mol of oxygen taken up (Harrison & Loveless, 1971);

$$Y_{O_2} = Y^{ATP} N$$

(g biomass.mol O_2^{-1} = mols ATP .mol O_2^{-1} x g biomass mol ATP^{-1}).

If M is the maintenance ATP requirement (units mol.$g^{-1}h^{-1}$, Harrison & Loveless, 1971): then

$$M_{O_2} = M/N$$

$$\therefore \quad Q_{O_2} = \frac{\mu}{Y^{ATP} N} + \frac{M}{N}$$

$$\text{and} \quad N = \frac{\mu}{Y^{ATP} Q_{O_2}} + \frac{M}{Q_{O_2}} \qquad (32)$$

(viii) Transition States.

All concepts and observations pertaining to chemostat systems so far discussed have been concerned with steady state conditions. Indeed, there are very few publications which describe the behaviour of continuous cultures under transient state conditions. The few reports available have been primarily concerned with the theoretical implications of such experimental systems. Acknowledgement is made of the work of Maaløe & Kjeldgaard (1966) and of Pritchard and his co-workers (Pritchard, Barth & Collins, 1969) in which studies of transitions in batch cultures between different growth rate values have been made. These experiments, however, suffer from the inherent limitations of batch culture studies discussed previously. Moreover, the changes in growth rate described were brought about by altering important culture environmental parameters such as temperature or utilisable carbon source.

Unlike the study of steady states, the investigation of transient state conditions involves an interference with the balanced growth of the culture. This operation, known as a shift experiment, is a manipulation which brings about changes in the growth rate, μ. As outlined briefly in (ii) shift experiments may be of two types: "step-up" or "step-down".

When the steady state growth condition is suddenly changed, a period of time is often required for re-adjustment to occur in the physiological properties of the culture. According to Tempest, (1970a) organisms expressing a property (p), present in high concentrations in the initial population but almost absent from the steady-state population following the change

in growth conditions, should exhibit the following kinetics:

$$pt/_{po} = \exp(-Dt)$$

Thus:

$$\ln pt - \ln po = -Dt \qquad (33)$$

In the reciprocal case of the property showing an increase from pt to ps:

$$pt/_{ps} = 1 - \exp(-Dt)$$

i.e.

$$\ln(1 - Pt/_{ps}) = Dt$$

These simple kinetics apply to an experimental situation where p described the production of acetokinase by *Pseudononas oxalis* during a shift from acetate to succinate limitation. The changes observed appear to reflect a simple control mechanism for the enzyme and would be consistent with a single-event gene transcription control. Many enzymes in intermediary metabolism, however, are under allosteric control by a number of different and competing substances. Gross physiological changes brought about by the alteration of the activities of such enzymes would therefore exhibit transient state kinetics of much greater complexity. Mor (1969) and Gilley & Bungay (1967) have described the growth of *Saccharomyces cerevisiae* under transient state conditions brought about by changing the dilution rate of steady state continuous cultures. Complex oscillatory kinetics were observed when biomass, oxygen uptake and carbon dioxide release were determined in step-up and step-down transient states.

Young & Bungay (1973) derived kinetics to explain oscillation in terms of a systems engineering dynamics approach.

While the resulting dynamic terms became complex and voluminous, the models produced gave an accurate representation of experimental observations and its components could be related to actual physiological parameters.

II - 2. MATERIALS & METHODS.

(a) Determination of dry weights.

(b) Glucose determination

(c) Fluorescence microscopy

(d) Computer simulations of growth in batch culture

(i) The Monod model

(ii) The Logistic model

(e) Semicontinuous culture experiments

(f) Maximum dilution rate in continuous culture

(g) Transient states.

2. MATERIALS & METHODS

(a) Determination of dry weights.

Fungal biomass concentration was estimated from measurements of mycelial dry weights in 10ml culture samples. Samples were removed from the fermenter vessel using the procedure described in I.2(h).

The samples were rapidly filtered on sintered glass discs (porosity 2) and the mycelium washed twice with distilled water at $4^{o}C$. Mycelium was then collected from the surface of the filter and wrapped in a pre-weighed sheet of aluminium foil. After heating in an oven at 110° for 12 h., samples were weighed, replaced in the oven, then re-weighed at half-hourly intervals until a constant weight was obtained.

Biomass concentration was estimated from duplicate 10ml samples.

(b) Glucose concentration.

Samples for the determination of glucose concentration in the culture medium were taken at the same time as those for dry weight determinations. A sample volume of approximately 5ml was run into a 20ml bottle containing 5ml 0.2M perchloric acid. The contents of the bottle were filtered on a sintered glass disc and the volume of the filtrate measured. As a precaution against volumetric error, the volume was also estimated from the biomass concentration value and the dry weight of mycelium removed during filtration.

Glucose was determined using a glucose oxidase Blood sugar assay (Boehringer Ltd., Mannheim, Germany). Freshly prepared glucose solutions were used as standards for each series of

TABLE 2.

A BASIC programme for generating biomass concentration values using Monod's model for batch culture kinetics.

```
&TEXT

90  REM A=MUMAX;K=KS;T8=INC;T9=TOTALTIME;P=NO.PTS.;H=ELAPSED T
100 READ A,K,T8,T9,P,X,Y
110 DATA .2,.072,.01,30,30,2,25
160 LET T1=0
180 PRINT "T","BIOMASS","GLUCOSE CONC","TIME(HR)"
190 PRINT
210 PRINT 0,X,Y,20
220 FOR 1=1 TO P
230     FOR T=0 TO T9/P STEP T8
240   LET X=X+(A*Y*X/(K+Y))*T8
250   LET Y=Y+((-A*Y/(K+Y))*X*2.33)*T8
255 LET T1=T1 +T8
265 LET H=T1+20
266 NEXT T
270 PRINT T1,X,Y,H
280 NEXT I
900 STOP
&
```

determinations (concentration range 0-1 gl^{-1} and 0-10 gl^{-1}).

(c) Fluorescence microscopy.

Mycelium was prepared for fluorescence microscopy using the technique of Gull & Trinci (1974). Samples (5ml) from the fermenter were run into a sampling bottle containing 5ml of buffered glutaraldehyde (1% $^{v}/_{v}$ glutaraldehyde in 0.1M Na_2HPO_4/ NaH_2PO_4 buffer, pH 6.8) which rapidly stops growth. Mycelium was then collected by filtration and resuspended in buffered glutaraldehyde solution. Samples were stored at $4^{o}C$ for periods of up to six months prior to staining and observation. Specimens were stained in a 0.05% ($^{w}/_{v}$) solution of Calcafluor White M2R New (The American Cyanamid Company, Organic Chemicals Division, N.J., U.S.A.) for 5 min. Stained hyphae were viewed under ultra-violet illumination using a Zeiss photomicroscope. Micrographs were taken on Ilford HP4 film which was developed in Microphen (Ilford Ltd., Ilford, Essex).

(d) Computer simulation of growth in batch cultures.

(i) The Monod model.

The BASIC programme shown in Table 2 was used to determine the change of biomass with time in a batch culture following Monod kinetics.

Lines 230-255 compute values of δx and δs from equations (11) and (18),

i.e.

$$dx/_{dt} = \frac{x.\mu max\ s}{Ks + s}$$

$$ds/_{dt} = \mu max \frac{s}{Ks + s} \cdot \frac{x}{Y}$$

using an incremental of T_8 for δt. In Table 2 T_8 is shown equal to 0.01 h. As the length of the batch cycle to be

simulated is about 50 h, lines 220 and 280 are used to print biomass values in steps of T_9/P (1 hour) thereby avoiding the necessity of producing an unmanageable amount of data, in this case, 5000 biomass values. In operating the programme, the user specifies values for A, the maximum specific growth rate; K, the glucose saturation constant (Ks); T_8, the incremental value for δt; T_9, the total time to be computed; P, the number of values to be output and H, the elapsed time since inoculation, when the specified initial values of biomass (X) andsubstrate (Y) have been reached.

(ii) The logistic Model.

Table 3 shows a similar programme for out-putting values of biomass concentration in the batch cycle using equation (13);

i.e. $$dx/_{dt} = C\ (X_E - x)\ .\ x$$

Line 240 is analogous with lines 240 and 250 in Table 2. In 240, X_E has been given the value of maximum biomass concentration, $13gl^{-1}$, and C has been given the value of $\mu max/X_E$ ($0.2/13gg^{-1}h^{-1}$)

(e) Semicontinuous culture experiments.

Surface cultures of *Aspergillus nidulans* (13 mel) were grown on the solid medium described in I.2(b), in a series of 250ml conical flasks with cotton wool bungs. Spores were harvested after 5 days by washing with a solution of the wetting agent Manoxol OT (0.05% $^w/v$), (British Drug Houses, Poole, Dorset). Sterile glass beads (diameter, 4mm) were introduced into the flasks which were then shaken with the wetting agent (20ml. per flask) for 10 min to dislodge the spores.

The spore suspension thus obtained was diluted with growth

TABLE 3.

A BASIC programme for generating biomass concentration values using the Logistic equation for batch culture kinetics.

```
&TEXT

100 READ T8,T9,P,X,Y
110 DATA .01,90,10,2,25
120 LET T1=0
130 PRINT "T","BIOMASS","TIME(HR)"
210 PRINT 0,X,20
220 FOR I=1 TO P
230 FOR T=0 TO T9/P STEP T8
240 LET X=X+(.2/13*(13-X)*X)*T8
255 LET T1=T1+T8
260 LET H=T1+20
266 NEXT T
270 PRINT T1,X,H
280 NEXT I
900 STOP

&
```

medium (See I.2(b)) to give a final concentration of 10^7 spores ml^{-1} when introduced into a 3 l. culture vessel.

The specific growth rate of the culture produced after inoculating the spore suspension into synthetic medium (glucose concentration, 10 gl^{-1}) was measured over a period of 24 h in which growth conditions were maintained as described in I.2(e). Approximately 90% of the culture volume was then harvested and the remaining material used as a vegetative inoculum for a new culture. This "subculturing" procedure was then repeated 6 times and the specific rate measured in each case.

(f) Maximum dilution rate obtained in continuous culture.

The critical dilution rate (Dcrit), i.e. the maximum steady state dilution rate at which the culture may be grown without washing out, was estimated by raising the value of D in increments of 0.01 h^{-1}, starting at a dilution rate equivalent to 85% μ_{max}. The value assigned to μ_{max} was that measured in batch culture experiments.

The maximum specific growth rate also was measured in the chemostat using the wash-out method of Jannasch (1969). The rationale for this technique is based on the following theory:

Considering a special case of equation (19) wherein $\mu = \mu max$,

$$dx/_{dt} = x\ (\mu max - D) \quad (34)$$

if D became greater than μ max, then $dx/_{dt}$ would assume a negative value.

integrating (34);

$$\ln \left(\frac{xt}{xo}\right) = (\mu max - D)_t$$

$$\therefore \quad \mu max = D + 1/_t \ln \left(xt/_{xo}\right) \quad (35)$$

Thus, a graph of ln x vs. t would have a slope of

$$-\ln\left(\frac{xt}{xo}\right) \cdot \frac{1}{t}$$

In the present study, the dilution rate of a culture growing at a steady state, with a growth rate equivalent to 85% of Dcrit was changed to 120% of Dcrit. The decline in biomass concentration was then measured over a period of 20 h and a graph of ln x vs. t plotted. The maximum specific growth rate could then be calculated from (35).

i.e.

$$\mu max = D + (-A)$$

where D is the final dilution rate and -A is the slope of the graph.

(g) Transient states.

Cultures growing in a steady state (defined by constant biomass, RQ and RNA concentration) of 0.175 h^{-1} had their dilution rate changed to 0.125 h^{-1}, by adjusting the medium metering pumps to a preset value. Samples for transient state studies were removed until all parameters under observation be became constant.

Similar experiments were carried out for transition states between 0.125 h^{-1} and 0.175 h^{-1}.

II - 3. RESULTS & DISCUSSION

(a) Computer simulations of batch culture

(b) Semicontinuous culture experiments

(c) Effect of growth rate on biomass concentration in continuous culture

(d) Maximum specific growth rate

(e) Minimum growth rate

(f) Effect of dilution rate on glucose uptake

(g) The effect of growth rate on respiratory parameters

(i) The specific rates of oxygen uptake (Q_{O_2}) and carbon dioxide evolution (Q_{CO_2})

(ii) The respiratory quotient (RQ) and the energy yield (Y^{ATP})

(h) Transient States.

3. RESULTS & DISCUSSION.

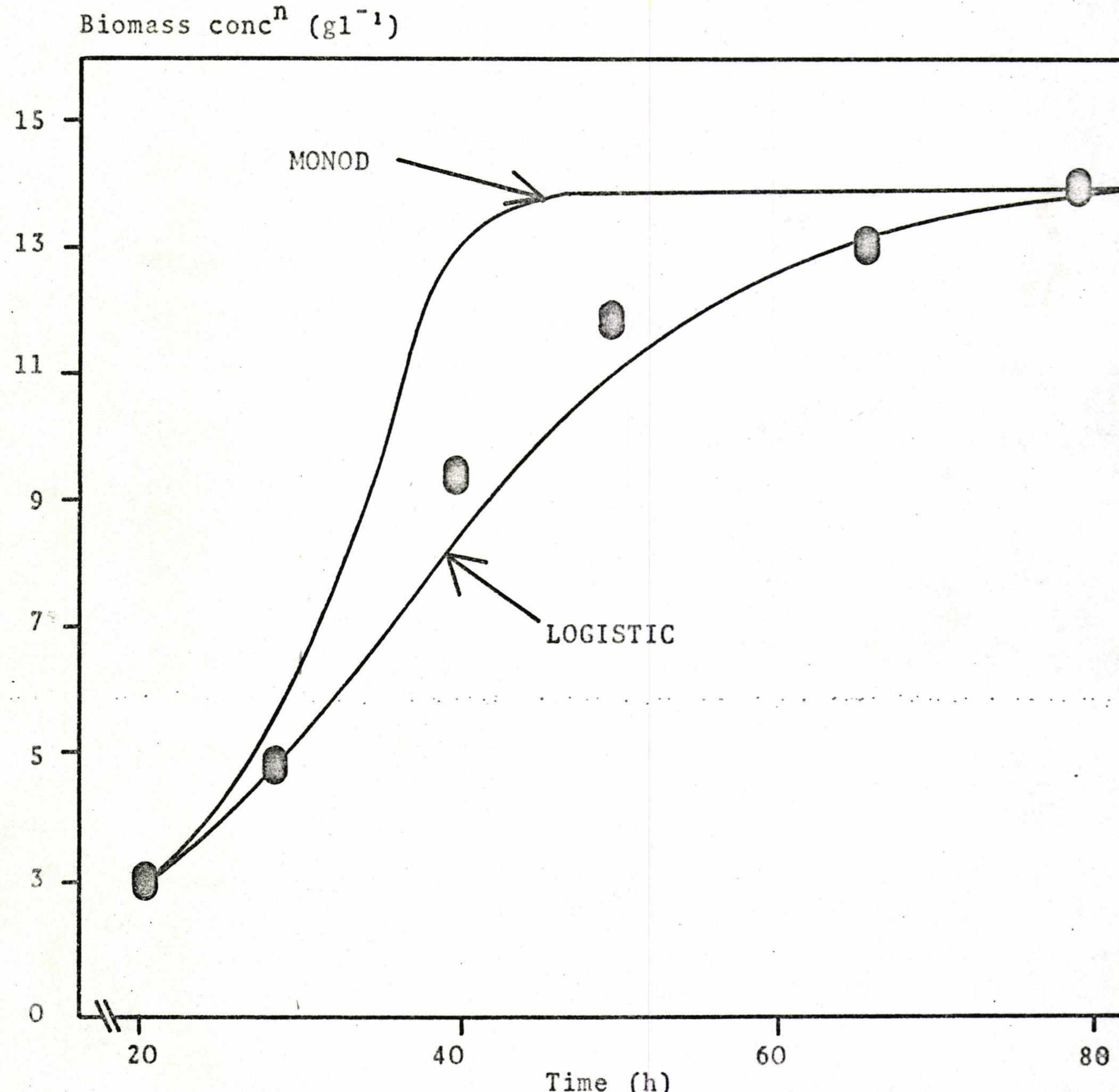
(a) Computer simulations of batch culture.

Two computer simulations of the growth of *Aspergillus nidulans* BWB224 in batch culture using the data of Carter & Bull (1969) are shown in Figure 3. The Monod model appears to depart markedly from the experimental data; indicating either that the rate of substrate utilisation has been over estimated, or that a proportion of the carbon utilised is being used to produce an extra-cellular product. The former explanation suggests that the value of Ks (0.72 gl^{-1})which was derived from continuous culture experiments, does not describe substrate utilization under conditions of batch growth. The latter hypothesis of extra-cellular product formation appears to be feasible, however, as extra-cellular melanin is produced by strain BWB224 as a secondary metabolite (Carter & Bull, 1969).

The logistic curve provides a fairly close fit, indicating that the second order negative growth rate term in equation (12), which is based upon the maximum population density achieved in the culture, provides an adequate description of the rate of decrease of μ in a batch culture.

(b) Semicontinuous culture experiments.

Values of μ obtained from the experiment described in II.2(d) were estimated using equation (10) from semilog plots of x against t as described in II.1(a). The results, shown in Table 4, indicate that growth in a batch culture with a spore inoculum is no faster than that from a vegetative inoculum. The culture derived from spores was morphologically heterogeneous,



Biomass conc^{n} (gl^{-1})
15
13
11
9
7
5
3
0
MONOD
LOGISTIC
20
40
60
80
Time (h)

Figure 3.

Computer simulations of the batch culture cycle of *Aspergillus nidulans* BWB224. Experimental values, O; and computed curves for the Monod & Logistic models are shown.

containing both filamentous mycelium and pellets. With consecutive subcultures, the filamentous mycelium became predominant. This effect would have resulted in a change in growth kinetics from those described by equations (12) and (14) in heterogeneous cultures to a simple exponential growth rate in filamentous cultures. This would have the consequence of masking any enhancement in growth rate by spore-derived nutrients as observed by Trinci (1970).

The distribution of values for μ in Table 4 shows a maximum spread of $\pm$ 5.2% about a mean of 0.193 h^{-1}.

The environmental conditions described in I.2(e) were maintained throughout the experiment. An empirical study of the critical conditions required for spore germination to form a ; filamentous culture, however, suggested that a low aeration rate was required for a minimum lag phase. Batch cultures grown as precursors to chemostat runs were aerated using the following procedure.

Spore suspensions (10^7 spores ml^{-1}) were stirred at low speed (250 rpm) with no air inflow for the first 12 h. The airflow was then increased in 50 ml min^{-1} steps and the stirring rate by 500 rpm increases every 1-1.5h to give an air inflow of 250 ml min^{-1} and a stirring rate of 1250 during active batch growth before starting continuous flow (as reported in Rowley & Bull, 1973). A stirring rate of 1000 rpm was then used for continuous cultures and air inflow rate adjusted to suit the experimental conditions required. The initial low aeration rate was found to prevent carbon dioxide from flushing out the culture and interpretations of these procedures in metabolic terms will be made in Section IV.

TABLE 4.

Experiment	Duration of experiment (h)	Specific growth rate (μ) (h^{-1})
1. Spore inoculum	12(+ 12 h lag phase)	0.183
2. Vegetative inoculum using 10% of material from (1).	5	0.193
3. Second subculture, inoculated from (2)	1	0.188
4. 3rd subculture	13	0.202
5. 4th subculture	3	0.198
6. 5th subculture	3	0.193
	mean	0.193

Semicontinuous culture of *A.nidulans* (13 mel) to test whether spore-derived nutrients influence the value of the observed culture growth rate.

When the complexity of the above procedure had been realized, it was decided to use vegetative inocula as seed cultures for continuous runs as their use caused little or no lag phase and medium flow could be applied to a newly-inoculated culture almost immediately.

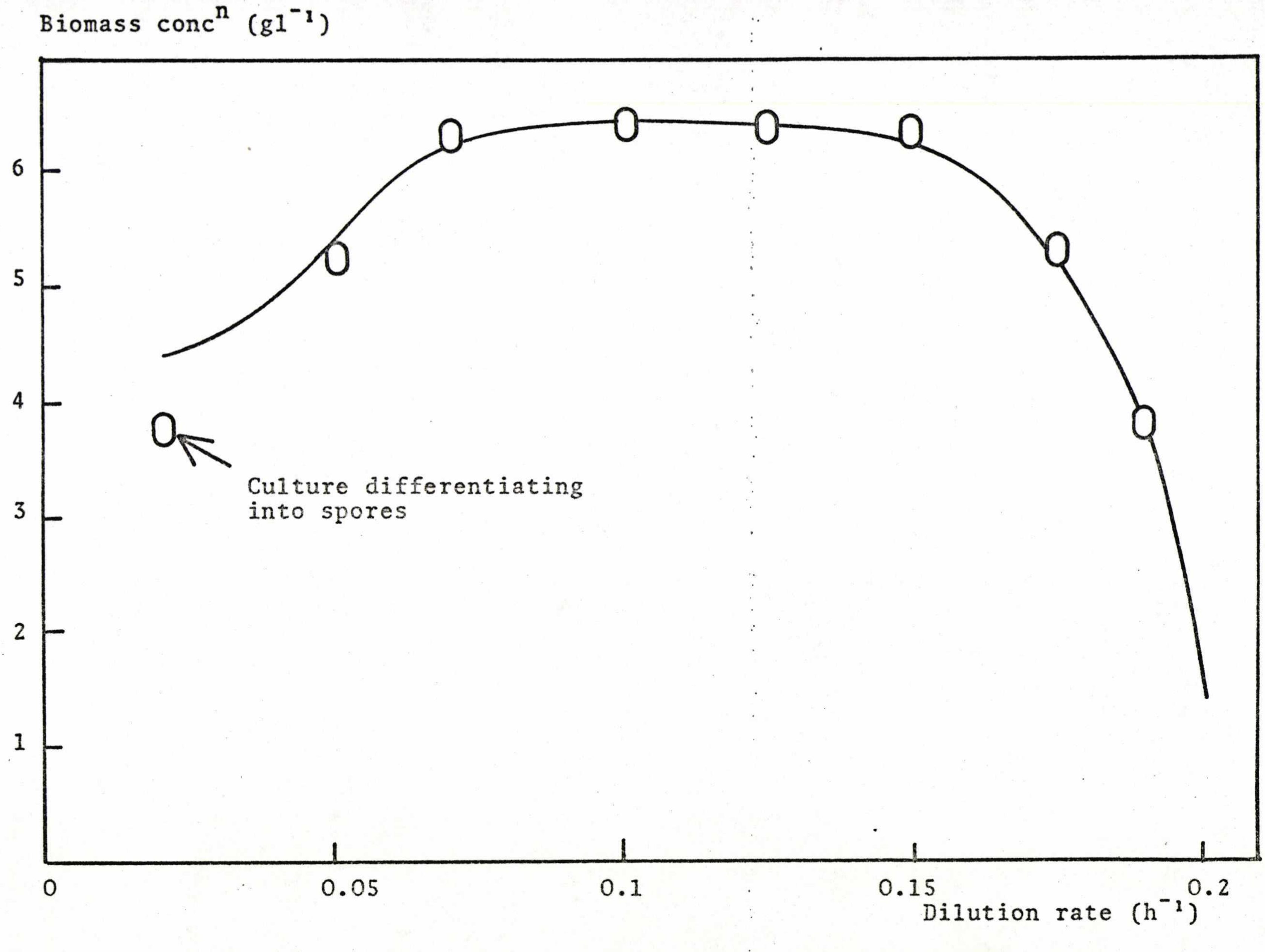
(c) Effect of growth rate on biomass concentration in continuous culture.

Figure 4 shows the effect of growth rate in continuous culture on biomass concentration. Steady states were assumed when biomass concentration, RQ and $\bar{s}$ reached constant values; cultures were run at a particular dilution rate for a period of $\frac{3}{D}$ hours (3 x culture mean residence time) before these parameters were measured. Cultures consisted of filamentous mycelial suspensions at all dilution rates below 0.19 h^{-1}. At 0.19 h^{-1}. At 0.19 h^{-1}, which was the highest steady state growth rate attained, the culture was heterogenous, consisting of pellets as well as filamentous hyphae.

The data points in Figure 4 represent experimental values obtained and the curve shows a theoretical plot of biomass concentration against growth rate using equation (31), i.e.

$$\bar{x} = \frac{DYg}{mYg + D} \qquad S_R - \frac{DKs}{\mu max - D}$$

Good agreement was obtained between the theoretical curve and the data points at dilution rates above 0.05 h^{-1}. As the growth rate approached 0.02 h^{-1}, sporulation occurred (see below, subsection (e)) and the biomass values predicted by equation (31) are not coincident with the observed dry weights of differentiating mycelium. The results may, therefore, be



Biomass conc^n (gl^{-1})
6
5
4
3
2
1
0
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})
Culture differentiating into spores

Figure 4.

The effect of dilution rate upon biomass concentration (observed and computed).

interpreted as proof that the kinetic model, derived in II.1(c), provides an adequate description of the growth of filamentous mycelium provided that the growth is morphologically homogeneous.

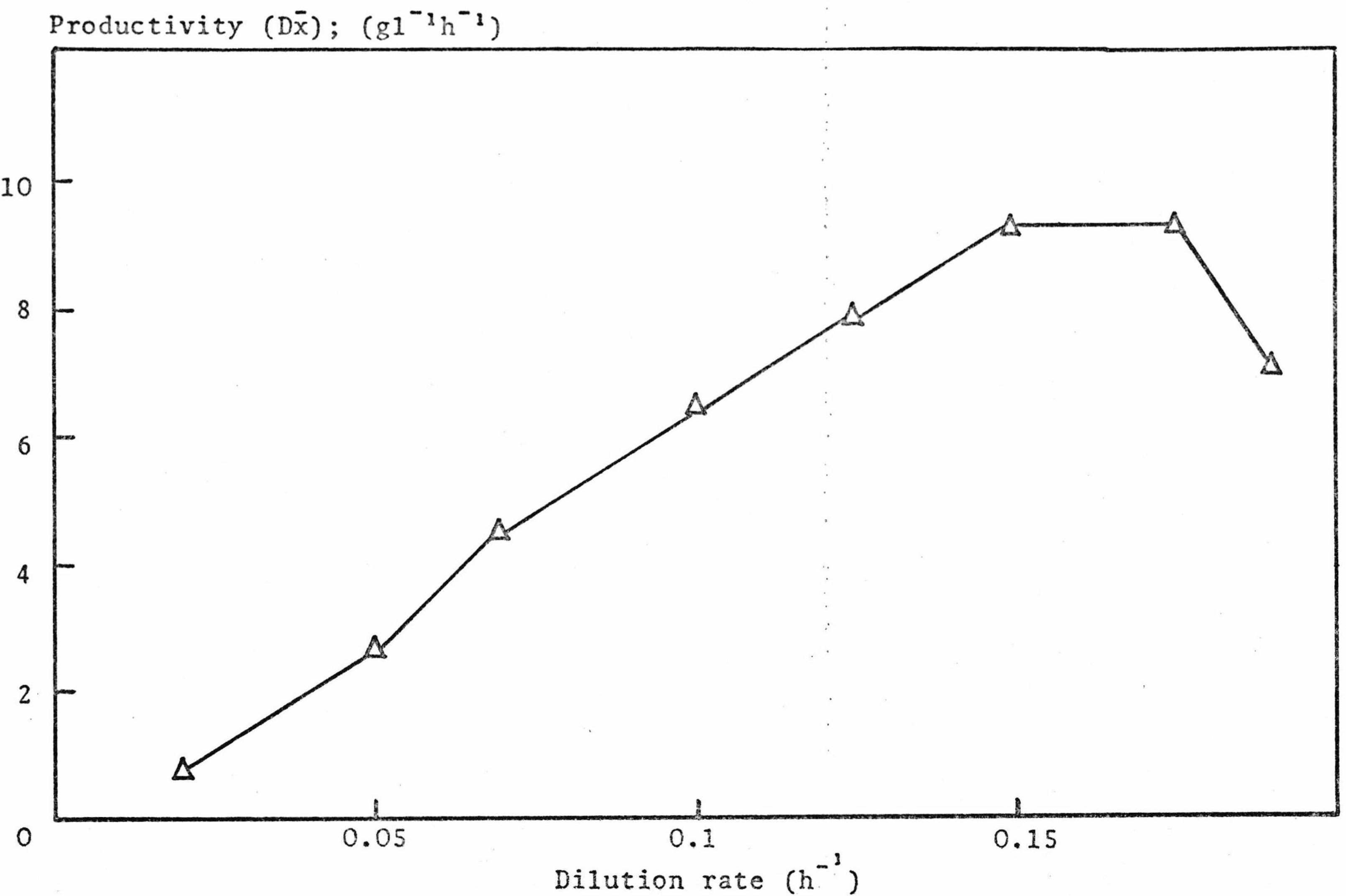
Figure 5 shows the effect of growth rate on culture productivity ($D\bar{x}$) which is the rate of output of mycelium from the fermenter (units: $gl^{-1}\ h^{-1}$). The shape of the curve produced is similar to that observed by Tempest, Hunter & Sykes (1965) with carbon-limited cultures of *Aerobacter aerogenes*. The explanation given may be summarised as follows:-

from equation (26)

$$\text{Productivity} = D\bar{x} = DY\left[Sr - Ks\left(\frac{D}{\mu max - D}\right)\right]$$

usually Ks is small relative to Sr. Thus at dilution rates very much less than Dcrit, productivity is approximately equal to DYSr and will thus increase with increasing values of D. As the dilution rate approaches Dcrit, however, the value of D approaches that of μmax and $Ks\left(\frac{D}{\mu max - D}\right)$ approaches infinity. This being the case, the value of $D\bar{x}$ will then approach zero.

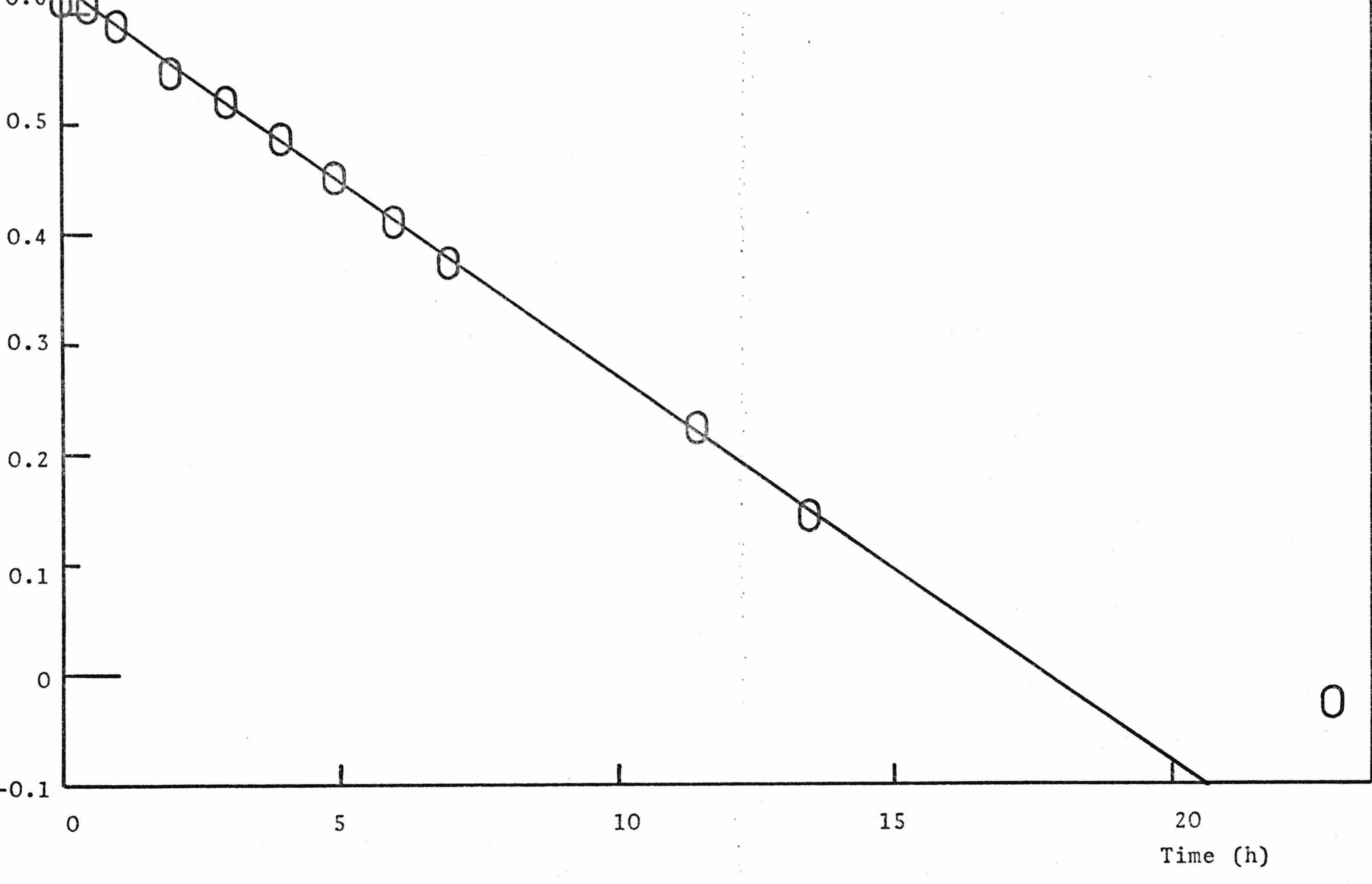
The similarity between the results in Figure 5 and those reported by Tempest et al suggests that this theory may also be applied to filamentous moulds. A computer analysis of the present data showed that the value of $D\bar{x}$ reached a maximum at a growth rate of $0.165\ h^{-1}$. This is therefore, the growth rate at which the highest rate of biomass output is possible under conditions of glucose limitation.



Productivity (Dx̄); (gl⁻¹h⁻¹)
10
8
6
4
2
0
0.05
0.1
0.15
Dilution rate (h⁻¹)

Figure 5.

Productivity curve for *Aspergillus nidulans*(13 mel)



0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
Time (h)

Figure 6.

Estimation of μmax using wash-out parameters.

Wash-out rate	=	-0.0692 h^{-1}
μmax	=	0.205L h^{-1}

(d) Maximum specific growth rate.

The value of Dcrit, obtained using the procedure described in II.2(e) was found to be 0.2 h^{-1}. The maximum specific growth rate, determined using the washout method described, was found to be 0.205 h^{-1}. Figure 6 shows the linearity obtained between log biomass concentration and time during washout. The upper limits of growth of *Aspergillus nidulans* (13 mel) may therefore be summarised as follows:

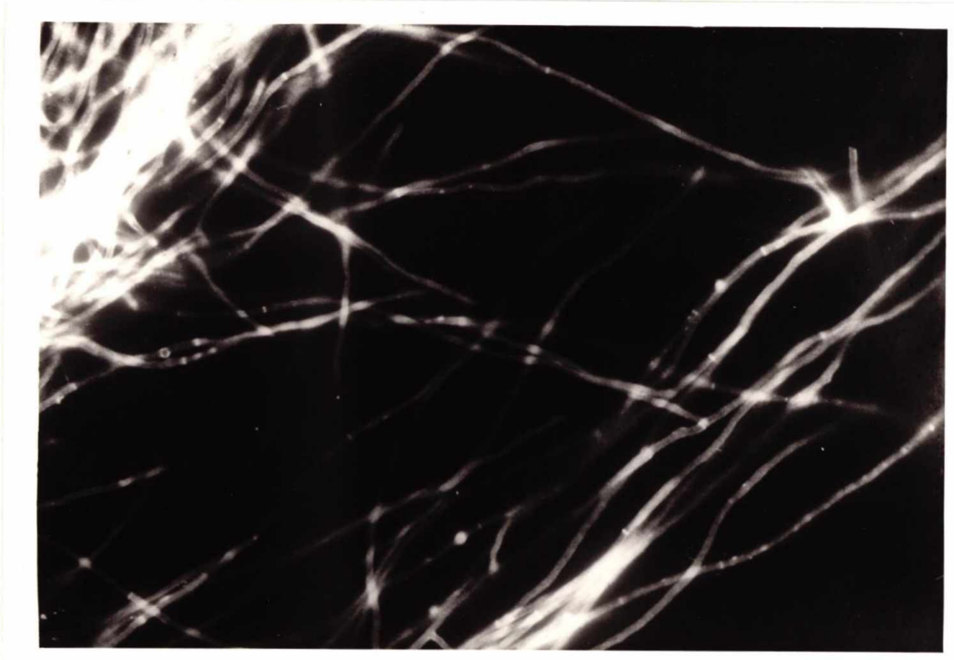
Specific growth rate; μ, (batch culture)	0.193 h^{-1}
Maximum specific growth rate; μ max, (continuous culture)	0.205 h^{-1}
Critical dilution rate; Dcrit (continuous culture)	0.2 h^{-1}

These may be compared with the results of Carter & Bull (1969) and others (see Section II.1(c).iv) who found that it was not possible to obtain values of Dcrit higher than half of the value of the specific growth rate observed in batch culture.

Further experiments with strain 13 mel by the present author demonstrated that batch cultures could be produced, under conditions of low spore inoculum ($<10^5.ml^{-1}$) and high stirring rate (>1300 rpm) which would begin to wash out at dilution rates in excess of 0.07 h^{-1}; the critical dilution rate observed by Carter & Bull with *Aspergillus nidulans* strain BWB224.

Microscopic examination of cultures of strain 13 mel with Dcrit values of (a) 0.20 h^{-} and (b) 0.07^{-} showed that the hyphae of type (a) cultures were very long and virtually unbranched (Plate 2a) whereas those from type (b) contained a high

PLATE 2a.



Highly branched mycelium;
Dcrit = 0.09 h^{-1} (approx)

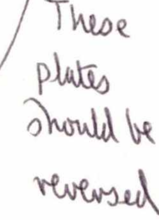
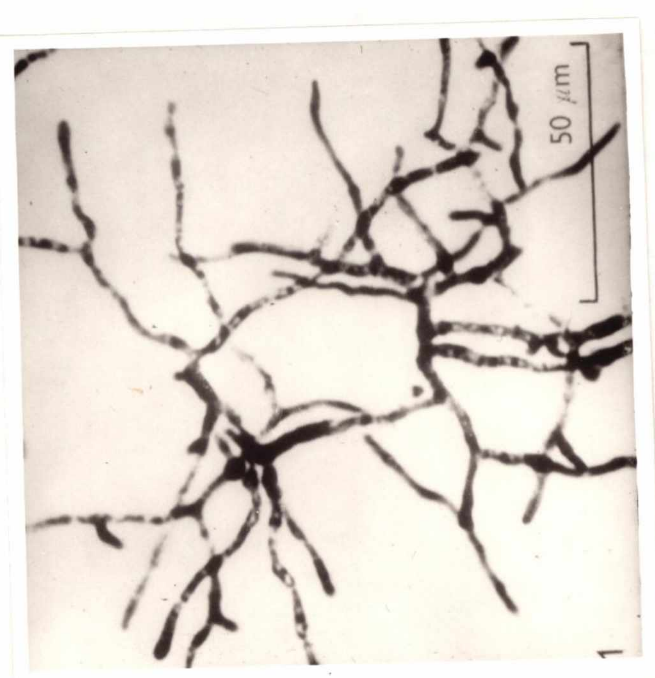


PLATE 2b



Non-branching mycelium ; Dcrit = 0.2 h^{-1} (approx).
Magnification; x 700

density of lateral branches (Plate 2b). In the appendix to this thesis are presented two models which attempt to explain this phenomenon. Evidence for a metabolic basis for this effect will also be discussed in II.3(g)ii. The first model described predicts that differential maximum rates of translocation of nutrients from the hypha to the growing tip will occur in branched and unbranched mycelium. Translocation rates in branched hyphae will reach a maximum and will therefore be growth rate limiting, whereas rates in unbranched hyphae will become a constant function of growth rate and will therefore increase as μ is increased. The second model makes the assumption that more carbon source is required to increase biomass by means of forming new branches than to simply elongate existing hyphae. In a chemostat, exponential branch formation will require the consumption of growth limiting substrate at a greater rate than will simple hyphal extension. The proposal of Solomons (1972) that this phenomenon is a result of a growth rate dependant co-factor requirement (see Section II.1.(c)iv) would not be inconsistent with this model if one or more of the enzymes responsible for branch initiation had a co-factor requirement which could not be met by intracellular material when branching frequency increased beyond a certain rate.

The hypothesis of Novak & Fencl (1973) that the accumulation of a growth-limiting 'intermetabolite' occurs after a few generations in continuous culture could be incorporated into the model if the growth limiting substance was a precursor to a co-factor dependant branching enzyme.

Light micrographs of strain BSB224 growing in continuous cultures (Bainbridge, Bull, Pirt, Rowley & Trinci, 1971)

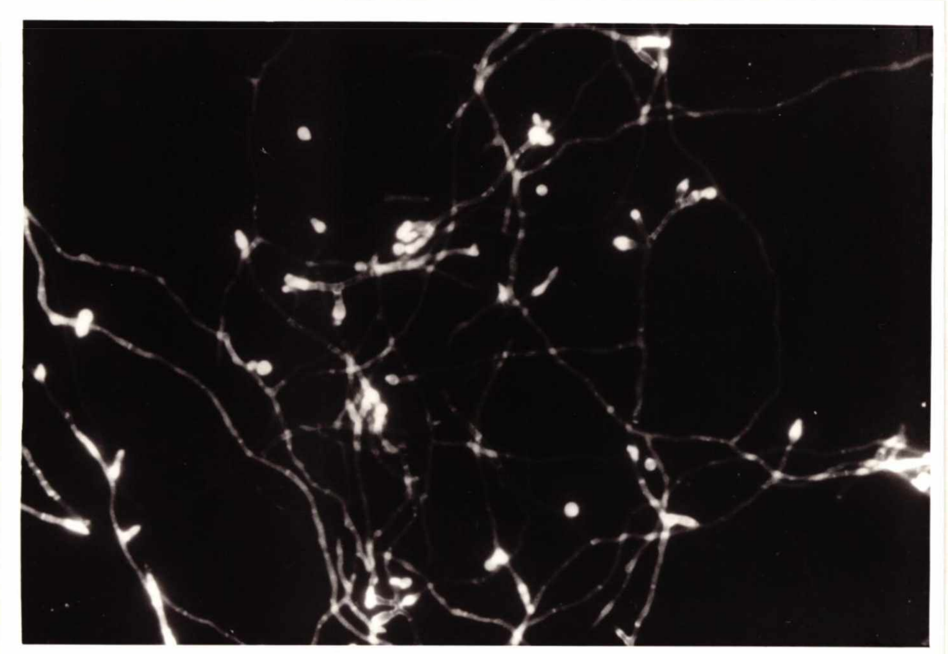
(Dcrit < 0.08 h^{-1}) show hyphae with a highly branched morphology; similar to that in Plate 2(a). To determine whether the correlation between Dcrit and hyphal morphology was valid for strain BWB224, cultures of this strain were prepared using a vegetative inoculum. The technique of semicontinuous culture, described in II.2(d), was carried out, altering the stirring rate or aeration rate with each subculture until a culture with a very low hyphal branching rate was obtained (950 rpm, 100 ml min^{-1}). Glucose-limited steady-state cultures with dilution rates of 0.7, 0.1, 0.15 and 0.175 h^{-1} were thus obtained using strain BWB224. The value of μ max was then determined using the technnique described in II.2(e) and a value of 0.23 h^{-1} recorded.

Experiments to further elucidate the differing growth kinetic limits of branched and non-branched mycelial cultures are suggested in the appendix. A complete explanation of the observed phenomenon, however, would entail an investigation into the metabolic events determining the attainment of each alternative type of hyphal morphology as a response to physical conditions. The conditions in question presumably affect the oxygen transfer r rate in batch cultures. In this context, it is also cognate to consider the observation that the particular type of hyphal morphology, attained in batch culture, will remain stable once continuous flow conditions are applied thence influencing the growth rate range of the culture.

(e) <u>Minimum growth rate (μmin)</u>

In Figure 4, it was it was indicated that the culture obtained at a dilution rate of 0.02 h^{-1} consisted of a differentiating mycelium. This was composed of spores as well as veget-

PLATE 3.



Appearance of culture at D = 0.02 h^{-1}.
The fluorescent brightener (II.2.c) shows regions of differentiation.

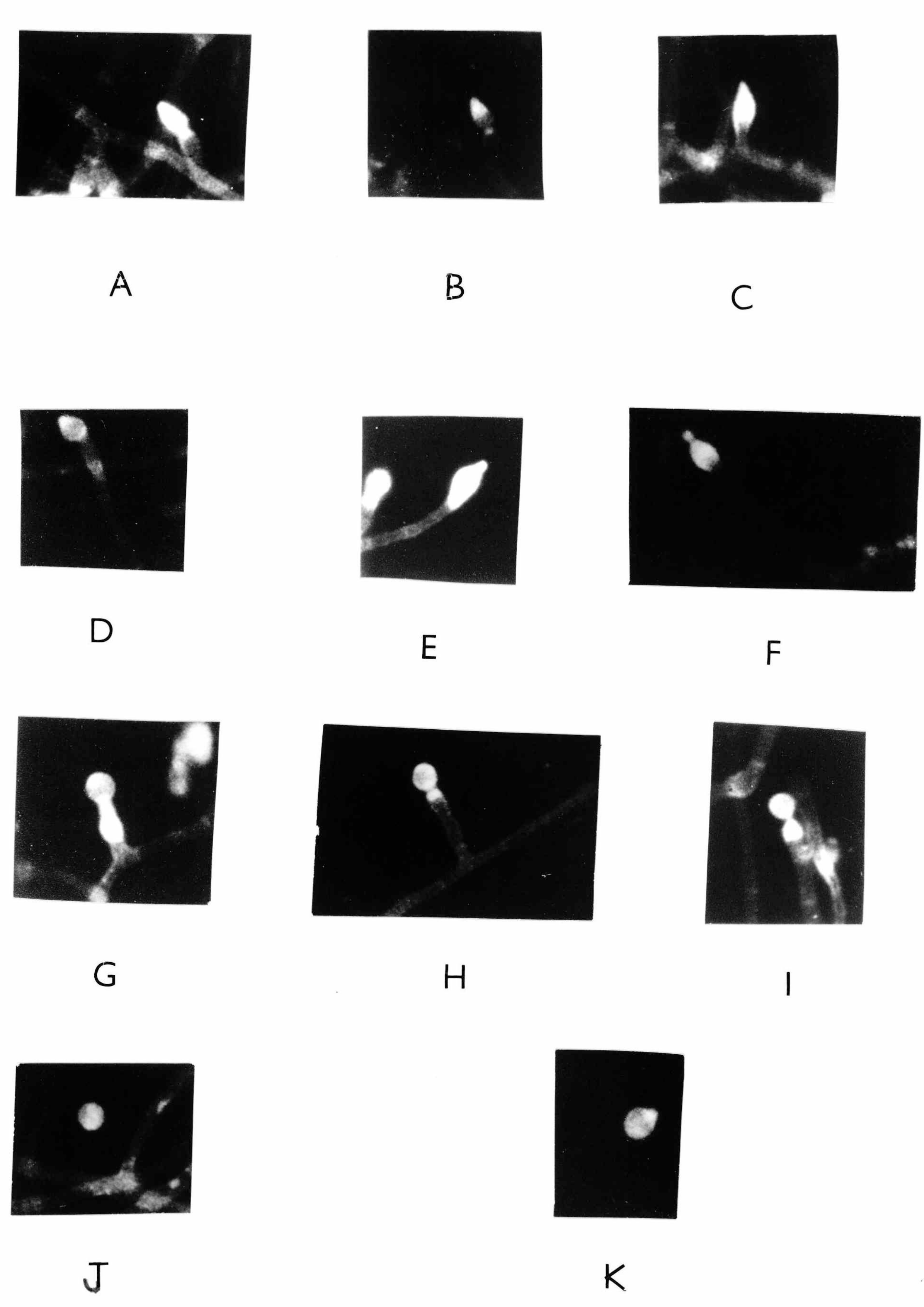
Magnification ; x 700

ative hyphae. Plate 3 shows the appearance of the culture under these conditions. The sample for observation was photographed in ultra-violet light using the technique described in II.2(c). This procedure, which results in the binding of the fluorescent brightner to β-linked polysaccharides in the cell wall gives an indication of the extent of differentiation in the mycelium. Regions of hyphae undergoing differentiation are areas where the formation of β-linkage is taking place at a higher rate than other parts of the mycelium. Areas of differentiation are thus selectively labelled by the fluorescent brightner.

Plate 4 shows the morphological events which appear to be taking place in a repeating sequence at a dilution rate of 0.02 h^{-1}. In the turbulent conditions of the fermentation vessel, the normal conidiophore is not formed and the conidia are borne singly on phialides at the hyphal tips. On the left of Fig.4a a short hyphal branch can be seen with fluorescence at the tip, indicating the onset of differentiation. Figures 4b-4d show the development of the phialide and, in Figure 4e, the spore precursor can be seen at the phialide tip. The development of the spore proceeds in Figures 4f-4i, ending in the release of the mature conidium (4j). In Figure 4k, localised fluorescence in the spore wall can be seen, indicating the imminent emergence of the germ tube. The germ tube would then itself differentiate, producing a repetition of the cycle of events.

The continuous cycle of morphogenesis observed at 0.02 h^{-1} may be compared with observations of Anderson & Smith (1971) that normal conidiophores were produced from newly-germinated

PLATE 4 - Morphogenesis at a steady state dilution rate of $0.02\ h^{-1}$



Magnification ; x 2000

conidia in submerged cultures of *Aspergillus niger*. When grown at an elevated temperature, conidiophores were produced directly from spores, in the absence of vegetative growth.

The continuous, stable rate of spore production at 0.02 h^{-1} suggests the existence of a minimum growth rate, as described in II.1(c), with a value slightly higher than 0.02 h^{-1}, i.e. in excess of 10% of μmax. A minimum growth rate equivalent to 5% of μmax was found in continuous cultures of *Penicillium chrysogenum* (Rhigelato et al, 1969) but no differentiation into spores was observed in *A.nidulans* strain BWB224 (Carter & Bull, 1969) at low dilution rates.

The concept of a minimum growth rate will be discussed in metabolic terms in the light of results obtained in Section III.

(f) Effect of dilution rate on glucose uptake.

Figure 7 shows the variation in steady state residual glucose concentration $\bar{s}$ with dilution rate. To analyse the validity of the Monod model for substrate utilisation by a filamentous mould, the experimental results were plotted in an alternative form. If the Monod model is applicable, then equation (11) may be assumed to provide an adequate algebraic description of growth-rate dependent substrate utilisation.

Rearranging (11);

$$1/_{\mu} = 1/_{s} + Ks/_{\mu max} + 1/_{\mu max} \tag{36}$$

which is of form y = mx + c.

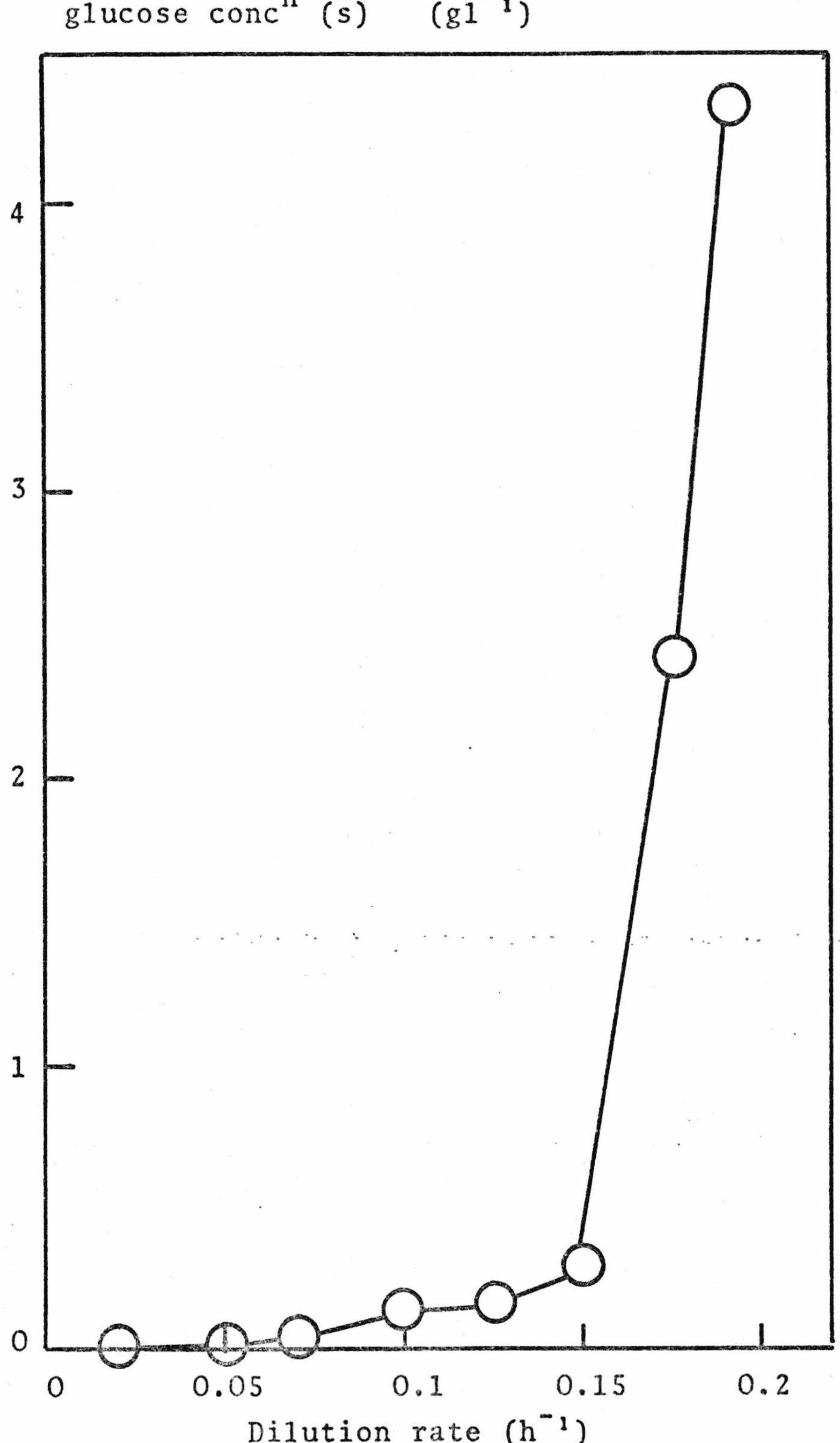
A double reciprocal plot of $1/_{\mu}$ against $1/_{s}$ for *Aspergillus nidulans* (13 mel) is shown in Figure 8. From (11), when:

$$1/_{\mu} = 0,$$

$$1/_{s} = -1/_{Ks}$$

Figure 7.

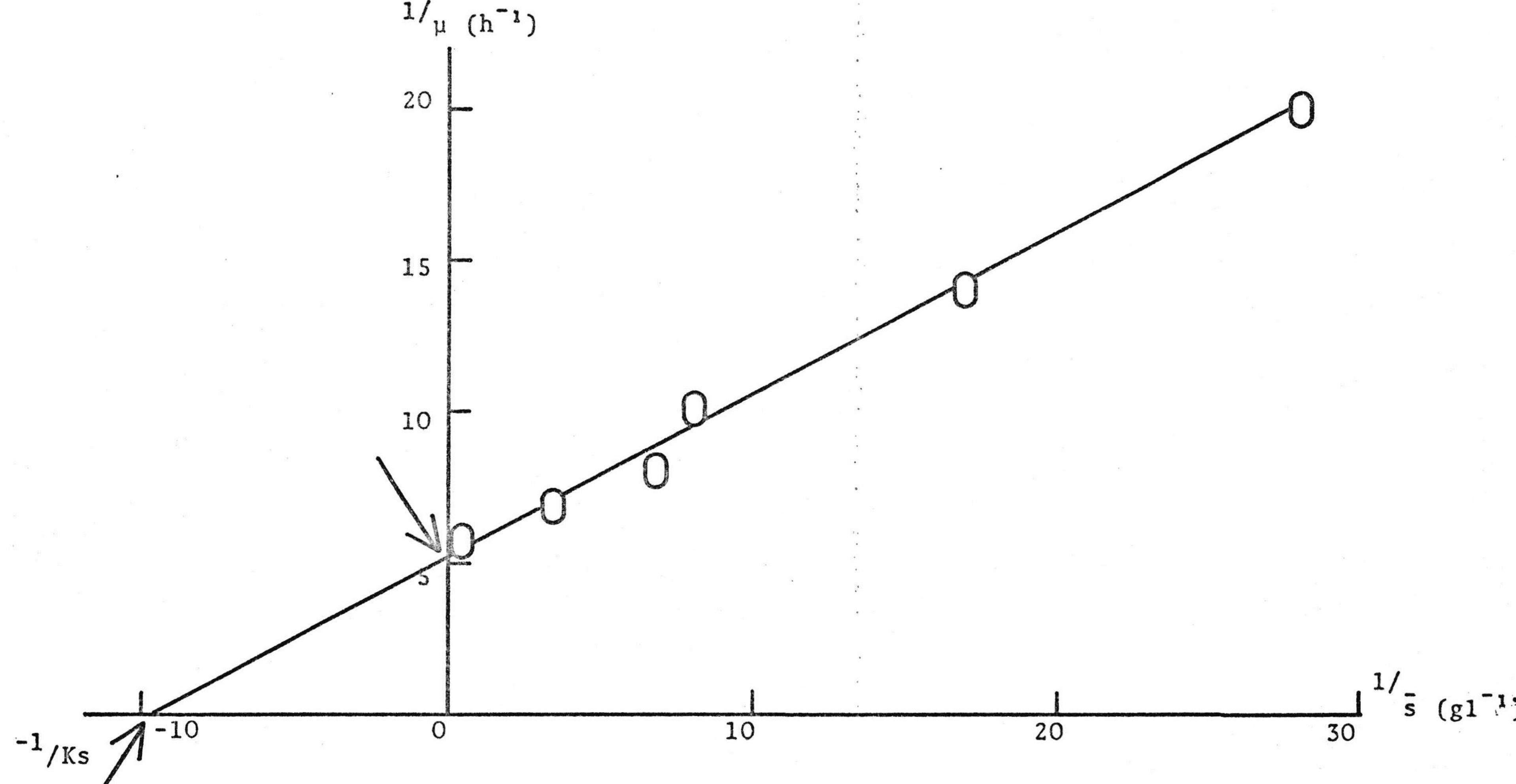
Effect of dilution rate upon steady state glucose concentration.



glucose conc^n ($\bar{s}$) (gl^{-1})
4
3
2
1
0
0
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})

Figure 8.

Double reciprocal plot of growth rate and steady state glucose concentration.



1/μ (h⁻¹)
20
15
10
5
1/s̄ (gl⁻¹)
-1/Ks
-10
0
10
20
30

The value of Ks from Figure 8 is 0.11 g.glucose 1^{-1}. The value of μmax, 0.19 h^{-1}, obtained from equation (36) using the value from Figure 8, is within ±0.07% of the observed value of 0.205 h^{-1}. These results appear to confirm the validity of the Monod model for the continuous culture of a filamentous mould.

Figure 9 shows the variation of $q_{glucose}$ (defined in equation 30) with dilution rate. The maintenance coefficient given by the intercept of the q axis is 0.0194 g.glucose $biomass^{-1}$ h^{-1}. This value is in close agreement with those derived for *Aspergillus nidulans* (strain BWB224; (Carter <u>et al</u> 1971) 0.018 $g.g^{-1}$ h^{-1} and *Penicillium chrysogenum* (Righelato <u>et al</u> 1968) 0.022 $g.g^{-1}$ h^{-1} which were both derived from glucose limited chemostat cultures growing at $30^{o}C$. The discontinuous linear relationship between q and D observed by Carter <u>et al</u> (1971) was not observed with strain 13 mel. This disparity between the two strains will be discussed in Section II.3(g).

(g) <u>The effect of growth rate upon respiratory parameters.</u>

(i) <u>Specific rates of oxygen uptake (Q_{O_2}) and carbon dioxide evolution (Q_{CO_2}).</u>

Figure 10 shows that the steady state uptake rates and rates of carbon dioxide evolution are dependent on the dilution rate; Q_{O_2} exhibiting a linear relationship with dilution rate. An oxygen maintenance coefficient of 0.13 $mmol.g^{-1}biomass$ h^{-1} was calculated from these data. This value is considerably lower than that observed for strain BWB224 (Carter <u>et al</u>, 1971) i.e. 0.55 $mmol.g^{-1}$ h^{-1}. As previously stated, the experimental conditions used by Carter <u>et al</u> were such that the culture had

Figure 9.

Effect of dilution rate upon the specific
rate of glucose utilisation

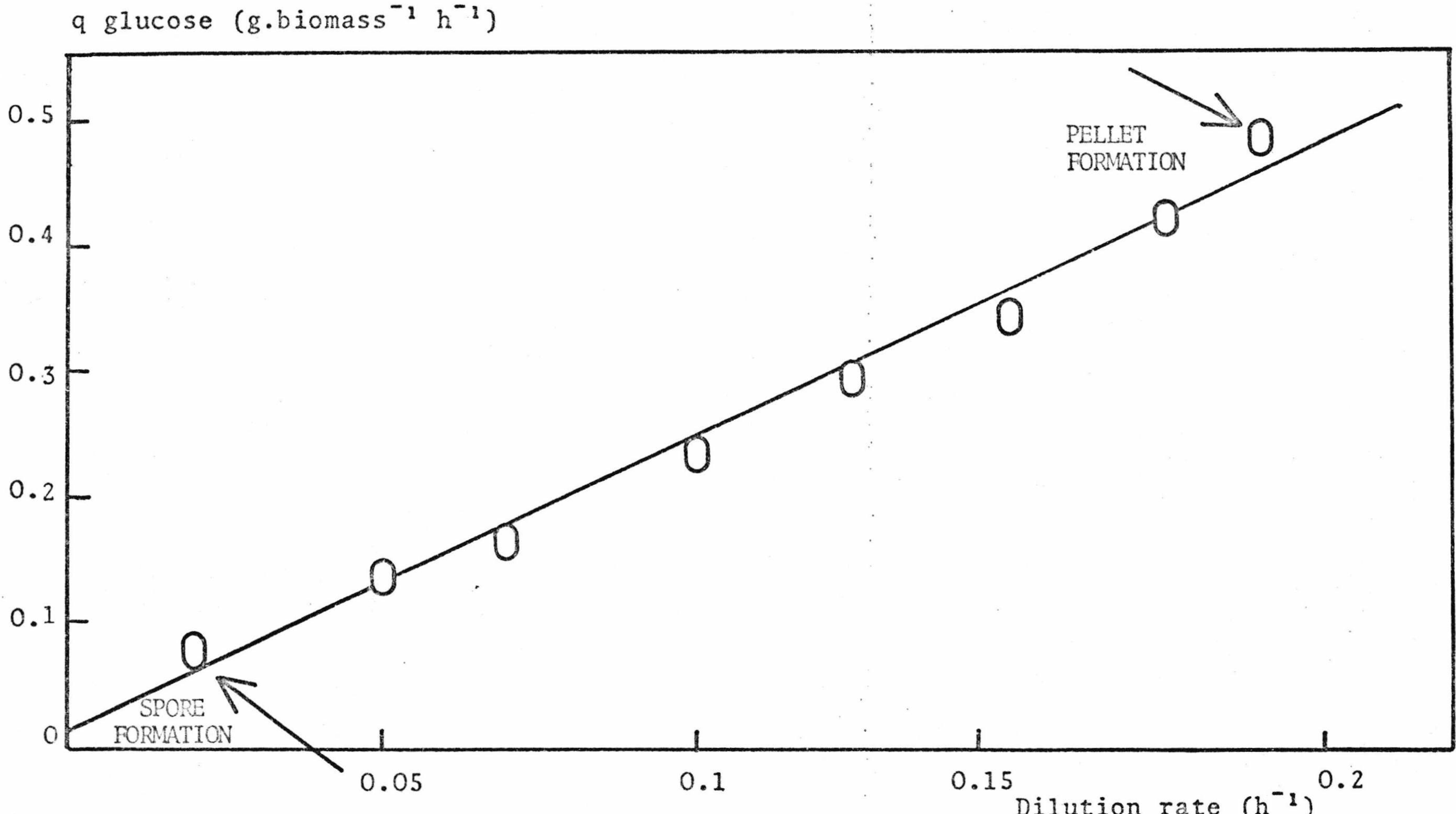
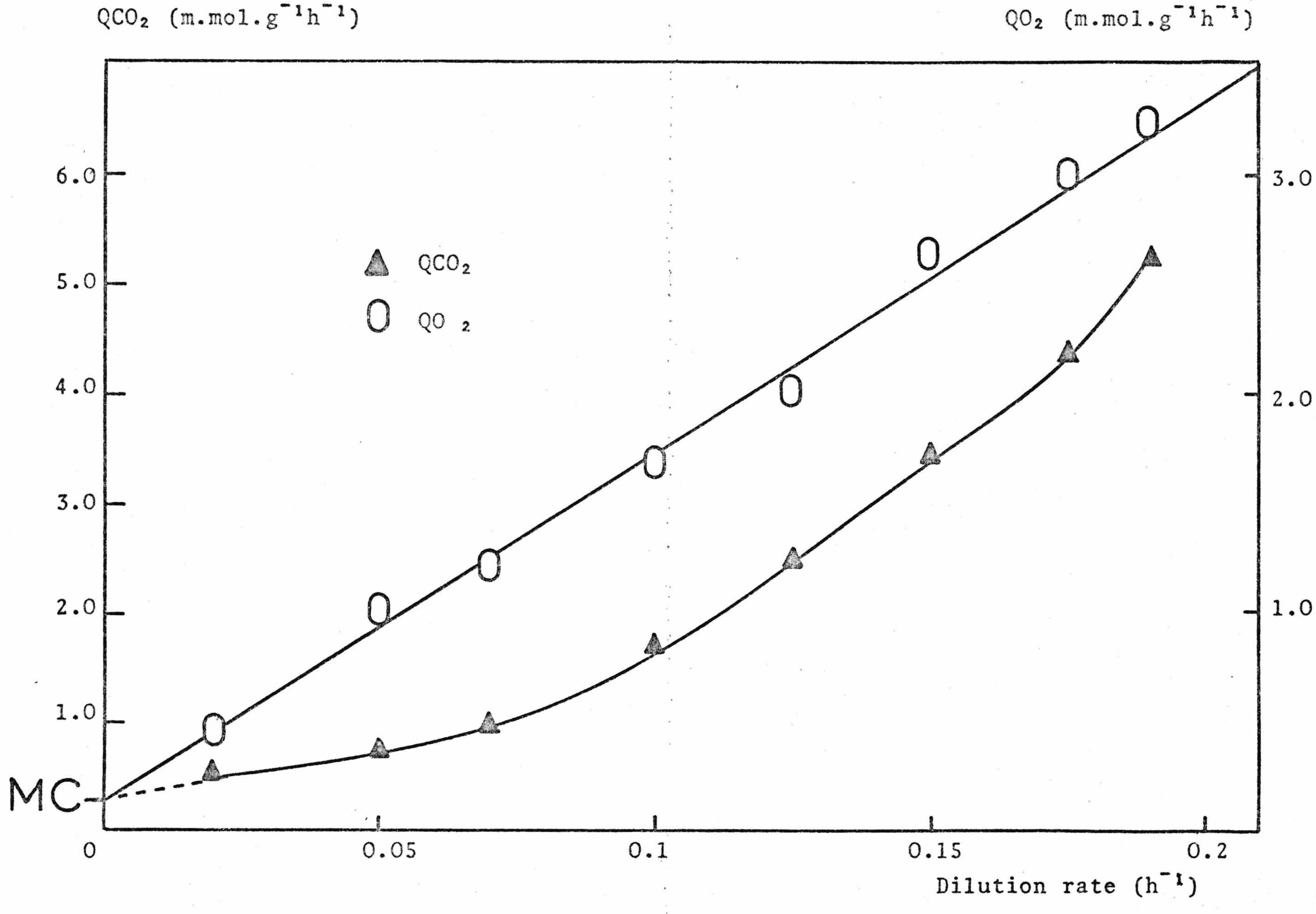

q glucose (g.biomass^{-1} h^{-1})
0.5
0.4
0.3
0.2
0.1
0
PELLET
FORMATION
SPORE
FORMATION
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})

Figure 10.

Effect of dilution rate on respiratory parameters.



QCO_2 (m.mol.g^{-1}h^{-1})
QO_2 (m.mol.g^{-1}h^{-1})
6.0
5.0
4.0
3.0
2.0
1.0
MC
3.0
2.0
1.0
QCO_2
QO_2
0
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})

a Dcrit value of less than half μmax and the fungus was growi
with a highly branched morphology. Respiratory parameters we
therefore estimated using the non-branching cultures of strai
BWB224, described in II.3(d), in which Dcrit and μmax were
0.23 h^{-1} and 0.23 h^{-1} respectively. An oxygen maintenance
coefficient of 0.24 $mmol.g^{-1}$ h^{-1} was calculated from data
derived from oxygen uptake measurements in steady states of
0.07, 0.1, 0.125, 0.15 and 0.175 h^{-1}. (Figure 10).

It would appear, therefore, that the oxygen maintenance
requirement for unbranched cultures (Dcrit 0.23 h^{-1}) is consi
erably lower than that of branched cultures (Dcrit, 0.08 h^{-1})
of strain BWB224; the values for maintenance coefficient bein
0.55 and 0.24 $mmol.g^{-1}$ h^{-1}, respectively for branched and non
branched cultures. Strain 13 mel requires still less oxygen
for maintenance, i.e. 0.13 $mmol.g^{-1}$ h^{-1}. The difference betw
strain 13 mel and BWB224 could be explained by an increased
maintenance requirement for the production of the pigment,
melanin, by strain BWB224, which is absent from strain 13 mel

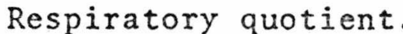
(ii) The respiratory quotient (RQ) and the energy yield (Y^{ATP}).

It was observed that the respiratory quotient varied betw
0.75 and 1.58 over the dilution rate range 0.02 h^{-1} to 0.19 h
(Figure 11) showing a gradual increase in RQ with dilution ra

Similar results were observed by Carter et al (1971) for
strain BWB224 in which RQ varied between 1.02 and 1.35 over t
range 0.025 h^{-1} to 0.075 h^{-1}. In the latter work this change
in RQ was accompanied by a progressive change in the value of
N in equation (32), i.e. the number of mol of ATP produced pe

Figure 11.

Effect of dilution rate on respiratory quotient


Respiratory quotient.
2.0
1.5
1.0
0.5
0
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})

mol of oxygen taken up. In contrast, the value of N, calculated for strain 13 mel, remained relatively constant at about 6.0 (see Table 5).

Apparently, the number of sites of oxidative phosphorylation operating in strain 13 mel is not a growth rate-dependent parameter. The observed change in RQ with growth rate is unlikely, therefore to reflect a qualitative change in glucose metabolism of the type observed with glucose limited chemostat cultures of strain BWB224 (Carter et al, 1971).

The increase in RQ as the dilution rate is raised suggests a progressive change to a more fermentative metabolism in both strains, which, as RQ is not a unitary parameter, is probably an additive effect, produced by the combination of a number of metabolic changes.

It is tempting to correlate the dissimilar responses in glucose metabolism with changing growth rate with observed differences in culture morphology in the two strains. If strain BWB224 achieves an increase in biomass by the formation of new branches, then its increased utilisation of the hexose monophosphate (HMP) pathway at growth rates near Dcrit (see Carter & Bull, 1969) may reflect an increased demand for *biosynthetic* energy in branch initiation. Under conditions of carbon limitation and a high dilution rate, glucose availability is low and there is a physical demand, imposed by the technique of chemostasis, for an increased growth rate; in this case, synonymous with an increased branching rate. This being the case, one could visualize a situation where the flow of glucose through the HMP pathway, and hence to biosynthesis, would become so great that glucose flow through the Embden-Myerhoff-

TABLE 5.

Values of N (number of sites of oxidative phosphorylation) for glucose-limited cultures of *Aspergillus nidulans.*

Strain BWB224

Dilution rate (h^{-1})	N
0.02	6.6
0.05	6.5
0.07	4.7

Strain 13mel

Dilution rate (h^{-1})	N
0.05	6.1
0.10	6.0
0.15	5.8
0.19	5.9

Parnas (EMP) pathway would not be sufficient to provide the essential energy pool derived from oxidative phosphorylation. This would account for the reduction in energy yield observed at higher growth rates. As the dilution rate is increased to the point where operation of the EMP pathway is insufficient, wash-out would result. In this case, the rate of energy-producing metabolism would not be sufficient to keep up with the increased rate of growth, imposed by chemostatic growth-rate control. Such a situation would not arise in batch culture, however, where glucose is always in excess.

(h) Transient states.

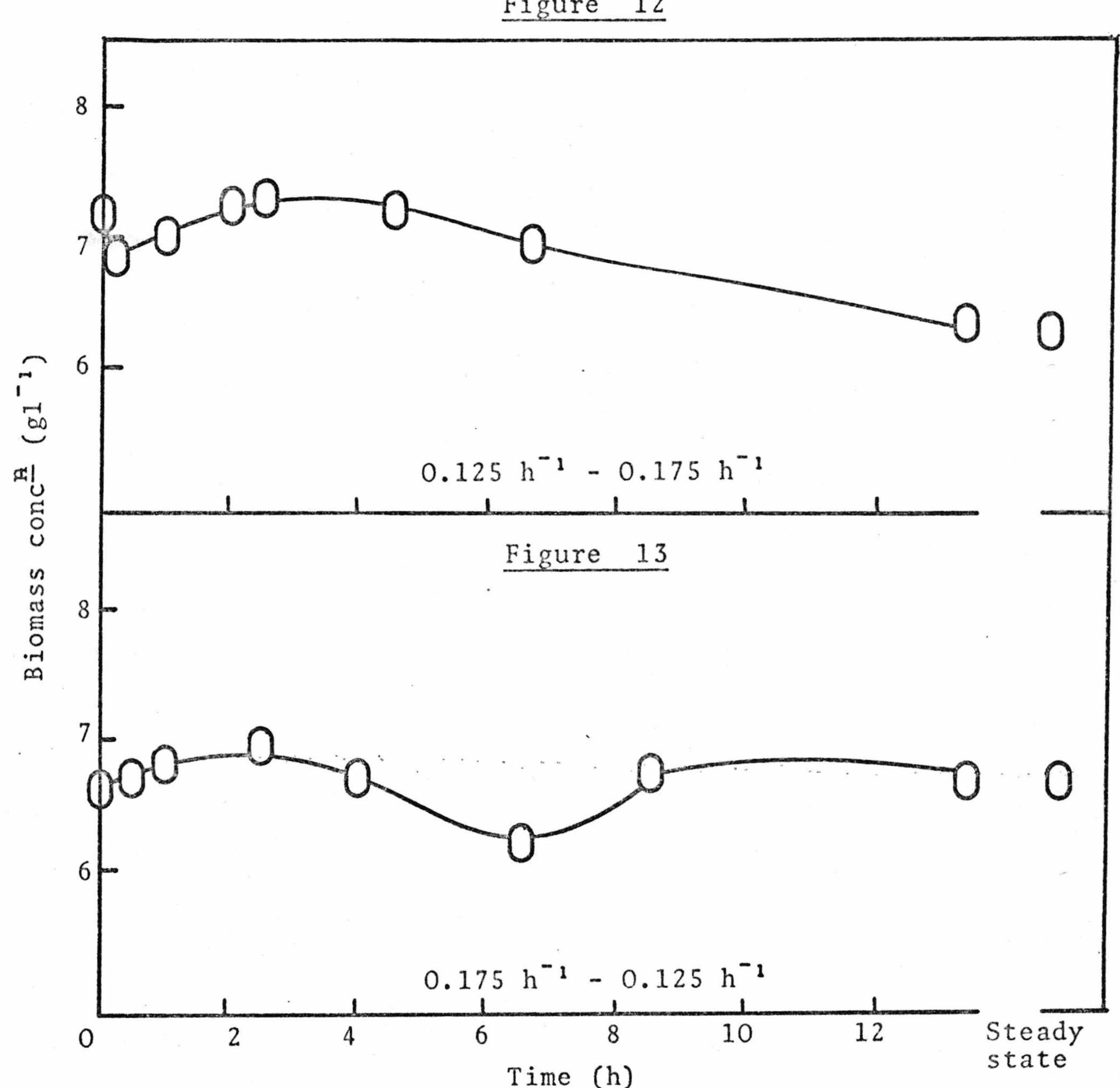
During transient states, oscillations in biomass were observed. Figure 12 shows the results obtained in the "step-up" experiment (0.125 h^{-1} - 0.175 h^{-1}). The dilution rate was changed to 0.175 h^{-1} at zero time on the graph. Similarly, Figure (13) shows the results of the "shift-down" experiment (0.175 h^{-1} to 0.125 h^{-1}).

Oscillations in fermentation parameters have been reported during transient states in many instances (see II.1(c)viii). The instantaneous responses to the changing conditions which are apparent in Figures (12) & (13) are also common observations (Herbert, 1962). Mor (1968) has commented that the rapid changes in growth rate reported for such experiments are indicative of the sensitivity of specific growth rate control mechanisms.

The oscillatory behaviour of cultures in a transient state has been explained by Sterkin, Chirkov & Samoylenko (1973) in terms of "biological inertia". A mathematical model was

Figures 12 and 13

Variation of Biomass with time during Step-up and Step-down Transient states



presented which resolves the growth rate, μ, of the culture into two components, one of which varies with time in a transient state. The other component varies according to an expression which simulate a time lag , resulting in an overall oscillation of specific growth rate which becomes progressively damped. The algebraic expressions involved, however, comprised 20 simultaneous equations, many of which had no apparent bioligical basis.

Young & Bungay (1973) used an analogue computer to "screen" a number of simulations for transient states. The components of the computer routine were varied until an out-put resembling the experimental data was obtained. These were then analysed and each component in the circuit was assigned a value as part of a biological variable. The growth rate, for example, was found to be modified by the functions G_T, GpM, and G_s; dynamic parameters brought about by changes in temperature, pH and substrate concentration.

These studies of Sterkin, Ghirkov & Samoylenko and Young & Bungay resulted in models which resembled the author's own experimental results. The oscillations obtained in both cases also resembled the overall pattern of results in transient states observed in the present study. The derivation of models to describe transient phenomena in *Aspergillus nidualns* using analogue techniques were, however, outside the scope of the present study.

SECTION III

THE BIOCHEMISTRY OF FUNGAL GROWTH.

1. INTRODUCTION

(a) Influence of growth rate on compositional biochemistry.

(b) The efficiency of protein synthesis during growth.

(i) Introduction

(ii) Ribosomal efficiency

(iii) Transient states

(iv) Summary

(c) Organic cations; the polyamines.

III. THE BIOCHEMISTRY OF FUNGAL GROWTH.

I. INTRODUCTION.

(a) Influence of growth rate on compositional biochemistry.

A study of the effect of growth rate on the macromolecular composition of micro-organisms was facilitated when reliable methods of chemostasis were developed. Herbert (1961b), observed that the proportions of intracellular macromolecules varied in response to changes in growth rate of carbon-limited continuous cultures of *Aerobacter aerogenes*. The percentages of protein and DNA changed very little with specific growth rate but both mean cell mass and the percentage of RNA increased markedly as growth rate was raised. Similar results were observed under nitrogen limitation, implying that the effect of growth rate on cell composition and mass is independent of the nutrient which limits growth.

Subsequent studies were made by Brown & Rose (1969) using carbon and nitrogen limited continuous cultures of *Candida utilis*, and Dalton & Postgate (1969) who studied similarly limited *Azotobacter chroococum* cultures. When these studies are compared, similarities in the patterns of results observed may be seen. The proportions of DNA and protein appear to remain relatively constant for any one organism, irrespective of growth rate or method of growth limitation. The amount of RNA, however, shows an increase with increasing growth rate. In contrast, the carbon-rich storage materials, polysaccharides and lipids, appear to be synthesised in greater quantities at low growth rates specifically under nitrogen limitation.

Results obtained with the protozoan *Astasia longa* (Morimoto & James, 1969) were somewhat different, however. Carbohydrate synthesis followed a pattern similar to those of the examples above and protein content was independent of growth rate. However, in contrast to the results of earlier analyses, the relative proportions of RNA and DNA decreased with increasing growth rate.

More recently, data describing the effect of changing growth rate (batch culture experiments) upon mould compositional biochemistry have been published. Griffen, Timberlake & Cheney (1974) found that the RNA content of *Achlya bisexualis* increased linearly with growth rate but its DNA and protein remained constant. These results are not in complete agreement with observations made by BuLock, Detroy, Hostalek & Munim-al-Shakarchi (1974). In a study of the regulation of secondary biosynthesis in *Gibberella fujikuroi*, BuLock et al, found that DNA, RNA and protein all increased to varying degrees as growth rate was increased.

When each type of macromolecule is considered in isolation, data from different organisms appears contradictory. It is only when differences in growth associated macromolecular synthesis are correlated with specific cellular functions that meaningful patterns of environmental effects on compositional biochemistry may be deduced.

(b) The Efficiency of protein synthesis during growth.

(i) Introduction.

Many attempts have been made to interpret the kinetics of microbial growth using alternative concepts to those described

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in Section II.1. The algebraic expressions describing growth such as those pertaining to the Monod model were, in many cases, originally derived to characterise the rates of chemical reactions. Therefore,a very attractive notion has been to analyse potentially rate-limiting events and to evolve mathematical models which can then be expanded to give descriptions of phenomena relating to overall rates of cell growth. A popular candidate for a rate-limiting biological process is the synthesis and action of ribonucleic acid both in micro-organisms (Edin & Broda, 1968; Maaløe & Kjeldgaard, 1966) and in animals (Temin, 1967; Todaro, Matsuya, Bloom, Robbins & Green, 1967). Because of the complexity of the molecular events involved, mathematical descriptions of protein synthesis at a fundamental level (usually in the form of esoteric computer programmes) are often unwieldy, inapplicable as general statements and contain concepts which are unfamiliar to the majority of other workers in this area of research. A situation has resulted in which many authors have published novel growth kinetic models which have never been referred to by other workers. Therefore, somewhat simpler models, relating protein synthesis dynamics to existing growth kinetics, should have a wider applicability and it is to such concepts that this discussion will be restricted.

(ii) Ribosomal efficiency.

Continuous cultures are ideal for the study of growth associated phenomena. However, a few experiments, described as "classics" in this field by many text-books, were carried out in batch culture. One such study is that of Maaløe & Kjeldgaard (1966) from which it was concluded that the efficiency of

Figure 14.

Possible lines to fit the data of Griffen *et al* 1974.

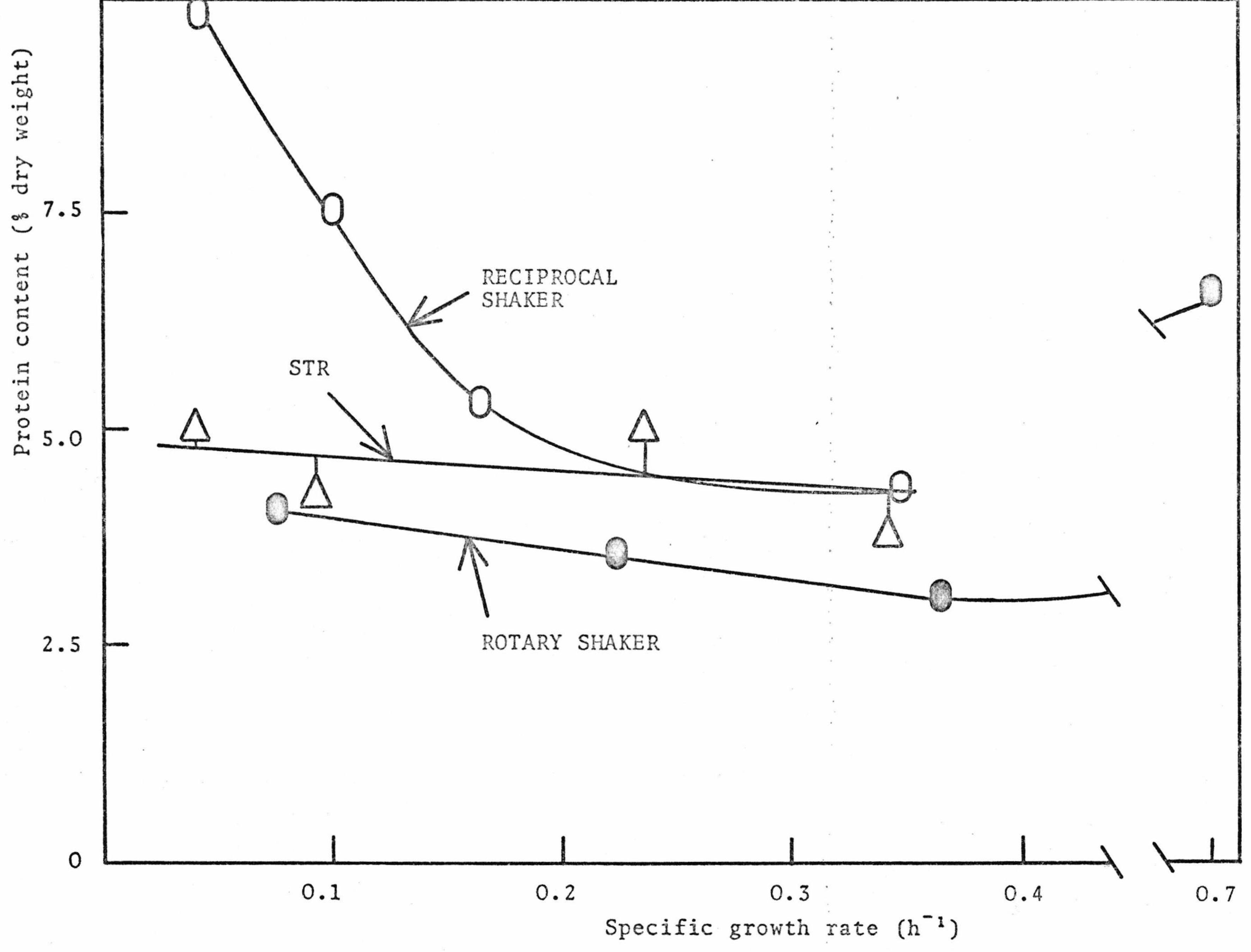
In the original publication, the authors stated that protein concentration remained constant as growth rate was varied.

KEY

O = reciprocally shaken

Δ = STR culture

● = rotary shaken



Protein content (% dry weight)
7.5
5.0
2.5
0
RECIPROCAL
SHAKER
STR
ROTARY SHAKER
0.1
0.2
0.3
0.4
0.7
Specific growth rate (h⁻¹)

ribosomes in protein synthesis remains constant over a range of culture growth rates. This notion was substantiated in a fungal system by Griffen, Timberlake & Cheyney (1974). Griffen et al grew cultures of *Achlya bisexualis* in reciprocally and rotary agitated flasks and in a fermenter vessel and concluded that the RNA concentration rose linearly with a growth rate, DNA and protein concentrations remaining constant. However, when the data are examined critically, a number of different trends in macromolecular concentration with growth rate are apparent, depending on the cultivation method used (Figure 14). As the value of μ is raised, protein concentration falls sharply in reciprocally shaken flask cultures, remains relatively constant in STR cultures and reaches a minimum at 0.4 h^{-1} in flasks which are agitated on a rotary shaker. Statistical analysis shows that the correlation between RNA concentration and growth rate is not satisfactory. Such criticisms are probably inevitable for an experimental system which seeks to provide comparability between batch cultures in which the organism is growing under a variety of different environmental conditions.

The highest growth rate obtained by Griffen et al was 0.815 h^{-1} which, the authors suggest, is the highest growth rate recorded for a eukaryote. Maynard-Smith (1969), in a discussion of the factors which limit microbial growth rate in enriched media, suggested that the requirements of protein synthesis determine the speed at which a culture can increase in biomass. He further concluded that the lower growth rates in eukaryotes compared to prokaryotes probably reflected the higher protein to RNA ratio found in the eukaryotes. This concept is supported by the results of Griffen et al who report a maximum protein concen-

tration of 6.5% for *A.bisexualis* with an RNA concentration of up to 10%.

Maynard-Smith (1969) also stated that limitations on growth rate occur when ribosomes and complexes between amino acids and transfer RNA reach limiting intracellular concentrations. In such a case, any further increase in levels of these components could actually slow down the rate of protein synthesis. Varricchio & Monier (1971), in studying the distribution of ribosomes, RNA subunits and polysomes in *E.coli*, found that the ratio of actively synthesising polysomes to DNA was proportional to the specific growth rate, whereas the concentration of RNA subunits decreased as the growth rate was raised. The authors concluded that the subunit pool acted as a reserve so that polysome concentration could be increased as a response to increased growth rate. This would result in an increase in the apparent efficiency of total RNA in protein synthesis as growth rate is increased.

Alroy & Tannenbaum (1973) have discussed a number of procedures for expressing the efficiency of protein synthesis from RNA in terms of growth rate. Thus,

$$E = \frac{P}{R} \times D \qquad (37)$$

where E is the ribosomal efficiency (units, g protein g RNA h^{-1}), P is the concentration of protein (%, w/w), R is the RNA conentration (%, w/w) and D is the culture dilution rate (h^{-1}). Results obtained with *Candida utilis* showed that ribosomal efficiency increased linearly with growth rate until it reaches a maximum at approximately 80% of μmax. More recently, Bu'Lock <u>et al</u> (1974) working with *Gibberella fujikuroi* and Solomons

(Solomons, G.L., 1973, personal communication) working with Fusarium species, have obtained similar results. Alroy & Tannenbaum suggested a means by which various micro-organisms, growing at different temperatures and growth rates, may be compared with respect to protein synthesis efficiency. The slope of the graph in which the ratio R/P was plotted against the "modified specific growth rate" was suggested as a "universal constant" - i.e.

$$\frac{R/P}{\left(\frac{\mu}{\mu max}\right)_T \left(\mu max\right)_{30}} = \text{a constant for all micro-organisms.}$$

where $\left(\frac{\mu}{\mu max}\right)_T$ is the ratio of specific growth rate to maximum specific growth rate at temperature T and $\left(\mu max\right)_{30}$ is the maximum specific growth rate at 30°C. Data from 15 different sources was plotted in this way and good linearity was obtained. From the graph produced, a ratio of 0.533 was obtained. Studying the dynamics of protein synthesis at the molecular level, Forchhammer & Lindahl (1971) found that the rate of polypeptide chain elongation on the ribosome increased linearly with growth rate at all values of μ below 40% μmax. At higher growth rates the velocity of chain elongation remained constant. It was concluded that protein synthesis efficiency changed with growth rate in two ways, as a result of a change in the number of polysomes and an alteration in the speed of amino acid incorporation into macromolecules.

Experiments by Muira, Tsuchiya, Nishikawa Obata & Okasaki (1974) provided further evidence that growth rate is controlled by cellular ribosomal RNA levels. Muira <u>et al</u> considered the

rate of protein production per unit cell mass per hour:

$$\left(\frac{p}{x}\right).D \quad = \quad \frac{1}{x} \cdot \frac{dp}{dt} \qquad (38)$$

which, in equation (38), is equal to the specific rate of protein synthesis. This was found to vary as a linear function of ribosomal RNA. When steady state ribosomal RNA concentrations were plotted against dilution rate, a straight line was obtained which gave a positive intercept on the d(rRNA) axis. This was found to be the same as the concentration observed in the stationary phase of batch culture and was taken by the authors to represent the minimum concentration of ribosomal RNA required for growth. An expression to describe cell growth in continuous culture was derived thus:

$$1/_x \ dx/_{dt} \quad = \quad k\left(\frac{R}{x} - \left(\frac{R}{x}\right)_c\right) \qquad (39)$$

where k is a constant (units, h^{-1}) and $\left(R/_x\right)_c$ is the minimum concentration of ribosomal RNA required for growth. This alternative for the Monod continuous culture model will only apply when the specific rate of synthesis of ribosomal RNA is constant at all growth rates. Therefore it would not apply to an organism which undergoes growth associated physiological differentiation.

Mirkes (1974) analysed protein synthesis in a number of germinating fungal spores. Several species were found to have functional polysomes present in their spores and thus were capable of protein synthesis upon germination. Polysomes were not detected in other genera, however, notably in species of *Aspergillus* and *Neurospora*. Mirkes concluded that, during sporulation induced by carbon source depletion, protein synthesis

is completely shut down in these organisms.

(iii) Transient states.

To elucidate the role of RNA and protein levels in determining growth rate, protein synthesis during transient state growth has been much studied. Maaløe & Kjeldgaard (1969) and Koch & Deppe (1971) found that RNA levels increased immediately when culture growth rate was raised by the addition of a nutrient-rich medium to continuous (low dilution rate) and batch cultures. Koch & Deppe also found that protein synthesis efficiency gave an immediate positive response to "shift-up". In an essay on the adaptive responses of *Escherichia coli* to the sporadic nutrient input encountered in the lower intestine, Koch (1971) stated that an evolutionary pressure has existed for enteric organisms to acquire the facility of rapid metabolic response to a changing nutrient environment. Similar observations to those of Koch and Maaløe & Kjeldgaard have been reported by Muira et al (1974) in "shift-up" experiments with *Bacillus subtilis*. A confirmation of Koch's hypothesis that excess protein synthesising machinery exists in slowly growing cultures has been produced by Varracchio & Monier (1971), Harvey (1973) and Mateles, Ryn & Yoshida (1965). In each case, the RNA subunit pool has been shown to undergo an abrupt decrease in response to a shift-up which is concurrent with a corresponding increase in the number of functional polysomes. This observation is in apparent conflict with results obtained by Young & Bungay (1973) who reported that ribosomal efficiency did not increase immediately after shift-up but did so after a measurable time lag. This discrepancy may be easily resolved if the response of

ribosomal efficiency to growth rate (as opposed to dilution rate) is followed (see III.3b vi).

(iv) Summary

(1) Microbial growth rates may be limited by the concentration of cellular RNA components.

(2) The RNA subunit pool decreases with increasing growth rate until all subunits have been converted to functional polysomes.

(3) Statement (2) may explain the apparent increase in ribosome efficiency with growth rate which reaches a maximum at or before μmax.

(4) An increase in the velocity of polypeptide chain elongation at the ribosome may also contribute to growth associated increase in protein synthesis efficiency.

(5) Transient responses to nutritional "shift-up" appear to confirm the existence of excess protein synthesising machinery at low growth rates.

(6) In some fungi, protein synthesis efficiency may become zero when sporulation is induced by carbon source depletion.

(c) Organic Cations; the polyamines.

The first published reference to the polyamines was, traditionally, made by Leeuwenhock (1678) in a letter to the Royal Society in 1677, describing crystals in semen which were most certainly the insoluble phosphate salt of spermine. Brief

references to spermine and spermidine may be traced throughout the 17th, 18th and 19th centuries. The first important observations, however, were made by Dudley, Rosenheim & Rosenheim (1924) and Dudley, Rosenheim & Starling (1927) who isolated and chemically characterised spermine and spermidine. The chemistry of the polyamines and their structural relationship to the diamine, putrescine, provided the first insight into the possible metabolic origins and functions of these bases:

$$NH_2(CH_2)_4.NH_2$$
Putrescine

$$NH_2(CH_2)_3NH(CH_2)_4NH_2$$
Spermidine

$$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$$
Spermine

The discoveries that polyamines were growth factors for the bacteria *Heamophilus parainfluenzae*, *Neisseria spp.*, and *Pasteurella tularensis* (Herbst & Snell, 1949; Martin, Pelczar & Hansen 1952; Traub, Mager & Grossowicz, 1955), the mould *Aspergillus nidulans* (Sneath, 1955) and certain tissue cultures (Ham, 1964; Bertossi, Bagni, Moruzzi & Caldavera, 1965) provided the stimulus for increased research upon these metabolites.

Interest in polyamines became more widespread when it was realised that strong associations exist between organic cations and nucleic acids. Polyamines were identified in T_2 bacteriophage particles (Hershey, 1957) and the association of spermine with DNA was characterised as a strong ionic interaction between the negatively-charged nucleic acid phosphate and the

positively charged base (Felsenfeld & Huang, 1961). Spermine, spermidine and putrescine have been shown to stabilize native DNA against thermal denaturation (Tabor, 1962; Mahler & Mehrotra, 1963) and X-ray analysis of the crystal structure of spermine hydrochloride has produced evidence for the molecular structures of the stabilised complexes (Liquori, Constantino, Crescenzi, Elia Giglo & Pulita, 1967). The conformation of the protonated polyamines suggested a steriospecific complex with ionised phosphate groups between pairs of nucleotide units in the narrow groove of helical DNA.

The stabilization of functional ribosome structures by polyamines has also been demonstrated (Cohen & Lichtenstein, 1960; Colbourn, Witherspoon & Herbst, 1961) and experiments describing the stimulation by polyamines of amino acid incorporation into polypeptides (Hershko, Amoz & Mager, 1961; Martin & Ames, 1962) and nucleotide incorporation into RNA (Fuchs, Millette, Zillig & Walter, 1967) have provided evidence for a broader participation of the organic cations in the biosynthesis and metabolism of proteins and nucleic acids.

The concept that spermine and spermidine are functionally interchangeable with magnesium has been suggested by many authors in *Escherichia coli* (Hurwitz & Rosano, 1967); *Pseudomonas*, Hurwitz, 1969); and phage MS2 (Takeda, 1969). During studies with the mould *Neurospora crassa*, Viotti, Bagni, Sturani & Alberghina (1971) observed enhanced synthesis of polyamines during magnesium starvation, RNA remaining undegraded until the cellular concentration of polyamines declined.

2. MATERIALS & METHODS.

(a) Analytical procedures, a discussion

- (i) Sampling
- (ii) Washing
- (iii) Storage
- (iv) The present technique for preparation of samples for chemical analysis.
- (v) Treatment of samples containing spores
- (vi) Extraction techniques
- (vii) Choice and standardisation of analytical procedures.

(b) Magnesium, polyamine and RNA determination

- (i) Extraction procedure
- (ii) Quantitative determinations
  1. Polyamines
  2. RNA
  3. Magnesium

(c) Protein determination

- (i) Extraction
- (ii) Quantitative determination

(d) Amino acid pool determination

(e) DNA determination

(f) Carbohydrate determination

(g) Lipid determination

(h) Determination of the percentage of hyphal wall in the mycelium.

(i) Whole mycelial ash determination

(j) Calculation of ribosomal efficiency in transient states.

2. MATERIALS & METHODS.

(a) Analytical procedures, a discussion.

(i) Sampling.

When material from a number of different steady states is to be compared by chemical analysis, the analytical procedures should, ideally, all be carried out at the same time using the same batch of reagents. It is, therefore, necessary to devise a sampling technique to effect the transfer of actively growing mycelia to a state suitable for storage, without altering their chemical composition. As a general principle, the method of preparing microorganisms for analysis depends upon the constituent to be determined and whether interfering substances are present in the suspending medium. In the present study, however, a number of different determinations were carried out on material from each steady state and there was a need to develop sampling techniques which would allow the detection and quantification of a number of different intracellular constituents.

Microbial cultures may be harvested by either filtration or centrifugation, the choice being determined by the amount of material to be processed and the equipment available. Speed is also important as changes in cell composition may occur during harvesting due to turnover of macromolecules and leakage of endocellular pool constituents. The amount of alteration will increase as the treatment time is extended (Strange, Dark & Ness 1961).

Tests carried out in the present study showed that the initial removal of mycelium (500ml culture volume) from the growth medium by centrifugation (800g for 10 min) could be accomplished in a minimum of 13 minutes. The same operation was carried out in less than 10 seconds using a filtration method.

(ii) Washing.

Once the mycelium have been separated from the growth medium they must be washed to remove all traces of nutrients and possible interfering substances in the medium. The use of cold washing materials decreases metabolic activity but may induce "cold shock" in rapidly growing populations, leading to leakage of soluble intracellular pool constituents (e.g. nucleic acid precursors, amino acids and ATP) (Strange & Dark, 1962).

The concentration of some endocellular materials depends on the concentration of these constituents in the environment. Washing microorganisms in solutions with differing composition to that of the growth medium may result in changes in the K^+, Na^+ and Mg^{2+}contents of the organisms (Tempest & Strange, 1966). This situation was monitored by testing buffer solution washings of *Aspergillus nidulans* mycelia for the presence of the constituents to be determined.

(iii) Storage.

Three methods of storage of microorganisms prior to chemical analysis are in common use. Harvested suspensions may be kept in the cold (1-5°C) or be frozen or freezedried. Washed suspensions of many microorganisms remain stable for several days at 1-5°C (Herbert, Phipps & Strange, 1971) although some leakage of endocellular material often occurs. Storage of cells in the frozen state prevents endogeneous metabolism and growth. However, lysis may occur during subsequent thawing leading to increased enzyme activity in the lysate. This may result in macromolecular degradation, particularly in the case of RNA (Herbert et al, 1971). The best method of storing microorganisms is in the freezedried.

state. Portions of the resulting material may then be weighed out for analysis and treated directly with the appropriate reagents.

(iv) <u>The present technique for preparation of samples for chemical analysis.</u>

With the culture in a steady state, as defined previously, 500ml samples were removed from the fermenter vessel. The tap on the sampling port was opened and a stream of culture run straight onto a sintered glass filter (porosity No. 2) of 15cm diameter. The filter was fitted with a Buchner flask connected to a high vacuum so that medium was removed as soon as the stream of culture hit the filter. When approximately 500ml had been removed from the culture vessel (indicated by graduations on the side of the vessel) the sampling port was closed and 500ml of 0.11M phosphate-buffered saline (pH 6.8) at 5°C was poured over the mycelium which had formed on top of the filter. When the buffer solution had been removed by means of the vacuum, the mycelium was collected and resuspended in 500ml of fresh buffer. The suspension was then transferred to a clean filter and the mycelium collected and freezedried. Using this procedure, less than two minutes elapsed between the removal of actively growing material from the fermenter and the transfer of washed mycelium to a freezing chamber at -20°C, prior to freeze-drying.

(v) <u>Treatment of samples containing spores.</u>

When the culture to be analysed contains spores the technique described in (iv) is unsuitable as the spores may clog or pass through the filter. The volumes and organism densities employed in this study dictated that membrane filtration techniques would be impractical. Solid material was therefore collected from differentiating cultures by centrifugation (1100g for 10 min.).

(vi) Extraction techniques.

Most of the published techniques for the extraction of endocellular materials from microorganisms were originally devised for analyses of bacteria. Many of these procedures are not suitable for fungi whose hyphal walls tend to confer greater resistance to chemical and mechanical disruption techniques. It is also noteworthy that an extraction technique which has been found to be satisfactory for a particular fungal species may not necessarily be universally applicable to moulds. Novacs, Martinez & Villaneuva (1967) found that different methods of disintegration were necessary for each of three species of Phycomycete which they studied.

The majority of the chemical analyses made in the present work were on material which had been treated chemically and mechanically. Time course experiments were carried out to determine the optimum treatment time for each constituent to be analysed.

Chemical treatment was carried out using various concentrations of perchloric acid, the concentration depending upon the nature of the analytical procedure. Mechanical disruption was effected by means of a motor-driven Teflon-glass homogeniser (Jencons Ltd., Hemel Hempstead) or using a Braun Tissue Disintegrator (B. Braun, Melsungen Apparatebau, Melsungen, Germany). The Teflon-glass homogeniser consisted of a Teflon pestle which could rotate inside a closely fitting tube. Mycelial suspension was forced between the rotating pestle and the walls of the glass tube, resulting in mild shearing of the hyphae into short lengths. The Braun homogeniser provided an alternative means of hyphal disruption using Ballotini (1.0mm) beads. Suspensions

were shaken in a glass bottle (capacity 65ml) placed horizontally in a chamber connected to an eccentric cam. The chamber was shaken at 2000-4000 oscillations per minute. A stream of liquid CO_2 delivered to the chamber cooled the assembly. Distintegration (100%) was rapid (3min) and it has been reported (Huff, Oxley & Silverman, 1964) that the temperature remains below $4^{o}C$ in the chamber under working conditions.

(vii) Choice and standardisation of analytical procedures.

In many cases, a number of different procedures are available for the determination of each cell constituent. When faced with a choice, the criteria of sensitivity, precision and cost of materials for each test must be assessed. In many cases, in the present work, a number of different procedures were tried for each constituent analysed. As a rule, however, only the most successful method is reported here. The comprehensive review of Herbert, Phipps & Strange (1971) on the chemical analysis of microbial cells was consulted, in the first instance, when selecting suitable analytical procedures.

The results presented are, in general, relative rather than absolute. Standardisation of the data observed for *Aspergillus nidulans* (13mel) was carried out against commercially obtained preparations of the cell constituents analysed. "Authentic" samples of the materials assayed were not available from *Aspergillus nidulans*.

(viii) Hyphal walls; criteria of purity.

When discussing growth associated variations in hyphal wall composition, a definition of the criteria of purity should be made.

The main contaminants likely to be present in wall preparations are cytoplasmic and debris from external sources (Work, 1971).

Examination by light microscopy gives an indication of the number of unprocessed cells in a wall preparation but provides little information regarding its purity. Microscopic examination of wall preparations stained with Trypan blue solution may give some indication of the extent of contamination with cytoplasmic debris, however. The presence of nucleic acids in wall preparations may indicate cytoplasmic contamination. Examination of phosphate buffer washings from walls for absorption at 260nm has been used to determine the presence of nucleic acids (Barkulis & Jones, 1957).

The following two sections refer to procedures based on published methods and containing modifications made by the present author. These techniques are described in full. All other analytical procedures used are identical to published methods in all respects except, in some cases, the extraction procedure. These latter are only described briefly and the appropriate references and modifications given.

(b) Magnesium, polyamine and RNA determination.

(i) Extraction procedure.

Freezedried mycelia were macerated at $0^{o}C$ in 0.2N perchloric acid (5mg mycelium ml^{-1}) by a motor-driven Teflon-glass homogeniser (six passes at 75 rev/min for 10 s). Samples were left for 10 min and then centrifuged at 800g for 10 min.

(ii) Quantitative determinations

1. Polyamines.

Polyamines in the perchloric acid extracts were assayed by the fluorescence method of Dion & Herbst (1970). Supernatant

(1ml) was added to dansyl-chloride (BDH, Ltd.) solution (30 mg ml acetone^{-1} containing 1.85 sodium carbonate). After leaving for 16 h in the dark to allow dansylation to proceed, excess dansyl chloride was converted to dansyl proline by the addition of 1ml proline solution (100 mg ml^{-1}). Fluorescent dansyl derivatives were then collected by vigorous shaking with 2ml benzene and separated by thin-layer chromatography (TLC) plates (Eastman-Kodak Co.). After development in ethylacetate-cyclohexane mixture (2:3v/v) plates were sprayed with triethanolamine-isopropanol mixture (1:4v/v) to enhance and stabilize fluorescence. TLC plates were dried in vacuo for 16 h in the dark at room temperature, equilibrated at atmospheric pressure for 1 h and then scanned with a fluorescent lamp to locate the spots. The fluorescent dansyl derivatives were eluted by cutting out the spots and extracting the silica gel three times with the ethyl acetate-cyclohexane mixture. Their fluorescence was measured in a Perkin Elmer MPF3 fluorescence spectrometer (wave length of activating light 365 nm, slit 8; fluorescence measured at 520nm, slit 8).

2. RNA

Aliquots (4 ml) of the perchloric acid homogenate were heated at 70°C for 15 min. The cell debris was collected by centrifugation (800g for 10 min) and extracted with hot perchloric acid (following further homogenisation) twice more. The supernatants were then combined and assayed for RNA by the Schneider orcinol procedure (Herbert, Phipps & Strange, 1971). Aliquots (1ml) of perchloric acid extract were added to 3.0ml of orcinol reagent (0.09% $FeCl_3$ in concentrated HCl mixed with 1% aqueous

orcinol solution, 4:1 v/v). This was then heated at 100°C, cooled, and the volume of each sample made up to 10ml with n-butanol. Absorption at 672nm was determined in a Unicam SP600 spectrometer.

3. Magnesium.

The magnesium concentration of the perchloric acid extract was measured by the Titan-Yellow method (Herbert, Phipps & Strange, 1971). Aliquots (1ml) of extract were added to 0.2ml Cum Ghatti (0.2%) (BDH), 1ml 3N NaOH, 0.3ml Titan-Yellow solution (BDH) and 1ml water. After mixing and standing for exactly 5 min, the absorbance of the samples was measured at 540nm.

4. Standardisation of assays.

Spermidine, spermine (Sigma), yeast RNA (BDH) and magnesium chloride were used as reference compounds.

c. Protein determination.

(i) Freezedried mycelium (100μg) was macerated with 1ml 0.5N NaOH in a Teflon-glass homogeniser. The homogenate was then heated for 8 min in a boiling water bath.

(ii) Quantitative determination.

Protein was estimated using the method of Lowry et al (1951). To the NaOH extract was added 2.5ml of a mixture of 5% Na_2CO_3 and 0.5% $CuSO_4.5H_2O$ in 1% NaK tartrate (50:1 v/v). After standing for 10 min, 0.5ml diluted Folin-Ciocalteu reagent (BDH) was added. Absorbance at 750nm was read after allowing 30 min for colour development.

Bovine serum albumin (Sigma) was-used as a reference compound.

(d) Amino acid pool determination.

The procedure of Herbert, Phipps & Strange (1971) which is an adaptation of the method of Yemin & Cocking (1955) was followed

without modification. Alanine (Sigma) was used as a reference material.

(e) DNA determination.

The extraction procedure described for RNA was employed. DNA in the extracts was assayed using the method of Burton (1956) Calf thymus DNA (Sigma) was used as a reference compound.

(f) Carbohydrate determination.

Freezedried mycelium was ground in a mortar and pestle to a fine powder. To determine total endocellular carbohydrates, the phenol method of Herbert, Phipps & Strange (1971) was followed without modification. Glucose was used as a reference material.

(g) Lipid determination.

The chloroform/methanol extraction procedure of Sutherland & Wilkinson (1971) was employed, preliminary extraction being carried out by homogenising 1g mycelium with 10ml chloroform and 20ml methanol in a Braun homogeniser at $0^{o}C$. The extracted lipid was dried *in vaccuo* at $50^{o}C$ for 24 h and then weighed.

(h) Determination of the percentage of hyphal wall in the mycelium.

Freezedried mycelium was re-suspended in Na_2HPO_4/KH_2PO_4 buffer (0.033 M. pH 6.1) at $0^{o}C$ and macerated in a Braun homogeniser for 6 min at $0-4^{o}C$. The contents of the chamber and the Ballotini beads were washed with buffer containing 0.1% Tween 80 into pre-weighed centrifuge tubes and the "wall fraction" collected by centrifugation (1400g for 10 min). The supernatant, which was turbid, was then discarded and the sediment washed thrice with distilled water. The pellets obtained were white. The centrifuge tubes were then placed in an oven at $105^{o}C$ and dried to a constant

weight. Purity of preparation was estimated, after the centrifugation step, using the techniques discussed in III.2a.

(i) Whole mycelial ash determination.

Weighed, freezedried mycelium was heated at 950°C for 3 h in a 'Hotspot' furnace (Gallenkamp Ltd.). The residue was weighed after equilibration at room temperature in a dessicator containing P_2O_5.

(j) Calculation of ribosomal efficiency in transient states.

The calculation of ribosomal efficiency as defined by Alroy & Tannenbaum (1971) requires a value for the specific growth rate, a quantity which varies continually in transition states. A polynomial expression for the variation of biomass with time in the transient state was obtained using the computer programme PLOFIT (see I. 2g). The equation thus derived was of the form

$$x = at^7 + bt^6 + ct^5 + dt^4 + et^3 + ft^2 + gt + h$$

where a-i are constants, output by the computer and x is the biomass (gl^{-1}) at time t (h) after changing the dilution rate. The growth rate μ which is equal to $\frac{dx}{dt} \cdot \frac{1}{x}$ was then calculated by differentiating the polynomial and multiplying througout at different values of t. Reuslts thus obtained were checked manually at intervals using the general equation for transient states;

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

which, on integration between t_o and t_t and reducing to natural log form gives;

$$\mu = \frac{(\ln x_t - \ln x_o}{t} + D$$

3. RESULTS & DISCUSSION.

(a) Variation of magnesium, polyamine and RNA concentration with growth rate.

- (i) RNA
- (ii) Polyamines
- (iii) Magnesium
- (iv) Inter-relationship between magnesium, polyamine and RNA levels.

(b) Effect of dilution rate on mycelial protein concentration.

- (i) Steady state protein concentration
- (ii) Effect of dilution rate on protein synthesis efficiency
- (iii) Role of polyamines in ribosomal efficiency
- (iv) The "universal" RNA to protein ratio
- (v) Transient states in ribosomal efficiency

(c) Effect of dilution rate on amino acid pool size

(d) Effect of dilution rate on mycelial DNA concentration

(e) Effect of dilution rate on mycelial "storage polymers"

- (i) Carbohydrate
- (ii) Lipid
- (iii) Energy requirement for carbohydrate and lipid synthesis

(f) Effect of dilution rate on mycelial wall content

(g) Accuracy of analysis procedures

3. RESULTS & DISCUSSION.

(a) Variation of magnesium, polyamine and RNA concentration with dilution rate.

(i) RNA

Mycelial concentrations of RNA as a function of growth rate are shown in Figure 15. The intracellular level expressed as a percentage of total dry weight, varied between 7% and 3% in continuous flow cultures. Over most of the growth rate range ($0.07-0.175\ h^{-1}$) however, the RNA concentration remained relatively constant, varying between 3% and 3.4%. These values are comparable with the concentration (3.75%) observed in batch culture. As the growth rate decreases below $0.07\ h^{-1}$ and presumably is approaching μmin, the concentration begins to rise but falls again when the culture differentiates(at $D=0.02h^{-1}$) into spores and vegetative mycelium.

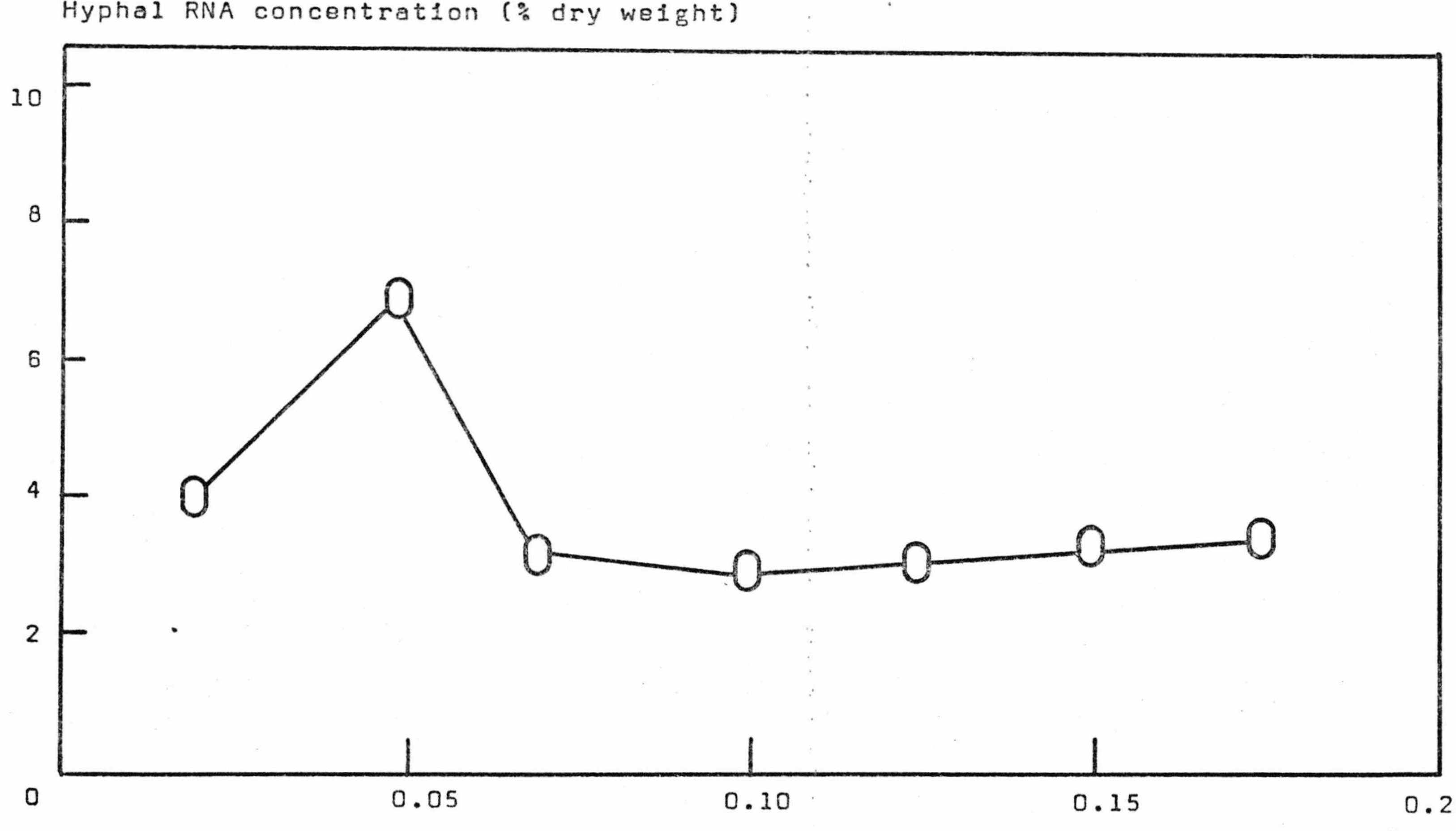
Bu'Lock, Detroy, Hostalek & Munim-al-Shakarchi (1974), when growing the mould *Gibberella fujikuroi* in a glycine-limited chemostat, observed a different pattern of RNA concentration. In this case, RNA concentration increased from 4.4% ($D=0.02h^{-1}$) to 8.2% ($D=0.12\ h^{-1}$); μmax was $0.18 \pm 0.02\ h^{-1}$). No morphological differentiation took place in these cultures, however.

Although the data of Bu'Lock *et al* and of the present author are dissimilar, a consideration of RNA in the context of its metabolic function, i.e. the synthesis of protein, reveals that the effect of changing growth rate on RNA synthesis and activity are comparable in the two organisms (see section 3(b) below).

Increases in RNA concentration with increasing growth rate

Figure 15.

Variation of hyphal RNA concentration with dilution rate.



Hyphal RNA concentration (% dry weight)
10
8
6
4
2
0
0.05
0.10
0.15
0.20
Dilution rate (h⁻¹)

growth rate have been observed by Griffin, Timberlake & Cheyney (1974) in batch cultures of the mould *Achyla bisexualis* and in continuous cultures by Muira, Tsuchiya, Nishikawa, Obata & Okazaki (1974); McMurrough & Rose (1967); Dalton & Postgate (1969; Khmel & Andreeva (1969) and Herbert (1961); (these authors were working with respectively, *Bacillus*, *Saccharomyces*, *Condida*, *Aztobacter*, *Azotobacter* and *Aerobacter* species).

The opposite effect of a decrease in RNA content with increasing growth rate, has also been described (Morimoto & James, 1969; Bendijkeit, 1966) in *Astasia longa* and *Escherichia coli* respectively.

The rise in RNA concentration as the dilution rate is decreased below 0.07 h^{-1}, in the present work, suggests that the rate of RNA synthesis does not decrease in synchrony with growth rate at lower values of D. This implys some form of "uncoupling" between gross RNA synthesis and cell division at low growth rates (doubling times greater than 10 h). The fall in RNA concentration when heterogeneous, sporing cultures are obtained is consistent with observations of Righelato, Trinci, Pirt & Peat (1968) that considerable turnover of RNA takes place during conidiation.

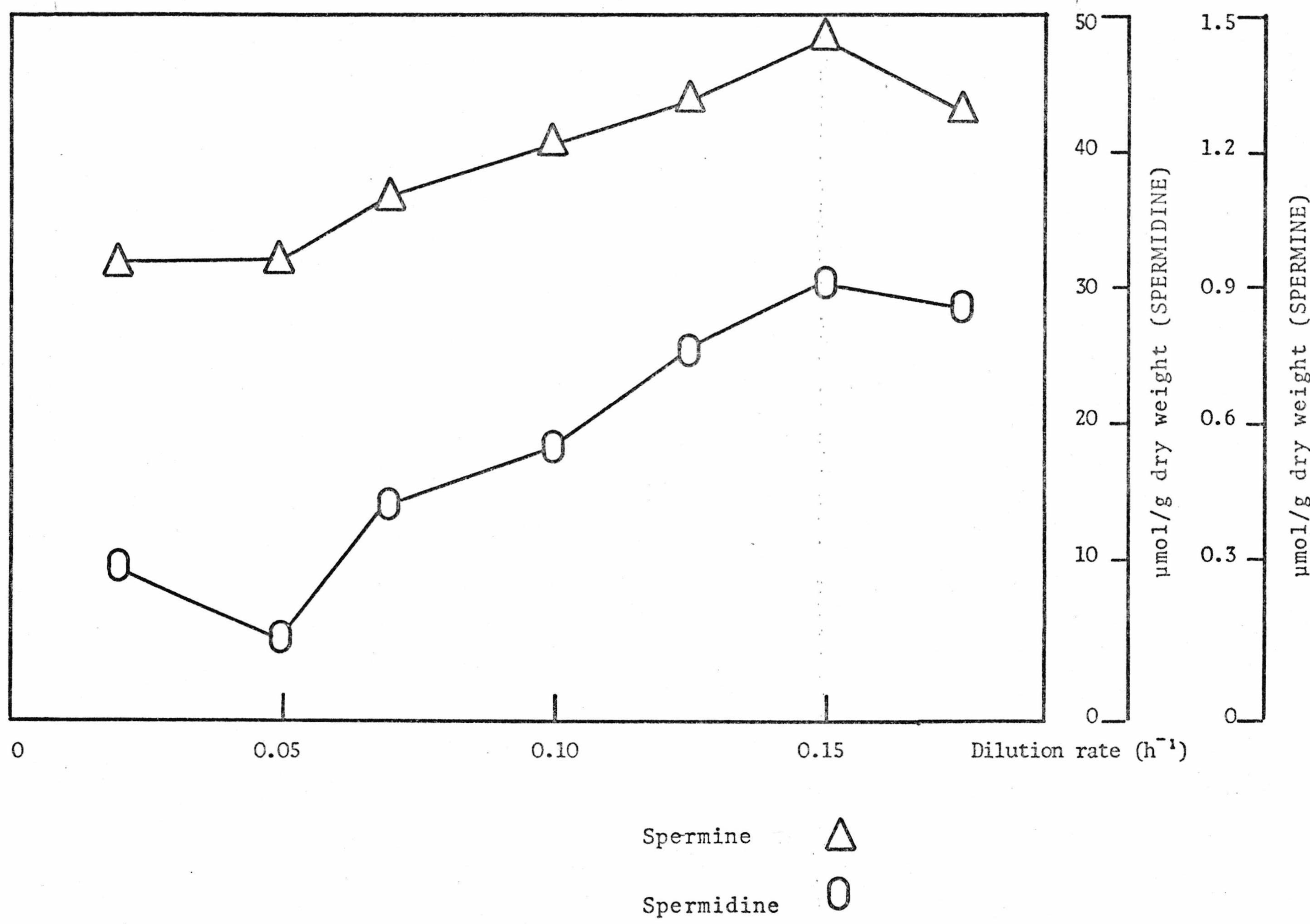
The high value (7%) observed at 0.05 h^{-1} is comparable with the values observed by Bainbridge, Bull, Pirt, Rowley & Trinci (1971) with *Aspergillus nidulans* (BWB 224) growing at a dilution rate of 0.042 h^{-1} (6.6% RNA).

(ii) Polyamines

Figure 16 shows the variation of spermine and spermidine intracellular concentrations with growth rate. Concentrations of both polyamines increased as the dilution rate was raised.

Figure 16.

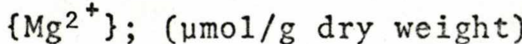
The effect of dilution rate on polyamine concentration.



0
0.05
0.10
0.15
Dilution rate (h^{-1})
0
10
20
30
40
50
μmol/g dry weight (SPERMIDINE)
0
0.3
0.6
0.9
1.2
1.5
μmol/g dry weight (SPERMINE)
Spermine △
Spermidine O

Figure 17

The effect of dilution rate on hyphal magnesium concentration


{Mg2+}; (µmol/g dry weight)
80
60
40
20
0.05
0.10
0.15
0.20
Dilution rate (h-1)

Spermidine increased from 6μmol/g dry weight (2.9mM) to 32μmol/g dry weight (15.4mM) and spermine from 1.0mmol/g dry weight (0.4mM) to 1.5μmol/g dry weight (0.59mM). Polyamine assays were not made on *Aspergillus nidulans* batch cultures. Comparable values for exponentially growing *Neurospora crassa* batch cultures, however, were 16mM spermidine and 0.22mM spermine (Viotti, Bagni Stuvani & Alberghina, 1971).

(iii) Magnesium.

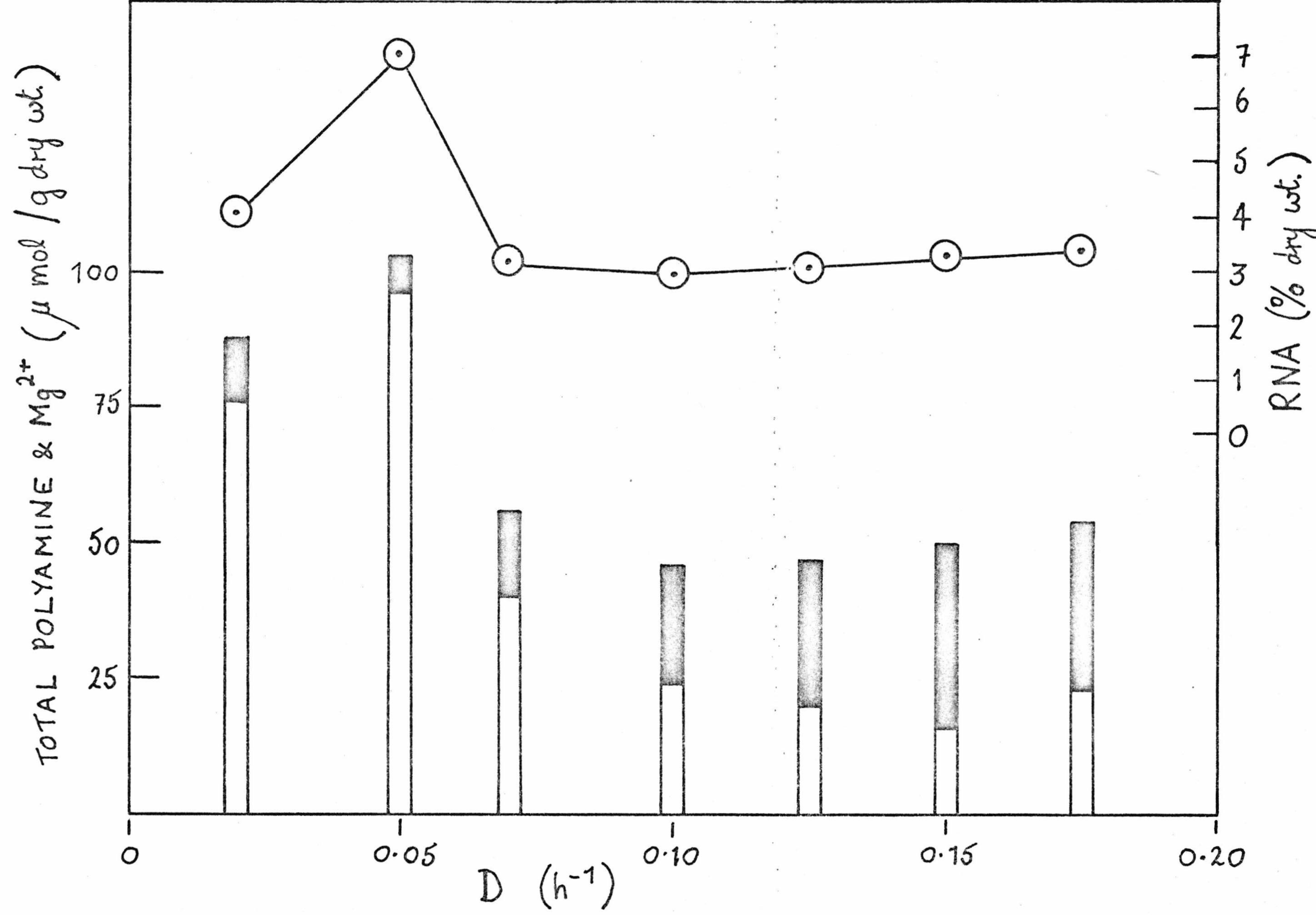
Magnesium concentrations varied nearly six-fold (18 to 97.5μmol/g dry weight) and fell with increasing dilution rate (Figure 17). An average value of 97.5μmol/g dry weight for the magnesium concentration of vegetative bacteria is evident from Tempest's (1969) data and this is equivalent to an approximate molar concentration of 150mM. The internal water of *Aspergillus nidulans* mycelium was not estimated, but, using the value obtained by Viotti, Bagni, Stuvani & Alberghina, (1971) for young *Neurospora crassa* mycelia, the magnesium concentration of *Aspergillus nidulans* varied over the approximate range 6 to 37mM. This compares with the magnesium concentration of exponentially growing *N.crassa* (Viotti et al,1971) and *A.nidulans* (the present work) which were both 16mM.

(iv) Inter-relationship between magnesium, polyamine and RNA levels.

Figure 18 shows the variation of total polyamine concentration, (solid histograms) magnesium concentration (open histograms) and total RNA concentration (open circles) as a function of dilutation rate. The molar ratio of magnesium to RNA does not vary with dilution rate in carbon-limited cultures of

Figure 18

The effect of dilution rate on hyphal RNA concentration (circles), spermine plus spermidine concentration (solid histograms) and magnesium concentration (open histograms)



TOTAL POLYAMINE & Mg^{2+} (µ mol / g dry wt.)
100
75
50
25
RNA (% dry wt.)
7
6
5
4
3
2
1
0
0
0·05
0·10
0·15
0·20
D (h^{-1})

bacteria (see Tempest, 1969) but data from *Aspergillus nidulans* show marked variations. However, when the polyamines are considered, the molar ratio of polyamines plus magnesium to RNA remains constant at approximately 2:1. It appears, therefore, that the synthesis of the polyamines and spermidine in particular, changes in response to growth rate dependent fluctuations in magnesium concentration in such a way that the cation to RNA ratio is maintained. It is difficult to interpret this data in molecular terms. The strong correlation between the relative concentrations of magnesium and polyamines with the RNA concentration suggests a functional inter-changeability between magnesium and polyamines with respect to RNA. The necessity for polyamines in the stabilisation of functional ribosomal RNA and in RNA synthesis has been described in III.1.c. The correlations between the gross levels of these intracellular constituents suggests that polyamines are primarily concerned with the maintenance of cell RNA levels although the techniques employed were not specific enough to elucidate any relationship between polyamine and DNA concentrations. The significance in the ratio of magnesium to polyamine in protein synthesis efficiency will be discussed in III.3.b.iv.

The ratio of total cation (polyamine plus magnesium) to RNA remained constant only under conditions of vegetative growth and changed significantly in the heterogeneous culture of spores and vegetative mycelium (D=0.02 h^{-1}). This effect would seem predictable when one considers the large turnover of RNA during conidiation (Righelato, Trinci, Pirt & Peat, 1968) and the change in ribosome efficiency which occurred when the dilution rate was decreased to 0.02 h^{-1} (see III.3.b)

(b) Effect of dilution rate on mycelial protein concentration.

(i) Steady state protein concentrations.

The variation of mycelial protein concentrations with dilution rate is shown in Figure 19. The relationship is not simple, protein concentration reaching a peak value at $D=0.10\ h^{-1}$ (48% total dry weight). The lowest concentration obtained in vegetative mycelium was 30% of total dry weight ($0.05\ h^{-1}$). These data contrast with those of Herbert (1961); Dalton & Postgate (1969); Bendigkeit (1966); Alroy & Tannenbaum (1973); and Muira, Tsuchiya, Nishiskawa, Obata & Okazaki (1974) who found that cellular protein concentrations varied very little with growth rate.

Maaløe & Kjeldgaard (1960); McMurrough & Rose (1967); and Bu'Lock, Detroy, Hostalek & Munim-al-Shakarchi (1974), however, found that protein concentration decreased with increasing growth rate. Kmel & Andreeva (1969) observed a discontinuous relationship, similar to the present results.

(ii) Effect of dilution rate on protein synthesis efficiency.

An interpretation of the data in Figure 19 may be made when protein and RNA concentrations are compared. Figure 20 shows the variation of ribosomal efficiency defined as $\frac{P}{R} \times D$ (Alroy & Tannenbaum, 1973) with dilution rate (see II.1.b(ii). Similar plots of data from *Candida utilis* (Alroy & Tannenbaum, 1973) and *Gibberella fujikuroi* (re-calculation of data from Bu'Lock et al, 1974) are also shown for comparison. In each case, ribosomal efficiency increases linearly with D, the curve starting to flatten off at high dilution rates, in the case of

Figure 19

The effect of dilution rate on hyphal protein content.

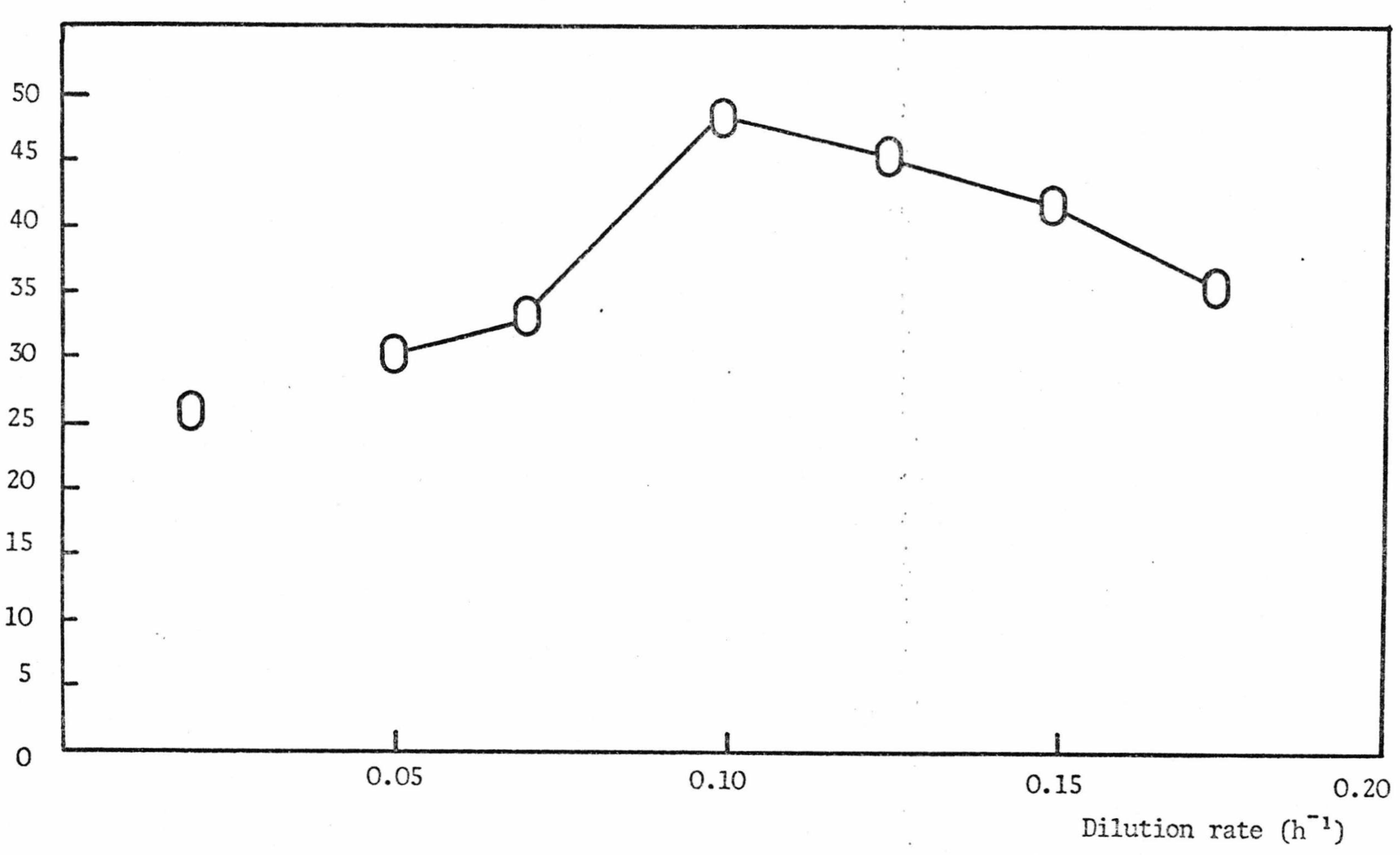
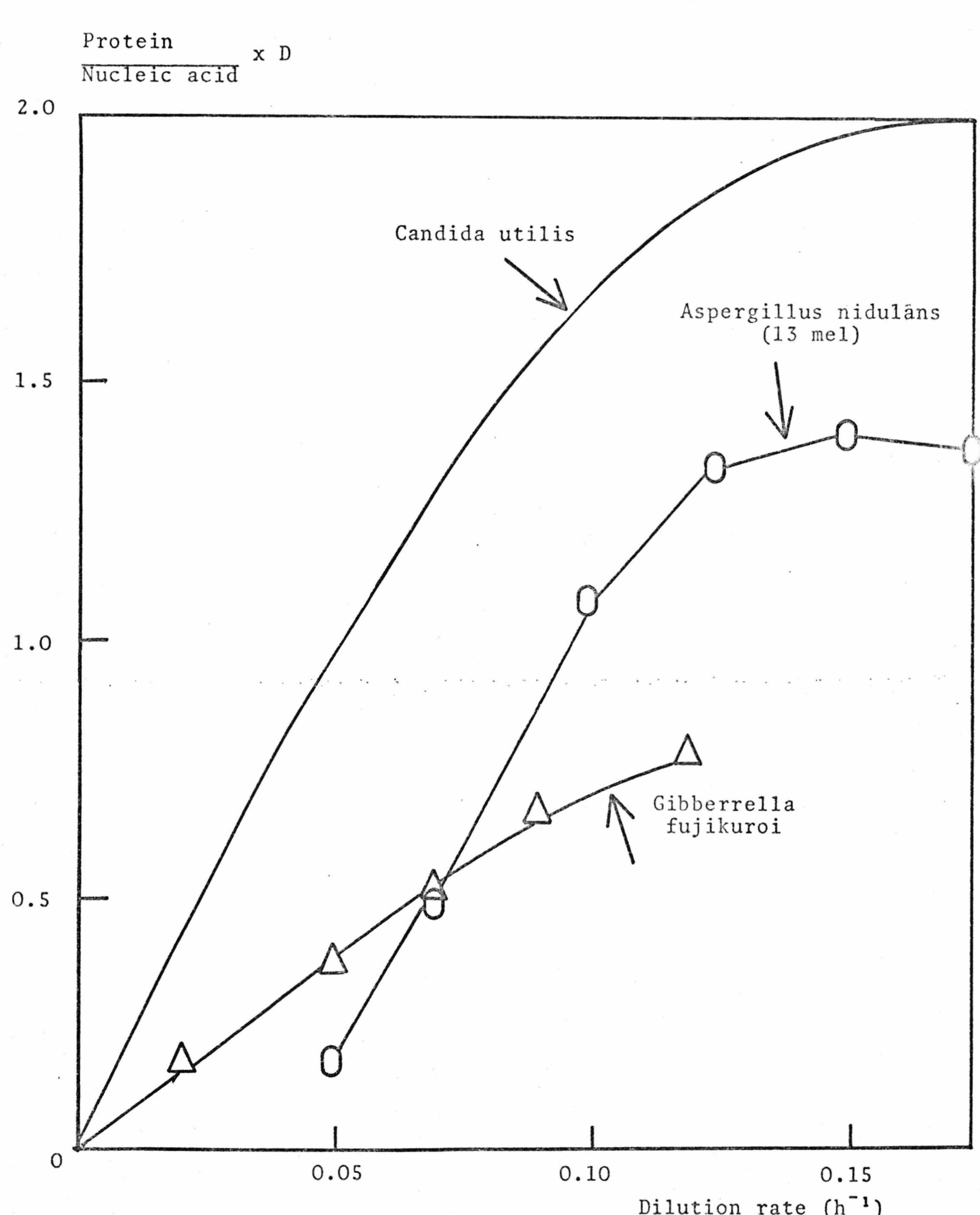

Hyphal protein content (% total mycelial dry weight)
50
45
40
35
30
25
20
15
10
5
0
0.05
0.10
0.15
0.20
Dilution rate (h^{-1})

Figure 20.

The effect of dilution rate on protein synthesis efficiency in *Candida utilis* (Alroy & Tannenbaum, 1973), *Aspergillus nidulans* (present work) and *Gibberrella fujikuroi* (calculated from the data of Bu'Lock et al 1974)


Protein
Nucleic acid
x D
2.0
1.5
1.0
0.5
0
0.05
0.10
0.15
Dilution rate (h⁻¹)
Candida utilis
Aspergillus nidulans
(13 mel)
Gibberrella
fujikuroi

Aspergillus nidulans, at 65% of μmax. These results may be interpreted to indicate that all the ribosomal RNA subunits become converted to functional polysomes before μmax is reached. Alternatively, the rate of polypeptide synthesis may have reached a maximum.

If the linear part of the ribosomal efficiency curve for *Aspergillus nidulans* is extrapolated, it is found that zero ribosomal efficiency occurs at a dilution rate of approximately 0.04 h^{-1}. This may be taken as metabolic evidence for the existence of a minimum growth rate in this fungus (see II.3.e). With *G.fujikuroi* and *C.utilis* this is not the case, however, as zero ribosomal efficiency occurs at zero growth rate. In practise, zero ribosomal efficiency is never obtained in steady-state cultures of *Aspergillus nidulans* as the culture undergoes cyclic differentiation at dilution rates below 0.04 h^{-1} (see II.3.e). This observation is consistent with the hypothesis of Mirkes (1974) that protein synthesis is completely shut down in dormant *Aspergillus* spores (III.1.b.ii). Zero ribosomal efficiency would be expected to coincide with zero dilution rate in *G.fujikuroi* and *C.utilis* because no sporulation occurs in continuous cultures of these organisms.

(iii) Role of polyamines in ribosomal efficiency.

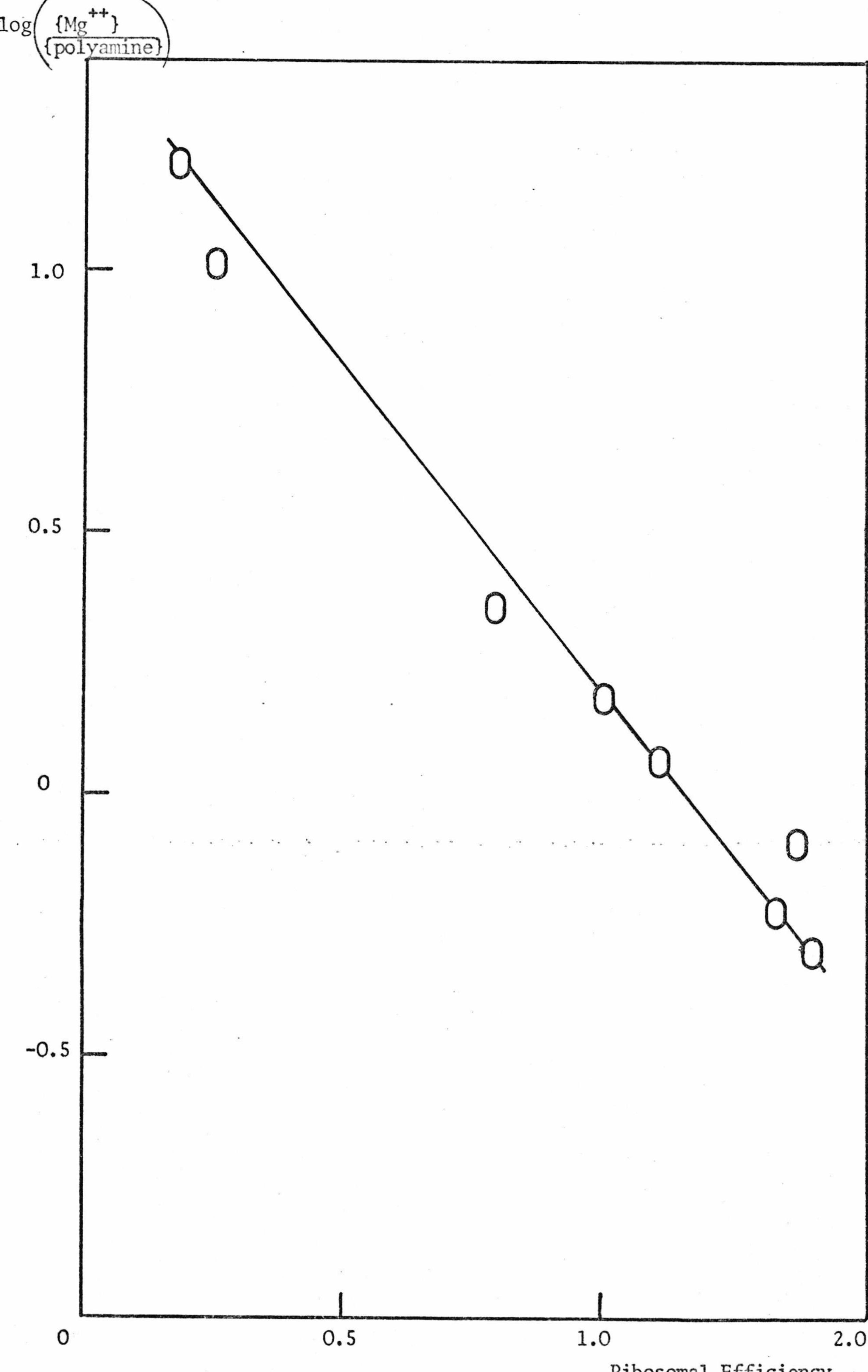
Figure 21 shows the logarithmic relationship between the ratio of mycelial magnesium concentration to polyamine concentration and ribosome efficiency. Broadly speaking, a larger proportion of polyamine is synthesised to increase ribosomal efficiency. This is consistent with the functional roles of the polyamines, described in the introduction (III.1.c), of stimulation of amino acids into polypeptides and of stabilisation

Figure 21

Logarithmic relationship between:

$$\frac{\text{magnesium concentration}}{\text{polyamine concentration}}$$

and ribosomal efficiency.



log({Mg++}/{polyamine})
1.0
0.5
0
-0.5
0
0.5
1.0
2.0
Ribosomal Efficiency

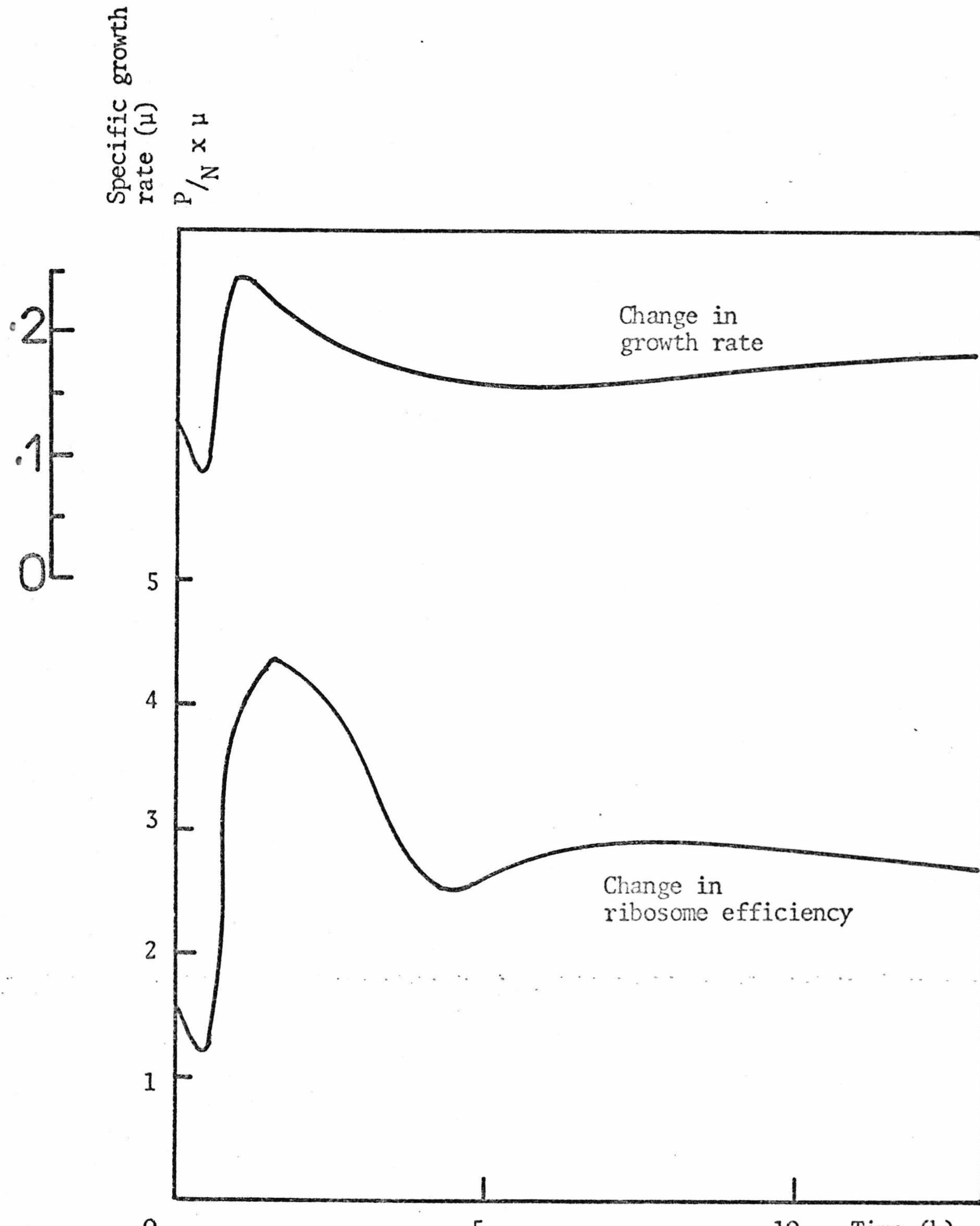
of functional ribosome structures. The logarithmic relationship shows that the relative requirement for polyamines increases with protein synthesis at a higher rate than the requirement for magnesium. This is, perhaps, predictable when one considers the number of processes, relevant to protein synthesis which require the participation of polyamines (see III.1.c). As ribosomal efficiency increases to a maximum, possible requirements for the polyamines are in the formation of transfer RNA, aminoacylation of transfer RNA, message binding to the 30s subunit and formation and stabilisation of the functional ribosome unit (Cohen, 1971; and section III.1.c.). If increases in ribosomal efficiency are due to the increase in formation of functioning ribosome units, as described in III.1.c, then it would appear conceivable that relative requirements for polyamines would rise at an accelerating rate.

(iv) The "Universal" RNA to protein ratio.

The "universal constant" proposed by Alroy & Tannenbaum (1971) (see III.1.b.ii), i.e.

$$\frac{R/P}{\left(\frac{\mu}{\mu max}\right)_T \left(\mu max\right)_{30}} = 0.533$$

was obtained from data derived from experiments with a number of bacteria and yeasts, and the protozoan *Tetrahymena pyriformis*. Computation of this value for *Aspergillus nidulans* showed that the universal ratio held for dilution rates between 0.07 h^{-1} and 0.175 h^{-1}. At growth rates below 0.07 h^{-1} however, the values obtained show significant departure from the theoretical ratio. Cultures at dilution rates approaching μmin may show an increased tendency towards sporulative metabolism. The change



Specific growth rate (μ)
P/N x μ
·2
·1
0
5
4
3
2
1
Change in growth rate
Change in ribosome efficiency
0
5
10
Time (h)

Figure 22

Step-up transient state (D changed from 0.125 to 0.175 h^{-1}).

Time scale shows time elapsed since the dilution rate was changed to 0.175 h^{-1}

Curves show change in growth rate (μ) and ribosome efficiency:

$$\frac{\text{Protein}}{\text{nucleic acid}} \times \mu$$

in the value of the ratio could, therefore, reflect a change in RNA metabolism. It has been established that marked changes in the species of RNA present in *Bacillus subtilis* occur at the onset of sporulation (Losick, Shorenstein & Soneusheim, 1970). The considerable turnover of RNA during sporulation of *Penicillium chrysogenum* (Righelato, Trinci, Pirt and Peat, 1968) and the alteration in protein synthesising capacity of many fungal species (Mirkes, 1974) upon sporulation suggests that similar effects may take place in moulds.

(v) Transient changes in ribosomal efficiency.

Figure 22 shows the variation of growth rate and protein synthesis efficiency (as defined by Alroy & Tannenbaum, 1971) with time in a step-up transient state (D changed from 0.125 h^{-1} to 0.175 h^{-1}). Growth rate oscillated during the transient state, varying between 0.08 h^{-1} and 0.25 h^{-1}. The oscillations presumably were produced by "biological inertia" described in (II.2.c).

Protein synthesising efficiency appears to respond immediately and positively to changes in growth rate. This observation is in complete agreement with the bulk of literature reviewed in II.1.b. In the examples cited, however, increases in growth rate were obtained by alteration of the culture medium, leading to a smooth transition between growth rates. Continuous cultures, which allow oscillations to take place in the transient phase, enable the study of the correlation between ribosomal efficiency and increasing, decreasing and zero growth rates. The time lag observed by Young & Bungay (1973)between change in dilution rate and change in ribosomal efficiency may be

explained by oscillations occurring in growth rate and ribosomal efficiency prior to the attainment of a steady state where growth rate is equivalent to dilution rate.

(c) Effect of dilution rate on "amino acid pool size".

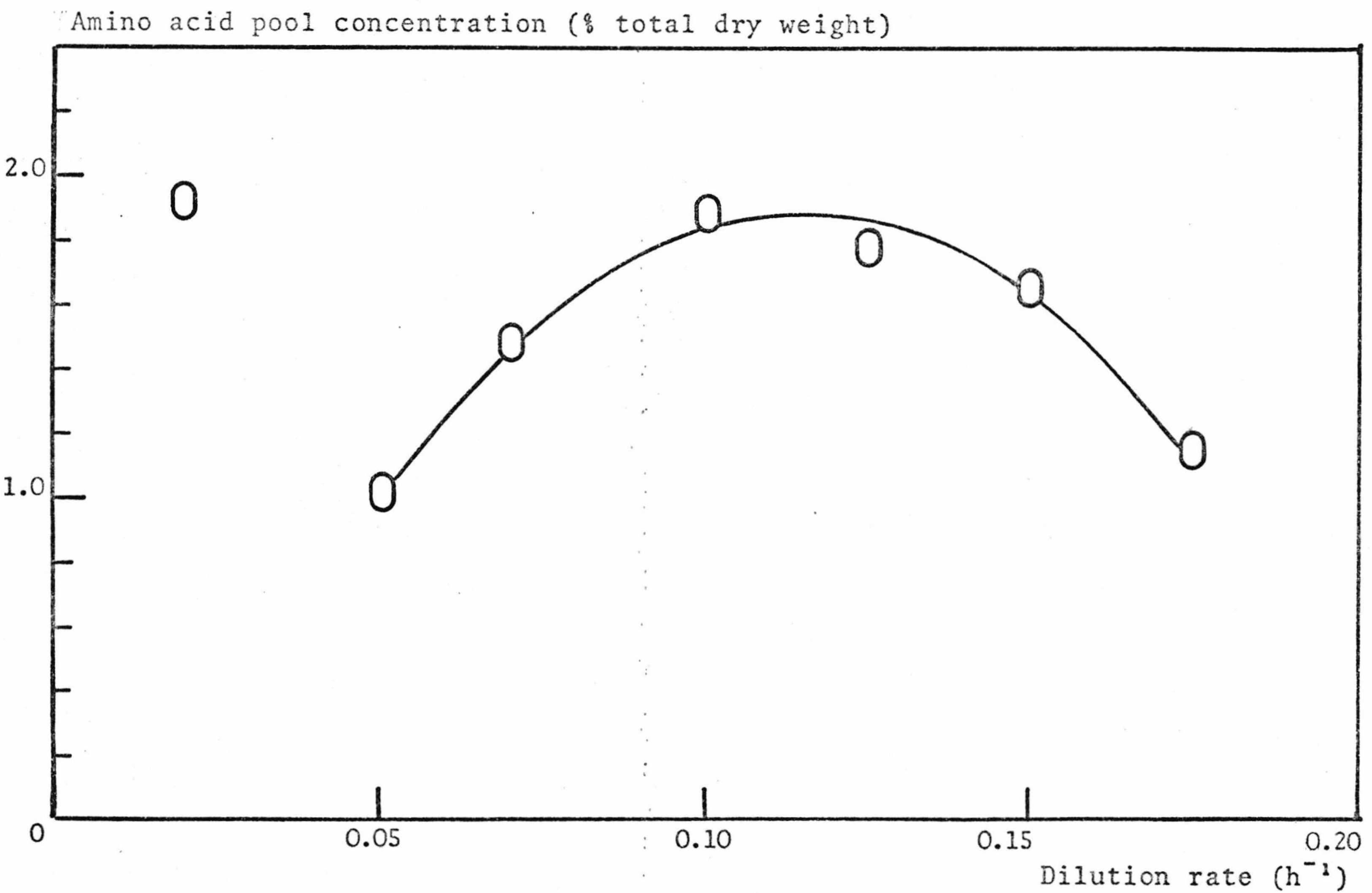
Figure 23 shows the effect of changing dilution rate on the amino acid pool size of *Aspergillus nidulans*.

The "pool" constituents of a microorganism comprise many soluble, low molecular weight compounds concerned with both anabolic and catabolic cellular functions. The amino acid pool provides amino acids which act as precursors to protein synthesis. The variation in amino acid pool size as a function of dilution rate in vegetative *Aspergillus nidulans* mycelium follows a similar pattern to the change in mycelial protein concentration with D. The curve in Figure 23 passes through a maximum (1.88%) at D = 0.10 h^{-1} and falls to 1.02% at 0.05 h^{-1} and 1.14% at 0.175 h^{-1}. The amino acid pool concentration in the culture containing spores was slightly higher than in vegetative mycelium (1.92%). This observation may be interpreted as being a parallel effect to the hypothesis of Mandelstam (1958) that "non-growing" bacterial cells undergo a process of simultaneous degradation and synthesis of protein; amino acid pool and cell protein exist in a state of dynamic equilibrium. As continuous protein degradation does not take place, to a great extent, in actively growing populations, one would expect lower concentrations of free amino acids than in "non-growing" cultures.

A higher amino acid pool concentration (2.3%) was detected in batch culture, indicating a restrictive effect of carbon limitation on amino acid synthesis.

Figure 23.

Effect of dilution rate on amino acid pool size.



Amino acid pool concentration (% total dry weight)
2.0
1.0
0
0.05
0.10
0.15
0.20
Dilution rate (h⁻¹)

(d) Effect of dilution rate on mycelial DNA concentration.

The mycelial DNA concentration of carbon-limited *Aspergillus nidulans* cultures varied very little with changing dilution rate (Figure 24). Endocellular concentration varied between 0 .9% and 1.4% of total dry weight. Similar observations have been recorded for many other micro-organisms in continuous culture (see III.1.a). If the data is recalculated, however, to give DNA productivity, i.e. the amount of DNA produced by 1g of mycelium in 1 h, (Figure 25) these data may be interpreted to mean that the rate of DNA production (per unit mycelial mass) remains constant at doubling times between 4 and 5.8 h. At longer doubling times, however, this rate falls as a linear function of growth rate. When Figure 25 is compared with Figure 20, it can be seen that similar trends occur in ribosomal efficiency and DNA productivity with changing growth rate. Figure 26 shows that, indeed, the relationship between ribosomal efficiency and DNA is linear. Possible interpretations of this relationship are; (1) that nuclear division rate is limited by ribosomal activity and (2) that the rate of nuclear division dictates the proportion of active ribosomes in the cell. In any event, an analogy exists with the concept that, in unicells, the rate of cell division is limited by (or has a rate-limiting effect on) RNA activity (see III.1.b). When this notion is applied to the filamentous organism, *Aspergillus nidulans*, the term "cell division" could be replaced by DNA productivity or nuclear division. These results indicate the desirability of performing experiments which correlate hyphal ultrastructural observations of nuclear division with DNA productivity at different growth rates.

Figure 24.

The effect of dilution rate on mycelial DNA content

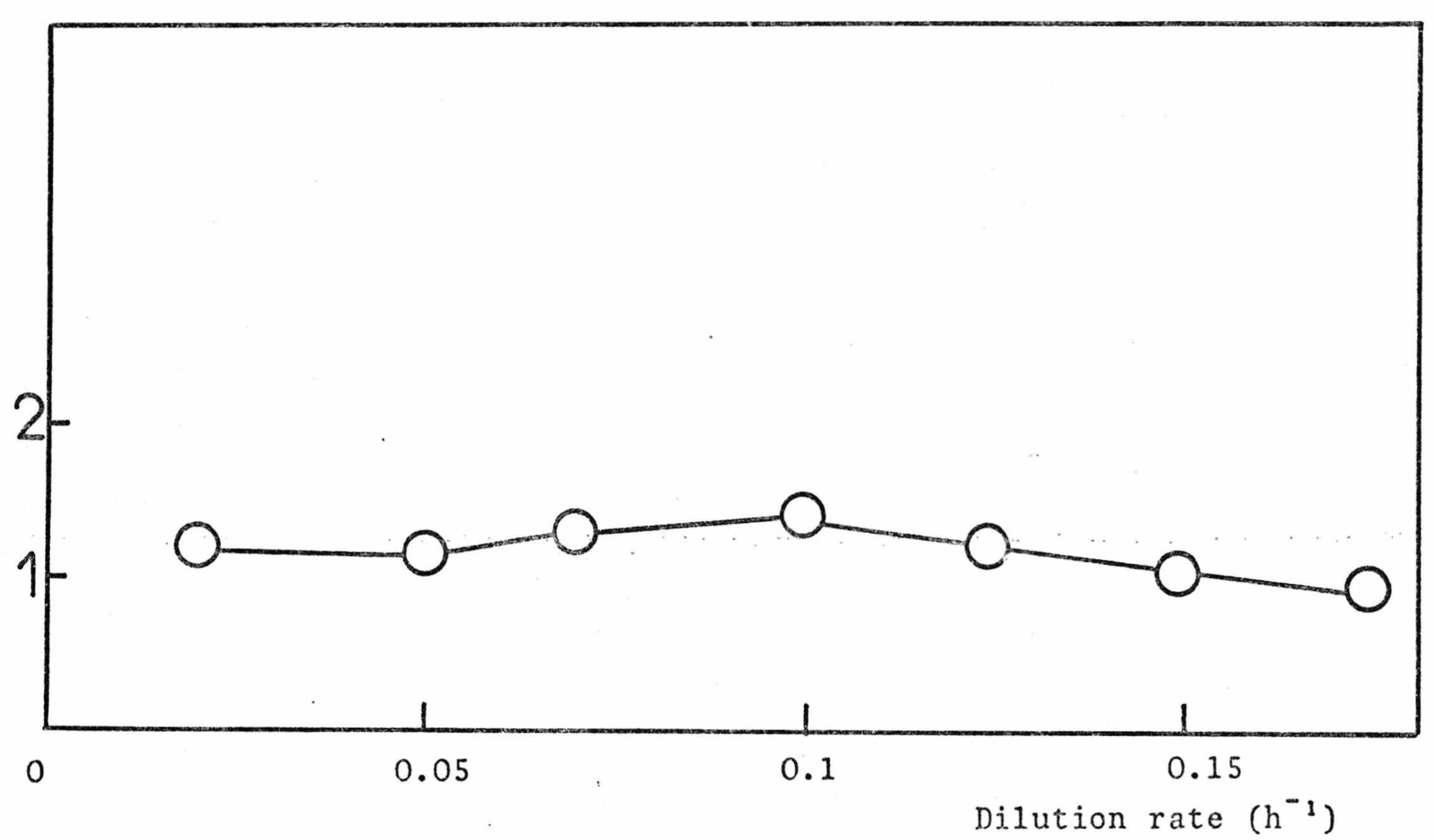


Figure 25.

The effect of dilution rate on DNA productivity

(DNA mycelial concentration x dilution rate)

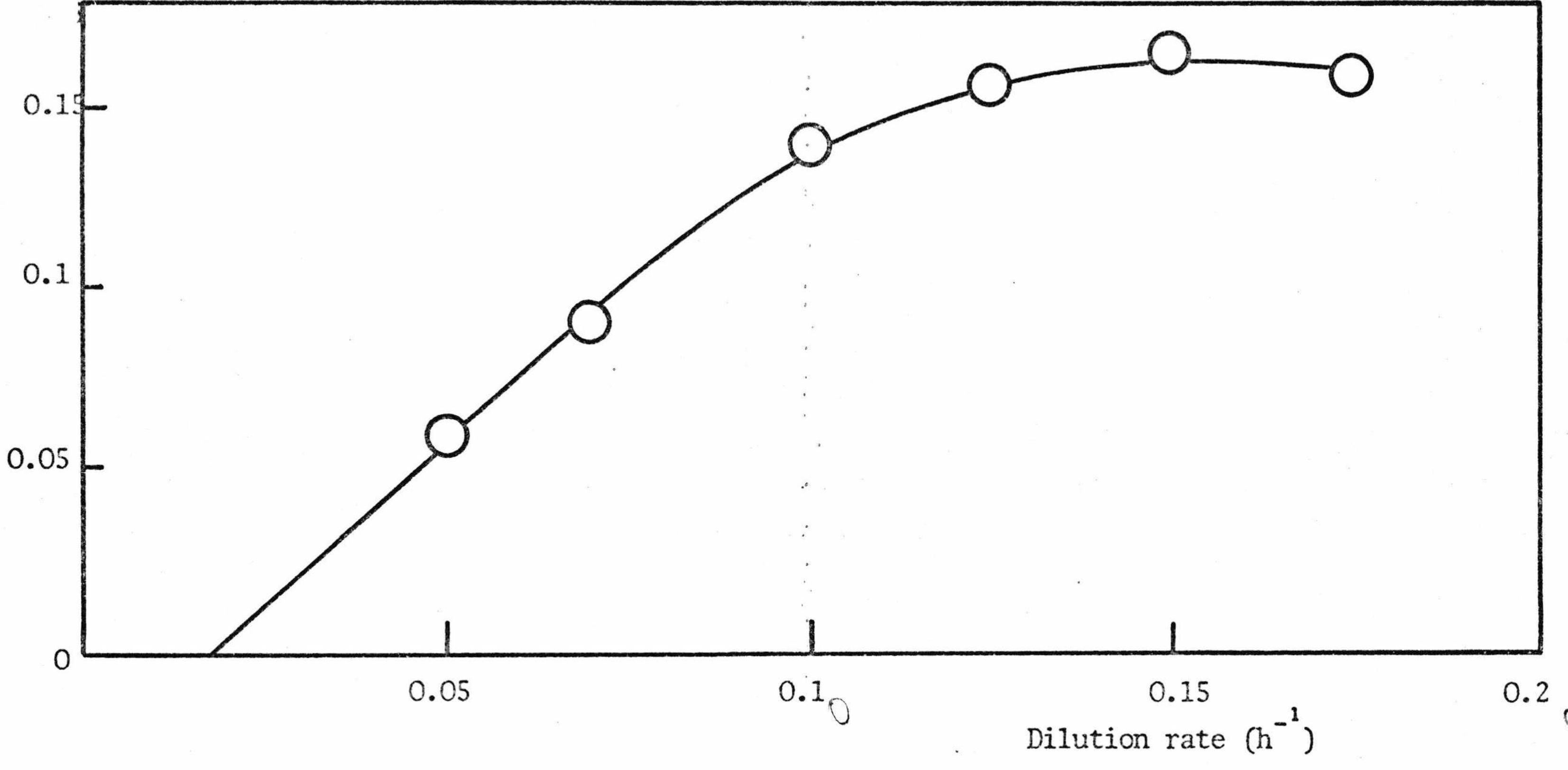

DNA productivity (g h^{-1})
({DNA} x D)
0.15
0.1
0.05
0
0.05
0.1
0.15
0.2
Dilution rate (h^{-1})

Figure 26.

The relationship between ribosomal efficiency

$\left(\frac{\text{Protein}}{\text{Ribonucleic acid}} \times \text{dilution rate}\right)$ and DNA

productivity (DNA x dilution rate)

Ribosomal efficiency (g g $^{-1}$h^{-1})

1.5

1.0

0.5

0

0.05

0.10

0.15

0.20

DNA productivity (g h^{-1})

(e) The effect of dilution rate on mycelial "storage polymers".

(i) Carbohydrate.

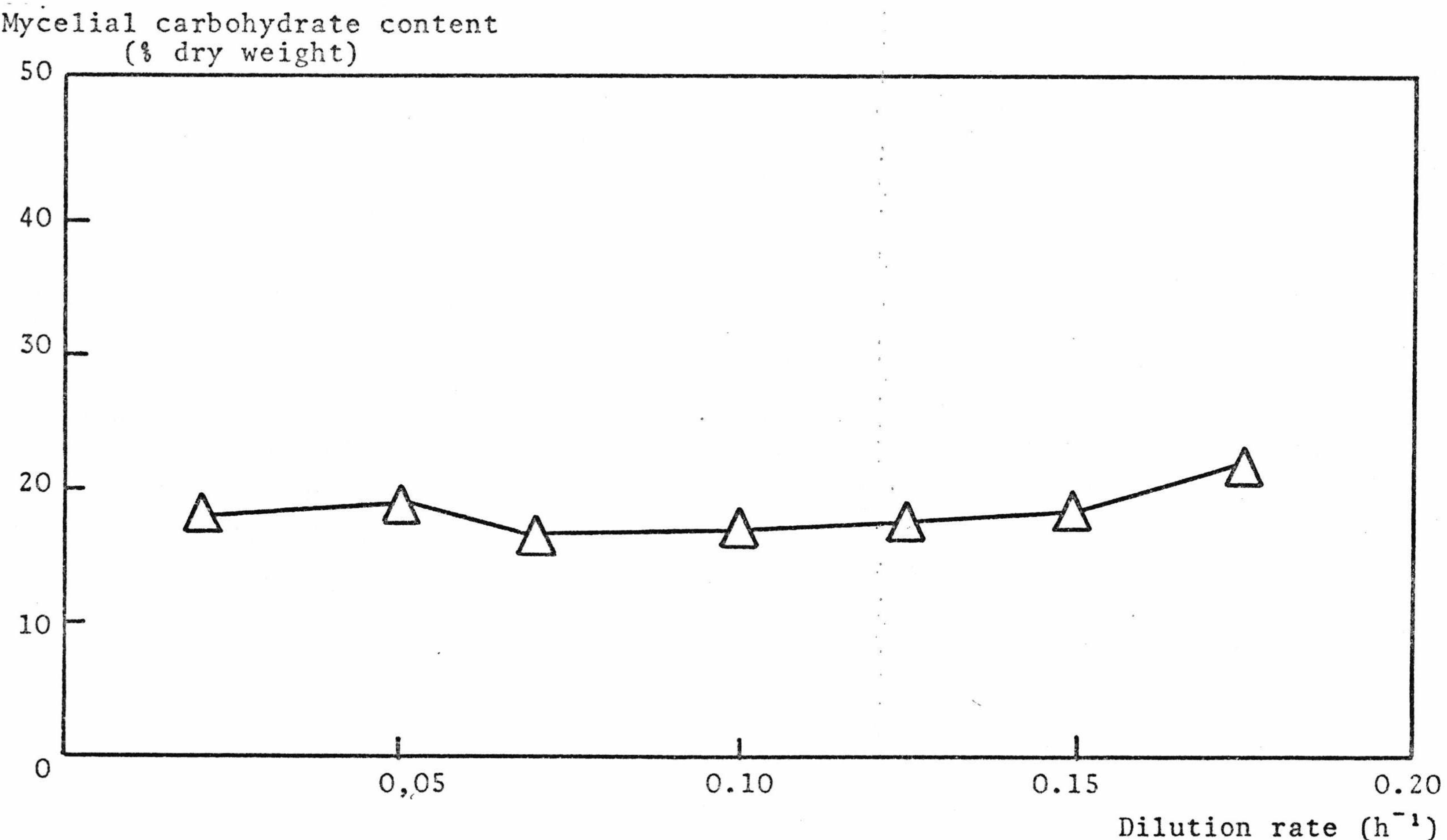
The variation of the proportion of total mycelial carbohydrate with dilution rate is shown in Figure 27. These results are in disagreement with similar studies made on other organisms (see, for example, Morimoto & James, 1969; Kuenzi & Fletcher, 1972; Bu'Lock, Detroy, Hostalek & Munim-al-shakarchi, 1974) in which carbohydrate content has been found to show a general decrease with growth rate. The carbohydrate content of *Aspergillus nidulans* increases slightly at high as well as low dilution rates. The variability of carbohydrate content is probably effected by two simultaneous processes; the accumulation cytoplasmic carbohydrate as energy storage polymers and the variability of hyphal wall carbohydrate content with growth rate. Analysis of hyphal wall preparations from batch cultures of *Aspergillus nidulans* revealed that 44.7% of the wall is composed of carbohydrate. Variability in the carbohydrate in carbon-limited yeast continuous cultures has been recorded (e.g. McMurrough & Rose, 1967; Reuvers, Tacoronte, Mendoza & Novaes-Ledieu, 1969). The levels of mycelial carbohydrate obtained under carbon limitation (16-22% total dry weight) are similar to the value determined in batch culture (19.8%).

(ii) Lipid

The variation of mycelial lipid content as a function of growth rate is shown in Figure 28. The lipid content of vegetative mycelium rose in a linear fashion as the dilution rate was increased. Hyphal lipid content varied between 24%(D = 0.05 h^{-1}) and 31.8% (D = 0.175 h^{-1}). A similar effect was

Figure 27.

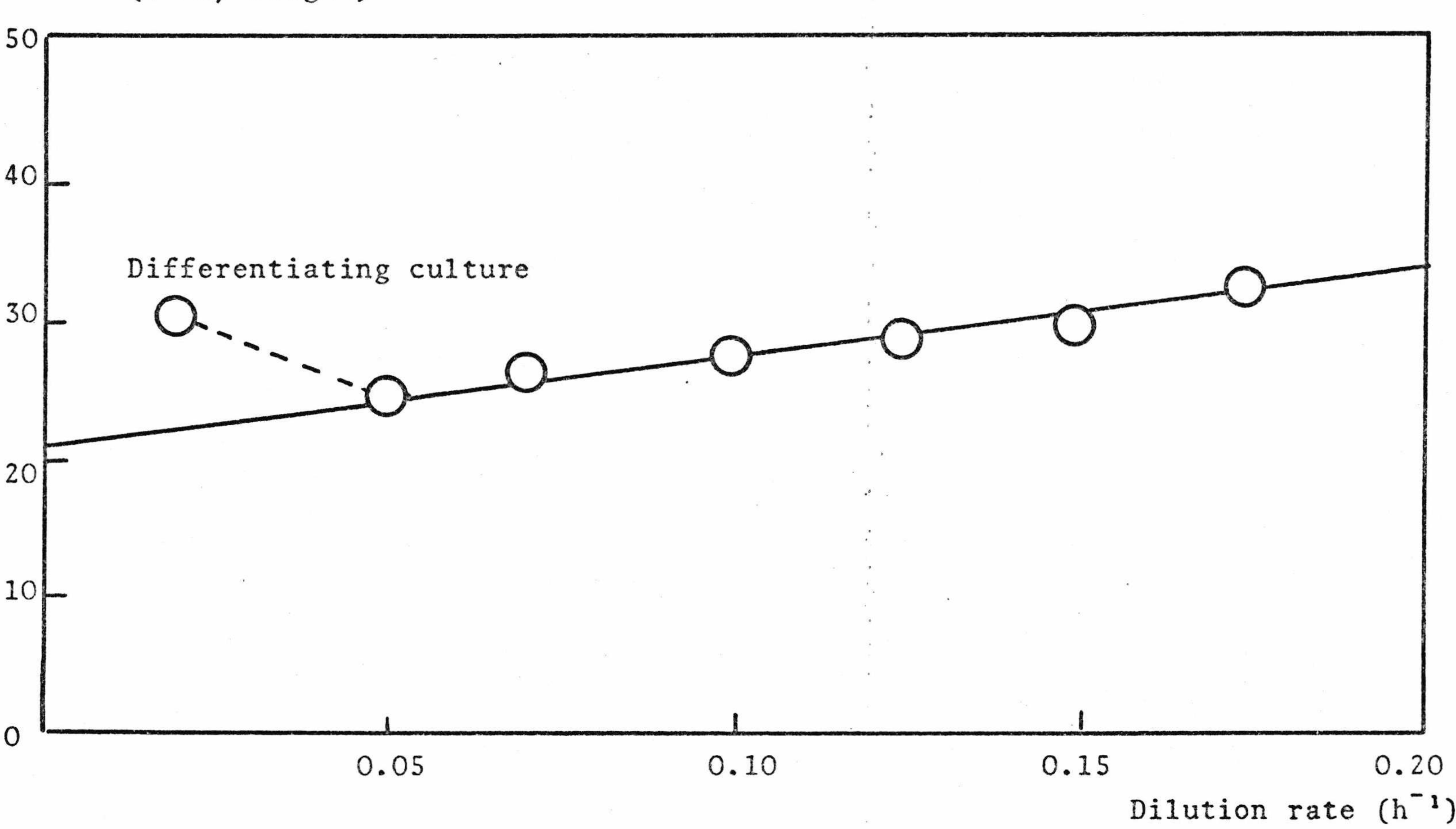
The effect of dilution rate on mycelial carbohydrate concentration.



Mycelial carbohydrate content
(% dry weight)
50
40
30
20
10
0
0,05
0.10
0.15
0.20
Dilution rate (h⁻¹)

Figure 28.

The effect of dilution rate on mycelial lipid concentration.


Mycelial lipid concentration
(% dry weight)
50
40
30
20
10
0
Differentiating culture
0.05
0.10
0.15
0.20
Dilution rate (h⁻¹)

observed by Moo-Young, Shimizu & Whitworth, (1971) who noted a rise in cellular lipid content with increasing dilution rate in *Candida lipolytica*.

(iii) Energy required for carbohydrate and lipid synthesis.

Figure 29 shows the energy requirement (as ATP) to synthesis the lipid and polysaccharide component of *Aspergillus nidulans* at different dilution rates. The ATP requirement was calculated using the theory and data of Forrest & Walker (1971). The energy available for synthesis appears to rise slightly as growth rate increases. This suggests a slight increase with specific growth rate in the activity of the metabolic pathways which produce energy for biosynthesis (e.g. the HMP pathway). A progressive increase in HMP pathway activity with dilution rate could explain the increase in respiratory quotient observed when dilution rate is raised, (section II.2.iii). The HMP pathway produces CO_2 and $NADPH_2$ whereas the alternative, $NADH_2$ producing, EMP pathway does not produce CO_2.

(f) Effect of dilution rate on mycelial wall content.

The proportion of mycelial dry weight composed of mycelial wall material is shown as a function of dilution rate in Figure 30. Over the dilution rate range 0.07 h^{-1} to 0.175 h^{-1}, the cell wall content varied very little, falling slightly with increased dilution rate from 22.6% to 20%. Between 0.05 h^{-1} and 0.07 h^{-1}, however, the wall content increased 2-fold as the dilution rate was increased. These data compare with a yield of 18% of total dry weight obtained by BUll (1970) in stationary phase (batch) cultures of the same organism. Experiments on the effected growth rate on bacterial wall content (e.g.

Figure 29.

Energy (ATP) required to synthesise the "carbon-rich storage polymers" (lipid and carbohydrate) of *Aspergillus nidulans* as a function of dilution rate.

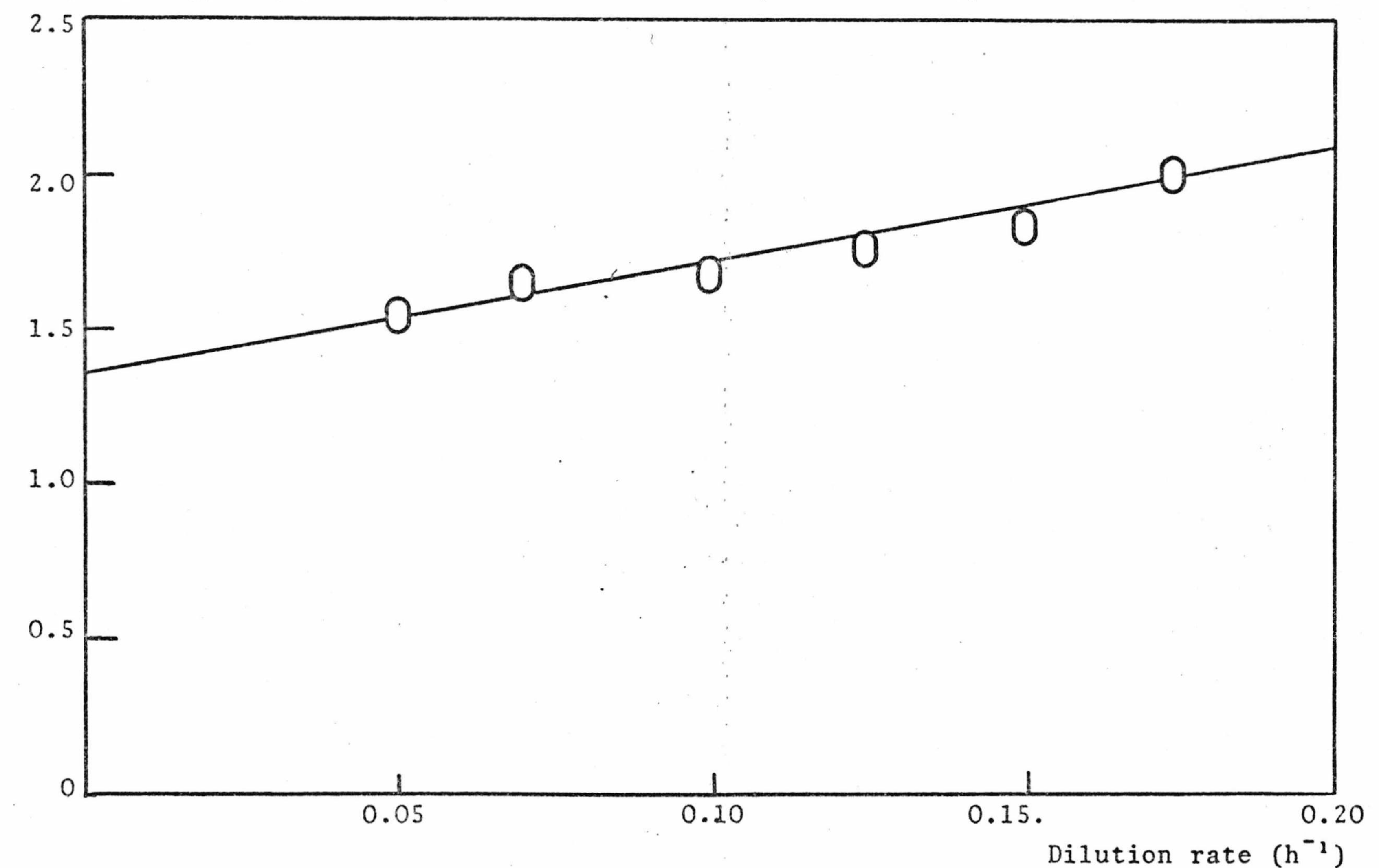
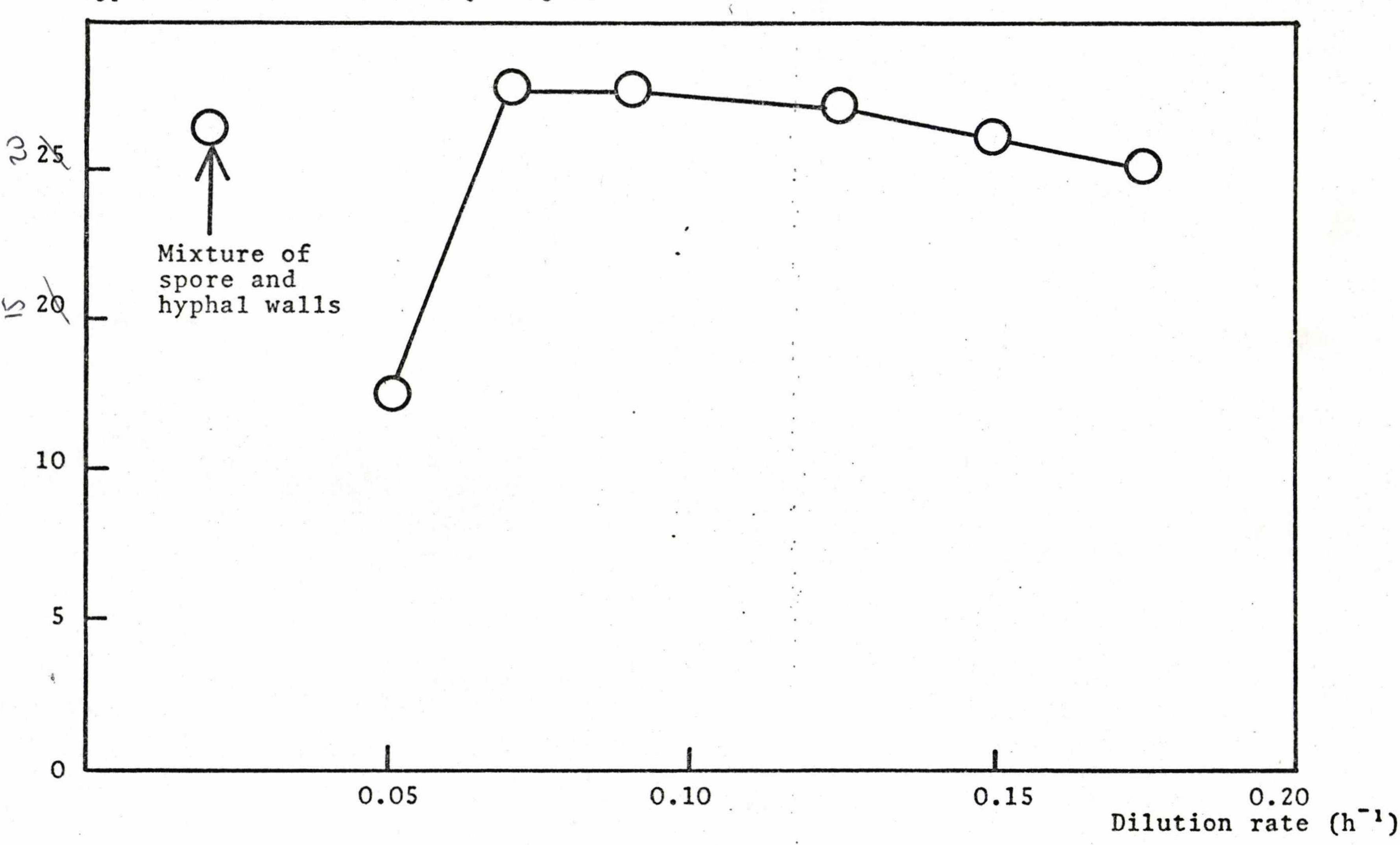

ATP (mol/100g) required to synthesise the carbohydrate and lipid fractions of *A.nidulans*
2.5
2.0
1.5
1.0
0.5
0
0.05
0.10
0.15.
0.20
Dilution rate (h^{-1})

Figure 30.

The effect of dilution rate on the hyphal wall content of *Aspergillus nidulans*.



Hyphal wall content (% dry weight)
25
20
10
5
0
Mixture of
spore and
hyphal walls
0.05
0.10
0.15
0.20
Dilution rate (h^{-1})

Ellwood & Tempest, 1967) are probably of little relevance in the elucidation of effects on fungal walls. A number of fundamental structural differences as well as the dissimilarity between hyphal elongation and binary fission complicate any attempts to correlate parallel observations of bacterial and fungal wall processes.

The drastic fall in hyphal wall content when the dilution rate was lowered from 0.07 h^{-1} to 0.05 h^{-1} may reflect the tendency for weakening of the wall structure at low dilution rates, often leading to the formation of swollen hyphae (Bainbridge, Bull, Pirt, Rowley & Trinci, 1971).

The walls prepared from the heterogeneous culture at D = 0.02 h^{-1} (mycelium differentiating into conidia) presumably were not comparable with those from purely vegetative mycelium as the preparation consisted of a mixture of spore and hyphal walls.

(g) Accuracy of analysis procedures.

When the quantities of the biochemical constituents determined were added together with mycelial ash, totals between 90% and 100% were obtained. This indicates that values obtained for each cell constituent were within at least 10% of absolute values.

SECTION IV.

ANAPLEROTIC METABOLISM.

1. INTRODUCTION.

(a) Anaplerotic pathways of metabolism.

(i) The glyoxalate cycle

(ii) CO_2-fixing enzymes.

(b) CO_2 fixation in fungi.

(c) Carbon dioxide as a fermentation parameter.

(i) Introduction

(ii) The influence of CO_2 on some fungal fermentations.

SECTION IV - ANAPLEROTIC METABOLISM.

1. INTRODUCTION.

(a) Anaplerotic pathways of metabolism.

The tricarboxylic acid (TCA) cycle fulfils a dual role in microbial physiology. It is the predominant metabolic route whereby the products of catabolism are combusted to water and carbon dioxide, producing, in each "turn" of the cycle, two molecules of CO_2 from one molecule of acetyl-CoA. The electrons thus released are used to generate ATP via a membrane bound electron transport system. The other function of the TCA cycle is the supply of its intermediates to provide the carbon skeletons for many of the macromolecular constituents of the cell (Krebs & Lowenstein, 1960; Mandelstam & McQuillen, 1968). Figure 31 shows the products derived from three major sources of biosynthetic precursors in the TCA cycle; oxaloacetate, succinyl-CoA and 2-oxoglutarate. All microorganisms with a functional TCA cycle, therefore, have a requirement for replenishment of its intermediate when these latter are used in anabolic pathways. The metabolic reactions which fulfil those requirement have been termed 'anaplerotic' (Kornberg, 1966a) to delineate their unique physiological role. The presence of anaplerotic enzymes enables the cell to synthesise all its cell constituents *de novo* when supplied with two or three carbon compounds, or their precursors, as the sole carbon source.

Anaplerotic metabolism may proceed by two means, via the glyoxalate cycle or by the fixation of CO_2.

Fig.31 The dual role of the TCA cycle

Sugars

Glucose 6-Phosphate

Phosphoenol pyruvate

Pyruvate

CO_2 CO_2

2H 2H

Acetyl-CoA

Citrate

CIS Aconitate

Isocitrate

2H

Oxalosuccinate

CO_2

α-Oxoglutarate

2H CO_2

Succinyl CoA

Succinate

2H

Fumarate

Malate

2H

Oxaloacetate

pyrimidines
aspartate
threonine
methionine
leucine
(lysine)

porphyrins

glutamate
glutamine
arginine
proline

(i) The glyoxalate cycle.

If a microorganism is grown on acetate as a carbon source, the TCA cycle will effect complete combustion of the acetate to water and CO_2. Growth will, therefore, only take place if ancillary reactions exist which can re-form the TCA cycle intermediates from acetyl-CoA. This is brought about by the sequential action of two enzymes; isocitrate lyase (EC.4.1.3.1.) and malate synthesase (EC.4.1.3.2.) which, together with certain of the TCA cycle enzymes form the glyoxalate cycle (Kornberg, 1966b). Isocitrate lyase catalyses the aldol cleavage of isocitrate to succinate and glyoxalate:

$$\text{Isocitrate} \rightleftharpoons \text{succinate} + \text{glyoxalate}$$

Malate synthase then causes the condensation of acetyl-CoA with glyoxalate to form malate:

$$\text{Acetyl-CoA} + \text{glyoxalate} + H_2O \rightarrow \text{malate} + \text{CoA-SH}.$$

In this way, the two Co_2-producing reactions (see Figure 31) are by-passed and the acetate input conserved.

Glyoxalate cycle activity is not restricted to situations where growth is on acetate but operates under many conditions when intermediates are drained from the TCA cycle (Kornberg & Elsden, 1961).

(ii) CO_2-fixing enzymes.

The replenishment of TCA cycle intermediates using carbon derived from CO_2 fixation takes place using either pyruvate or phosphoenol pyruvate (PEP) as CO_2 acceptors.

The direct carboxylation of pyruvate by means of the enzyme pyruvate carboxylase (EC.6.4.1.1.) has been observed in yeasts

Losada, Canovas & Ruiz-Amil, 1964); bacteria (e.g. Bridgeland & Jones, 1967); and fungi (Bloom & Johnson, 1962).

Carboxylation of PEP by phosphoenol-pyruvate carboxylase (EC.4.1.1.31) has been observed in a number of bacteria including members of the Enterobacteriaceae (Ashworth & Kornberg, 1966); *Thiobacillus thiooxidans* (Suzuki & Werkman, 1958); *Ferrobacillus ferroxidans* (Din, Suzuki & Lees, 1967); *Chlorobium thiosulfatophilum* (Evans, Buchanan & Arnon, 1966) and *Streptomyces aureofaciens* (Vorisek, Powell & Vanek, 1970). Pyruvate carboxylase catalyses, in the presence of biotin, the interaction of CO_2 and pyruvate to form oxaloacetate. The synthesis of the new carbon bond requires the expenditure of ATP.

Pyruvate + ATP + CO_2 $\rightarrow$ oxaloacetate + ADP + Pi. This reaction requires the presence of catalytic quantities of acetyl coensyme A which acts as an allosteric effector. The oxidation of pyruvate produces acetyl-CoA (Figure 31) which cannot enter the TCA cycle unless oxaloacetate is present to accept it. Acetyl-CoA thus provides positive feedback to the enzyme to produce the oxaloacetate required.

Phosphoenol pyruvate carboxylase acts in an analogous manner, catalysing the reaction: Phosphoenolpyruvate + CO_2 $\rightarrow$ oxaloacetate + Pi without, apparently, consuming ATP.

(b) CO_2 fixation in fungi.

A number of fungal intermediary metabolites are synthesized using fixed CO_2 as a major source of carbon. Experiments with radioactively labelled CO_2 demonstrated its incorporation into lactic, fumaric, citric and succinic acids in a number of species (Cochrane, 1958). The amino acids immediately associated

with the TCA cycle have also been shown to act as acceptors of CO_2. Experiments which have followed the incorporation of $^{14}CO_2$ showed that aspartate, glutamate and alanine are all primary products of CO_2-fixation in fungi (e.g. the uredospores of *Ustilago phaseoli;* Staples & Weinstein, 1959). Similarly, arginine (Gitterman & Knight, 1952), tyrosine (Budd, 1969) and isoleucine (Hartman, Keen & Long, 1972) have been identified as radioactive products following $^{14}CO_2$ fixation in *Penicillium chrysogenum*, *Neocosmospora vasinfecta* and *Verticillium alboatrum* respectively. With hindsight, it is obvious that pyruvate carboxylase (PC) or phosphoenol pyruvate carboxylase (PEPC) are responsible for CO_2 fixation in these organisms; the action of these enzymes would result in the labelling of TCA cycle-associated intermediates following $^{14}CO_2$ incorporation.

Some authors have detected phosphoenolpyruvate carboxykinase (E.C.4.1.1.32) in fungi and have concluded that it operates in a reverse manner to its normal function in gluconeogenesis (for example see Woronick & Johnson, 1960; Bachofen & Rast, 1967); however, the enzyme activities assayed in vitro have yet to be correlated with in vivo rates of fixation. The results of fixation. The results of Hartman & Keen (1973) who detected thos this enzyme in *Verticillium albo-atrum*, recently have been reinterpreted by the authors (Hartman & Keen, 1974) in the light of evidence that CO_2 is fixed by normal anaplerotic enzymes in this organism; in vivo phosphoenopyruvate carboxykinase apparently functions solely as a decarboxylase. The results of Rast & Bachofen (1967a) have suggested the presence of a further CO_2-fixing enzyme, β-methyl crotonyl CoA carboxylase (EC.6.5.1.5.) in

Agaricus bisporus. The activity of this enzyme resulted in the incorporation of CO_2 into acetone.

The aqueous dissolution of CO_2 results in the formation of bicarbonate at physiological pH values. A number of attempts have been made to distinguish between the utilisation of gaseous CO_2 and dissolved bicarbonate as substrate for carboxylase activities. The differential growth of fungi in soils of different pH has been attributed to the utilisation of bicarbonate as the sole C_1 source (Macauley & Griffen, 1969); the equilibrium between gaseous CO_2 and dissolved bicarbonate being strongly pH-dependent.

(c) Carbon dioxide as a fermentation parameter.

(i) Introduction.

A large proportion of the technical inovations in the design and operation of fermentation processes have been devoted to the manipulation of gas transfer and assimilation phenomena. Inevitably, oxygen, which occupies a central position in microbial physiology, has received the most attention; much effort having been directed towards the study and optimisation of culture aeration conditions. However, CO_2 has received comparatively little attention in this area. The effect of CO_2 on fungal morphogenisis is well established (for reviews see Tabak & Cooke, 1968; Smith & Galbraith, 1971). This role of CO_2 has lead to its consideration as a regulator of fungal growth and metabolism. Reviewing some early work, Rockwell & Highberger (1927) stated that CO_2 is an essential prerequisite to the growth of bacteria, yeasts and moulds. Subsequent studies have considered CO_2 as a fungal growth stimulent; in *Rhizopus* (Barinova, 1954); *Blastocladiella* (Cantino & Horenstein, 1956);

Fusarium (Stover & Frieberg, 1958); soil fungi (Macauley & Griffin, 1969)and a number of coprophilous pyrenomycetes (Harvey & Hodgkiss, 1972).

The effect of CO_2 as an inhibitor of fungal growth has also been examined. Tabak & Cooke (1968) concluded that gas mixtures containing in excess of 95% CO_2 are, in general, inhibitory. Lengyel & Nyiri (1967) found a concentration of 1% CO_2 to be optimal in the penicillin fermentation. Higher concentrations resulted in decreased growth rates and antibiotic production. The term "ventilation" has been coined by Nyiri & Lengyel (1968) in describing CO_2 transfer processes to distinguish them from aeration effects which are concerned with oxygen transfer and availability.

(ii) The influence of CO_2 on some fungal fermentations.

The importance of CO_2 fixation in the commercial production of organic acids by fungi is well established. Citric acid, which is formed from hexoses by *Aspergillus niger* (Cleland & Johnson, 1954); itaconic acid (from *Aspergillus terreus:* Bentley & Thiessen, 1957) and fumaric acid (from *Rhizopus nigricans:* Overman & Romano, 1969) all require the anaplerotic fixation of CO_2 for their synthesis. In practice, however, ventilation requirements in organic acid fermentations are arrived at empirically. Some quantitative studies have, however, been made with the penicillin fermentation. Nyiri & Lengyel (1965) found an "inhibitory critical level" of CO_2 above which penicillin production was impaired. In studies with a model system, Nyiri & Lengyel (1968) produced data describing the effects of culture viscosity, buffering and agitation on culture ventilation. The effect of increasing the mycelial concentration

or of raising the culture viscosity with sucrose-glucose solutions appeared to inhibit the hydration of CO_2, i.e. to slow down the rate of bicarbonate formation. Increasing the impeller speed affected the mass transfer dynamics of CO_2, resulting in an increase in the rate of hydration.

IV.2. MATERIALS & METHODS.

(a) Enzyme assays.

(i) Extraction procedure.

(ii) Phosphoenol pyruvate carboxylase assay.

(iii) Pyruvate carboxylase.

(iv) Isocitrate lyase.

(b) Estimation of *in vivo* CO_2 incorporation.

(c) Fractionation procedure.

(d) Two stage fermenter studies.

IV 2 - MATERIALS & METHODS.

(a) Enzyme assays.

(i) Extraction procedure.

All assays were carried out on crude extracts prepared as follows.

Actively growing mycelium was removed from the fermenter vessel and rapidly filtered as described in III.2.a.iv. For enzyme assays, however, mycelium was washed with distilled water at $0^{o}C$ instead of buffer. Resuspended mycelium was then homogenised in a Braun tissue disintegrator (III.2.a.vi) at $0^{o}C$ for 3 min. The supernatant was collected after centrifugation at 1000g for 10 min and combined with 2 further washings from the cell debris in the sediment. Extracts were either freezedried or tested immediately for enzyme activity.

Enzyme assays carried out on the sediment after centrifugation and washing indicated little or no activity of anaplerotic enzymes.

(ii) Phosphoenol pyruvate carboxylase (PEPC) assay.

When purified from the Enterobacteriaceae, this enzyme is activated by acetyl-CoA (Canovas & Kornberg, 1966) and fructose 1,6 diphosphate (Sanwal & Maeba, 1966). Addition of organic solvents, e.g. dioxan and methanol or polycations, e.g. poly-L-lysine and polyamines also activate the enzyme and protect it, to some extent, from inhibitors (Sanwal, Wright & Smando, 1968). PEPC from other bacterial sources is also activated by acetyl CoA and inhibited by aspartate (Din, Suzuki & Lees, 1967). No bound co-factors have been detected in this enzyme.

Spectrophotometric assays (e.g. Ashworth & Kornberg, 1966) have been described for PEPC but results are subject to interference from NADH oxidase in crude preparations.

In the present study, a radiochemical adaptation of the spectrophotometric method of Canovas & Kornberg (1966) was used. In the original procedure, the formation of oxaloacetate from the carboxylation of PEP was measured by coupling its reduction to malate (with malate dehydrogenase) with simultaneous oxidation of $NADH_2$; the rate of change of extinction at 340nm gave the initial rate of NADH oxidation and hence enzyme activity. Experiments were carried out on *Aspergillus nidulans* extracts with $NADH_2$/malate dehydrogenase system present, to monitor any feedback inhibition of PEPC by oxaloacetate.

The composition of the reaction mixture for PEPC assays is shown in Table 6.

Semicarbezide was included as a trapping agent for any pyruvate present. This prevented the activity of any pyruvate kinase or pyruvate carboxylase, thereby preventing the possibility of erroneous results from these enzymes.

"Back-ground" control mixtures lacking PEP were included in each set of assay tubes.

When experiments to determine the effect of omission of each of the assay mixture components were made, (see IV.3) it was found that acetyl CoA and the $NADH_2$/malate dehydrogenase system could be left out without causing significant decrease in enzyme activity. Therefore, these components were omitted in all subsequent determinations.

The reaction was started by the addition of enzyme extract and terminated by the addition of 0.2ml of a mixture of ethanol and acetic acid (19:1 (v/v).

The ^{14}C activity of dried-down reaction samples was estimated in a Packard "Tricarb" liquid scintillation spectrometer (green channel, zero amplification). Counting efficiency was estimated from external standardisation values and a quenching curve for the scintillator (6 gl^{-1} butyl-PBD dissolved in toluene-methanol mixture, 3:1 v/v) and assay components.

The assay procedure used is somewhat similar to that used for the gluconeogenic enzyme phosphoenol pyruvate carboxykinase (PEPCK) which is assayed when operating in the reverse direction to its physiological function. To differentiate between PEPC and PEPCK, the rationale of Vennesland (1962); Scrutton (1971); and Divjak & Mor (1973) was followed. These authors have found ADP requirement for PEPK in crude extracts and have used this criterion to distinguish between the two enzymes. Further evidence that PEPC is, in fact, being measured is provided by the finding that the bulk of $H^{14}CO_3$ is incorporated, initially, into protein (see IV.3). This result is highly suggestive of anaplerotic metabolism. Also, the pH optimum of 8.7 (IV.3) is consistent with reported values for other PEP carboxylases and is quite distinct from that of carboxykinases.

(iii) Pyruvate carboxylase.

Three types of pyruvate carboxylase can be distinguished depending on the requirement for acetyl-CoA. Enzymes of the first category are active in the absence of acetyl-CoA and include the PC from *Aspergillus niger* (Bloom & Johnson, 1962). The second type, isolated from various yeasts, is activated by the addition of acetyl-CoA but is partially active in its absence (Cazzulo & Stoppani, 1965). The third category of

TABLE 6.

Composition of the reaction mixture for the assay of phosphoenol pyruvate carboxylase.

Volume (ml)		Final quantity in reaction mixture (µmol)
0.5	Tris-HCl, pH 7.8[1]	50
0.1	MgCl	10
0.1	NaH CO (1 Ci mol^{-})	25
0.1*	Acetyl-CoA	0.5
0.1	Semicarbazide, pH 7.0	50
0.1*	Malate dehydrogenase (Boehringer)	2 units
0.1*	NADH	0.2
0.1	PEP (Sodium salt, Sigma)	10
0.3**	Crude enzyme extract	
1.5ml		

1. Payne & Morris, 1969

* omitted in later assays (see text)

** 0.5ml used in later assays, distilled water being added to bring the final volume to 1.5 ml.

of enzymes is inactive in the absence of acetyl CoA, e.g. PC from *Arthrobacter globiformis* (Bridgeland & Jones, 1967).

The assay procedure used here was identical to that employed for PEPC, except that semicarbazide and PEP were omitted and pyruvate (potassium salt, Sigma, London) and ATP (Boehringer, Mannheim) were added (10µmol and 0.5µmol respectively in 1.5ml final volume). In the crude extract, however, it was found that ATP could be omitted without significant loss in enzyme activity.

The assay conditions were established before the *in vitro* pH optima were estimated. The pH value (7.8) used for the buffer was, consequently, not optimal.

Enzyme activity for both carboxylases was defined as µmol of HCO_3 fixed per mg protein in 1 h.

(iv) Isocitrate lyase.

The reversible reaction catalysed by isocitrate lyase (ICL), (see IV.1.a.i) can be assayed in either the forward (succinate formation) or the backward (isocitrate formation) direction. Assay methods for measuring the forward reaction usually depend, in principle, on detecting the glyoxalate formed (Daron & Gunsalus, 1962). The back reaction is measured using a coupled system to assay the isocitrate formed (Daron, Rutter & Gunsalus, 1965).

The technique employed for the present study was similar to that of Daron & Gunsalus (1962). The formation of the phenylhydrazone derivative of glyoxalate was measured at 324nm. The rate of formation of glyoxalate from isocitrate in the presence of the enzyme could therefore be estimated. Details of the reaction mixture is given in Table 7.

TABLE 7

Composition of the reaction mixture for isocitrate lyase assay.

Volume (ml)		Final quantity in reaction mixture (μmol)
0.5	*Imidazole-HCl buffer, pH 6.8	100
0.2	MgCl	20
0.5	glutathione (reduced)	10
0.5	EDTA	25
1.0	phenyl hydrazine-HCl	1
0.2	isocitrate (Na salt, Sigma)	10
0.1	crude enzyme extract	
3.0ml		

*J. H. Slater, 1973; personal communication.

The reaction was started by the addition of isocitrate and phenyl hydrazine to the other components. Incubation took place at 30°C and the absorbance was measured in a Unicam SP600 recording spectrophotometer. Enzyme activities were estimated from the slope of the curve produced by the chart recorder.

Sodium glyoxalate (Sigma) was used as a standard.

(b) Estimation of *in vivo* CO_2 incorporation.

The rate of incorporation of CO_2 into *Aspergillus nidulans* mycelium, growing in steady state chemostat cultures, was estimated using a replacement culture technique.

Aliquots (9ml) of medium (I.2.b), without carbon source, were placed in 50 ml conical flasks, closed with vaccine bottle rubber seals, and equilibrated at 30°.

Actively growing mycelium was removed from the fermenter vessel and 1 ml quantities filtered, (II.2.a) washed with buffer (III.2.a.ii) at 30°C, and rapidly resuspended in the conical flasks.

After allowing 1 min for equilibration, 0.1 ml of ^{14}C-$NaHCO_3$ (specific activity, 0.1 μCi μmol^{-1}) was added. The final concentration of bicarbonate in the flask was exactly equivalent to that in the fermenter (estimated using the methods described in I.2.f). The reaction was stopped by pipetting 1 ml samples from the flasks into 2 ml quantities of ethanol-acetic acid mixture (19:1 v/v) in scintillation vials. The samples were evaporated to dryness and then solubulised by incubation in 1 ml concentrated formic acid. Incorporated $^{14}CO_2$ was estimated with the scintillator and technique described.

(c) Fractionation procedure.

Fractionation of the mycelium of *Verticillium albo-atrum* has been carried out using the method of Roberts, Abelson, Cowie, Bolton & Britten (1965) without modification (Hartman Keen & Long, 1972). The procedures of Roberts et al was, therefore, adopted to elucidate the assimilation of fixed $^{14}CO_2$ by *Aspergillus nidulans*. Filtered mycelium from the fermenter was resuspended in growth medium (I.2.b); containing 15g glucose l^{-1} in 600ml conical flasks fitted with magnetic stirrers. The stirred cultures were incubated at $30^{o}C$ in the presence of ^{14}C-HCO_3. Samples were removed from the flasks at intervals, filtered, and then frozen. The filtered growth liquor was acidified with 2N HCl (1.8ml medium plus 0.2ml HCl) and flushed with nitrogen to remove any residual ^{14}C-HCO_3. The radioactivity of this solution was then determined to monitor the ^{14}C labelling of any extracellular products.

The frozen samples were treated according to the scheme of Roberts et al (1965) and the radioactivity of each fraction measured.

(d) Two-stage fermenter studies.

A 1 L capacity fermentation vessel (L.H. Engineering Ltd., Stoke Poges, Bucks) consisting of a QVF glass jar and a stainless steel head-plate, was connected, via a 50 cm length (6mm internal diameter) of silicone tubing to the 3.5 L fermentation vessel described previously (see Plate I). When the two stage system was in operation, material from the 3.5 L unit was pumpled by means of a flow inducer (Watson-Marlow) into the second stage. At the dilution rates employed, material stayed

in the connecting tube for a maximum of 13 min. The take-off point for the culture leaving the 3.5 L unit was a 7 cm length of Teflon tubing (7mm internal diameter) which was held by a rubber bung in a port in the base-plate of the fermenter vessel. Culture entered the 1 L unit through a similar length of Teflon tubing, set in the head-plate, which dipped below the surface of the culture broth.

Agitation in the 1 L vessel was effected by means of a magnetic stirrer and temeprature was controlled by means of a probe which carried water at 31°C, heated and circulated by a Churchill pump.

The culture flowed out of the second stage vessel through a siphon set in the head-plate, using the same principle as that described for the overflow from the 3.5 L vessel (I.2.e.).

3. RESULTS & DISCUSSION

(a) Phosphoenol pyruvate carboxylase and pyruvate carboxylase assays; effect of reaction mixture components.

(b) Batch cultures.

(i) Glucose-grown cultures

(ii) Acetate-grown cultures

(iii) Complex and supplemented media

(c) The effect of dilution rate on anaplerotic CO_2^- fixing enzymes.

(d) The effect of dilution rate upon mycelial HCO_3^- fixing activity.

(i) Replacement cultures

(ii) Rates of HCO_3^- fixation at different steady states

(e) The effect of variation of the HCO_3 concentration of the medium upon HCO_3 incorporation

(f) Transient states

(g) Fractionation of mycelium

(h) Two-stage experiments

IV. 3. RESULTS & DISCUSSION.

(a) Phosphenol pyruvate carboxylase and pyruvate carboxylase assays; effect of reaction mixture components.

The activities of PEPC and PC were estimated in crude extracts from glucose grown batch cultures of *Aspergillus nidulans*. Mycelium was harvested 24 h. after spore inoculation. Results obtained using different combinations of reaction mixture components are shown in Table 8.

Acetyl CoA gave no significant increase in PEPC activity, probably due to the presence of excess coenzyme in the crude extract. The malate dehydrogenase/$NADH_2$ system was also, apparently, redundant in the radiochemical assay, and, like the acetyl CoA, was omitted from subsequent assay reaction mixtures.

The effects of $MgCl_2$ and PEP omissions are consistent with observations of Canovas & Kornberg (1966). The drop in counts observed in tubes lacking PEP show a background incorporation into cell sap of 2.1% of the total incorporation in the complete reaction mixture.

Acetyl CoA, malate dehydrogenase/$NADH_2$ and ATP appeared to have little effect upon PC activity in crude extracts. Accordingly, these components were omitted from subsequent assay mixtures.

(b) Batch cultures.

(i) Glucose-grown cultures.

Figure 32 shows the variation of PEPC and PC activities with time in the batch culture of *Aspergillus nidulans*. Cultures were grown in the defined medium (I.2.b) containing 15 gl^{-1} glucose. PEPC activity reached a maximum (1.1×10^{-2} µmol HCO_3

TABLE 8.

(a) PEPC assay; the effect of the reaction mixture components.

The complete reaction mixture is shown in Materials & Methods, Table 6.

Component omitted	Radioactivity incorporated (dpm)
none	168835
$-MgCl_2$	1664
-Acetyl CoA	166436
$-MDH/NADH_2$	171235
-PEP	3601

(b) PC assay; the effect of the reaction mixture components.

Component omitted	Radioactivity incorporated (dpm)
none	52875
$-MgCl_2$	977
-Acetyl CoA	57478
$-MDH/NADH_2$	48275
-pyruvate	5047
-ATP	56275

Figure 32.

The variation of anaplerotic CO_2-fixing enzyme activities with time in glucose-grown batch cultures, following spore inoculation.

O Phosphoenol pyruvate carboxylase activity

Δ Pyruvate carboxylase activity.

Enzyme activity (μmol HCO_3 fixed mg protein^{-1}h^{-1})

1.0 x 10^{-2}

5.0 x 10^{-3}

0

PEPC

PC

10 20 30 40

Time elapsed since spore inoculation (h)

fixed mg protein^{-1} h^{-1}) 21 h after spore inoculation. The activity then declined with time, reaching undetectable levels by 42 h. PC and PEPC were not detectable during the first 11 h following spore inoculation. During germ tube emergence from the spores (12 h after inoculation) PEPC became detectable (activity 4.9×10^{-3} µmol mg^{-1}h^{-1}). PC was also detectable at this time, showing very much lower activity from PEPC (1.5×10^{-3} µmol mg^{-1}h^{-1}). This activity was the maximum obtained for PC in batch culture and declined to undetectable levels 15 h after inoculation.

Maximum PEPC and total enzyme activity therefore, were obtained during the mid exponential phase of batch culture.

No isocitrate lyase activity was detected in glucose-growth batch cultures, indicating that the glyoxalate cycle was inoperative under conditions of glucose catabolism.

The data observed are not consistent with those reported by Divjak & Mor (1973) who noted a glucose repression of PEPC in cultures of *Saccharomyces cerevisiae*. This enzyme was undetectable until all the glucose in the culture had been exhausted. Similar experiments with *Candida tropicalis* and *Schizosaccharomyces pombe* were also described; in both cases intracellular PEPC levels increased as glucose in the medium became depleted. This lead the authors to conclude that PEPC was acting as a gluconeogenic enzyme in a manner analogous to that of PEPCK activity (IV.1.b.). Moreover, Divjak & Mor (1973) also stated that PEPCK acts as a CO_2 fixing enzyme in vivo, a statement which was based upon circumstantial in vitro evidence.

The observations of Divjak & Mor (1973) appear to conflict with the majority of available literature in this area, although

similar unsubstantiated hypotheses on PEPCK-catalysed CO_2 fixation in vivo have been offered (IV.1.b). The notion that PEPCK fixes CO_2 in vivo (believed by the present author to be erroneous) reflects a certain amount of confusion in the literature. In some early studies, the enzyme now designated PEPCK (EC.4.1.1.32) was originally referred to by the trivial name of phosphopyruvate carboxylase (e.g. Cannata & Stoppani; 1959,1963) a name which was later adopted for PEPC (EC.4.1.1.31) (e.g. Divjak & Mor, 1973) which is an anaplerotic enzyme, fixing CO_2 in vivo (IV.1.b). Further confusion has arisen because both PEPCK (EC.4.1.1.32) and PEPC (EC.4.1.1.31) are assayed in vitro by following bicarbonate incorporation into PEP to form oxaloacetate. This situation has been somewhat clarified by Torrontegui, Palacian & Losada (1966) a publication which, ironically, was cited by Divjak & Mor (1973) to support their hypothesis of PEPCK catalysed in vivo CO_2 fixation. Torrontegui et al quoted the original characterisation of PEPCK (Cannata & Stoppani, 1963) in which in vitro CO_2 fixation by the enzyme was thought to reflect its in vivo activity. A number of publications were then cited which described another enzyme (PC) responsible for anaplerotic CO_2 fixation. Torrontegui also presented convincing experimental evidence that PEPCK has a gluconeogenic function in vivo whereas CO_2 fixation is catalysed by PC. This conclusion was consistent with other evidence from the same laboratory (Ruiz-Amil, Torrontegui, Palacian, Catalina & Losada, 1965).

In the light of evidence from the present study, it appears that an alternative explanation for the glucose repression of PEPC observed by Divjak & Mor (1973) must be sought as this

enzyme shows partial glucose-induced repression in *Aspergillus nidulans* (see IV.3.c). In vitro rates of CO_2-fixation by PEPC, moreover, correlate well with in vivo CO_2 fixation by intact, *A.nidulans* mycelium (IV.3.c).

(ii) Acetate-grown batch cultures.

No PEPC or PC activity was observed when *Aspergillus nidulans* was grown in batch cultures with 0.1M acetate as carbon source. Cultures were harvested 12 h and 23 h after inoculation into defined medium (I.2.b). The activity of isocitrate lyase, (ICL) which is indicative of the glyoxalate cycle (IV.1.a) was 4.971 x 10^{-4} mmol glyoxalate formed per mg cell protein per hour in the 23 h sample. No enzyme activity was detected in the 12 h sample, however. The specific activity obtained was 0.3% of that obtained by McFadden (1969) from a high yielding strain of *Pseudomonas indigofera*, and 35% of the activity observed by Wegener & Romano (1964) in *Rhizopus nigricans* growing on a complex medium. Reported activities of 1CL in *Aspergillus niger* (Collins & Kornberg, 1960) are not directly comparable as enzyme activity units are expressed as µmol h^{-1}mg dry weight^{-1} in this publication. Assuming an equivalent yield of active protein in *A.niger* to that obtained in *A.nidulans*, however, the date obtai ned by Collins & Kornberg and the present author appear to be in close agreement.

The presence of 1CL indicates the activity of a functioning glyoxalate cycle in *Aspergillus nidulans* in acetate grown cultures. As no glyoxalate cycle activity was detected in glucose-grown cultures, these data appear to support the hypothesis that the glyoxalate cycle is repressed by glucose (Kornberg, 1966; Tokunga, Malca, Sims, Erwin & Keen, 1969).

(iii) Complex and supplemented media.

To elucidate the effect of growth medium on PEPC and PC activity, stirred flask cultures were prepared using defined medium (I.2.b) and yeast extract (Oxoid, London) medium with and without biotin supplementation (5μg ml^{-1}, final concentration). The enzyme activities obtained are shown in Table 9. Cultures were harvested when mycelial biomass had reached 4 gl^{-1} (mid exponential phase, 26 h after spore inoculation).

PEPC activity in defined medium was three times higher than that observed in yeast extract without supplementation. When the supplemented media were compared, it was found that activity in defined media showed a 5-fold increase over that in yeast extract. Considering the same media with and without additions, biotin supplementation gave a 2-fold increase in defined medium but activity was increased by only 9% when the yeast extract medium was supplemented.

In contrast, PC activity was greater in yeast extract than in defined medium. In non supplemented medium, the activity was 3.1×10^{-4} μmol $mg^{-1}h^{-1}$ in yeast extract but was undetable in defined medium. Addition of biotin to defined medium produced a PC activity of 1.16×10^{-4}μmol $mg^{-1}h^{-1}$ which was increased 3-fold in cultures grown on supplemented yeast extract. Supplementation of yeast extract medium with biotin gave an increase of only 18% over enzyme activity in cultures grown in the non-supplemented medium.

Yeast extract is a potent source of growth stimulatory substances, (Woodin, 1959) including biotin. The observation that further biotin addition gave little increase in PC activity in yeast extract grown cultures suggests that the stimulation of

TABLE 9.

Medium	Enzyme Activity	
	Phosphoenol pyruvate carboxylase	Pyruvate carboxylase
Defined medium	1.10×10^{-2}	-
Defined medium + biotin	2.11×10^{-2}	1.6×10^{-4}
Yeast extract	3.88×10^{-3}	3.10×10^{-4}
Yeast extract + biotin	4.28×10^{-3}	3.76×10^{-4}

The effect of medium composition upon anaplerotic CO_2-fixing enzyme activity (units; µmol bicarbonate fixed per mg protein per hour).

enzyme activity in yeast extract, compared with defined medium, is mostly due to its biotin content. If this is the case, then 5µg biotin ml^{-1} was not a saturating concentration; the enzyme activity in yeast extract was 3.6 times that in defined medium with biotin. This observation is consistent with all the available literature on PC activation, all pyruvate carboxylases so far examined having a requirement for biotin (Scrutton, 1971).

No bound co-factors (e.g. biotin) have been detected in PEPC, however (Scrutton, 1971). The slight stimulation produced by biotin may be due to the increased synthesis of an activation substance (e.g. acetyl CoA, Canovas & Kornberg, 1966) catalysed by a biotin-requiring enzyme. The decreased activity of PEPC in yeast extract is probably due to inhibition by amino acids (Maeba & Sanwal, 1965; Nishikido, Izui, Iwatani, Katsuki & Tanak, 1965) which are present in great abundance in this medium.

(c) The effect of dilution rate on anaplerotic CO_2-fixing enzymes.

Figure 33 shows the change in PC and PEPC activities of *Aspergillus nidulans* growing at different dilution rates. Enzyme activities were significantly greater in carbon limited continuous cultures than maximum activities observed in batch cultures. Maximum PEP activity in the chemostat was 1.1 µmol $mg^{-1}h^{-1}$ which is a one hundred fold increase over activity observed in non-supplemented batch cultures. PC activity showed a 17 fold increase over batch cultures, maximum activity in the chemostat cultures being 2.5×10^{-2} mol $mg^{-1}h^{-1}$.

The levels of activity and relative predominance of both enzymes were dilution rate-dependent. PC decreased in activity as the dilution rate was increased whilst PEPC activity peaked at a dilution rate of 0.11 h^{-1}.

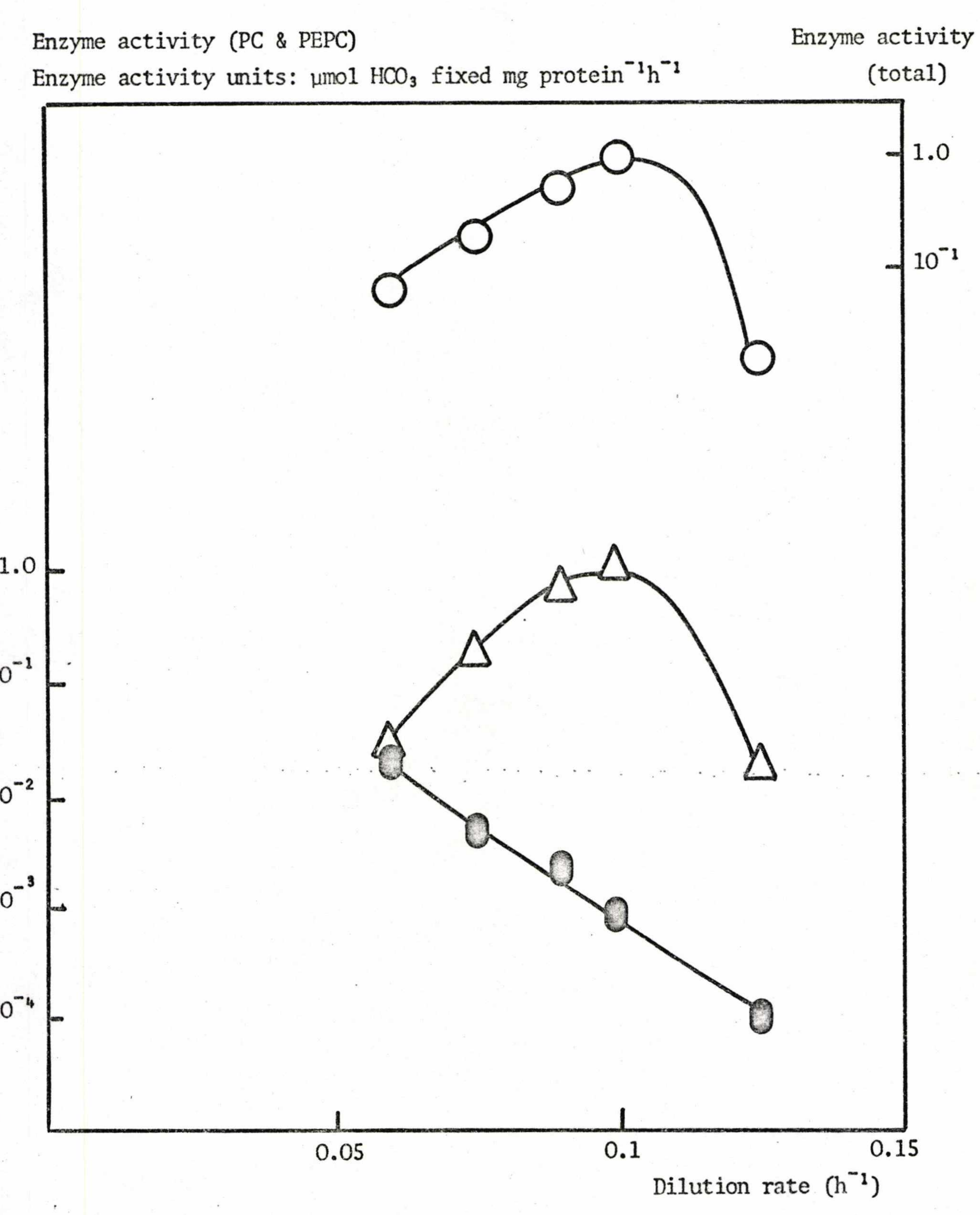
Figure 33.

Semilogarithmic plot of CO_2 fixing enzyme activity against dilution rate.

O Total enzyme activity (PC + PEPC)

Δ PEPC activity

● PC activity



Enzyme activity (PC & PEPC)
Enzyme activity units: µmol HCO₃ fixed mg protein⁻¹h⁻¹
Enzyme activity
(total)
1.0
10⁻¹
1.0
10⁻¹
10⁻²
10⁻³
10⁻⁴
0.05
0.1
0.15
Dilution rate (h⁻¹)

Changes in enzyme activity with changing dilution rate are not uncommon in the literature. Moreover, Dean (1972) has stated that this effect is observed far more frequently than constant enzyme levels with changing dilution rate, points of inflection in plots of enzyme activity against growth rate often being observed.

The decrease in anaplerotic enzyme activity at high dilution rates may be consistent with observations of Mian, Fencl & Prokop (1969) that the activity of the enzyme isocitrate dehydrogenase increased at high dilution rates in sucrose-limited *Candida utilis* cultures. Isocitrate dehydrogenase is thought to be a competitive regulator of anaplerotic metabolism by the glyoxalate cycle, competing with ICL for isocitrate (Holmes & Bennet, 1971). Increase in isocitrate dehydrogenase activity at high dilution rates is, therefore, indicative of a decreased requirement for replenishment of the TCA cycle by anaplerotic pathways.

An alternative means of rationalisation of these data is to compare anaplerotic enzyme activity with hyphal protein levels (Figure 19).

The curves in Figure (19) and Figure (33) both pass through maxima at approximately 0.11 h^{-1}, showing similar decreases as the dilution rate is raised or lowered from this value.

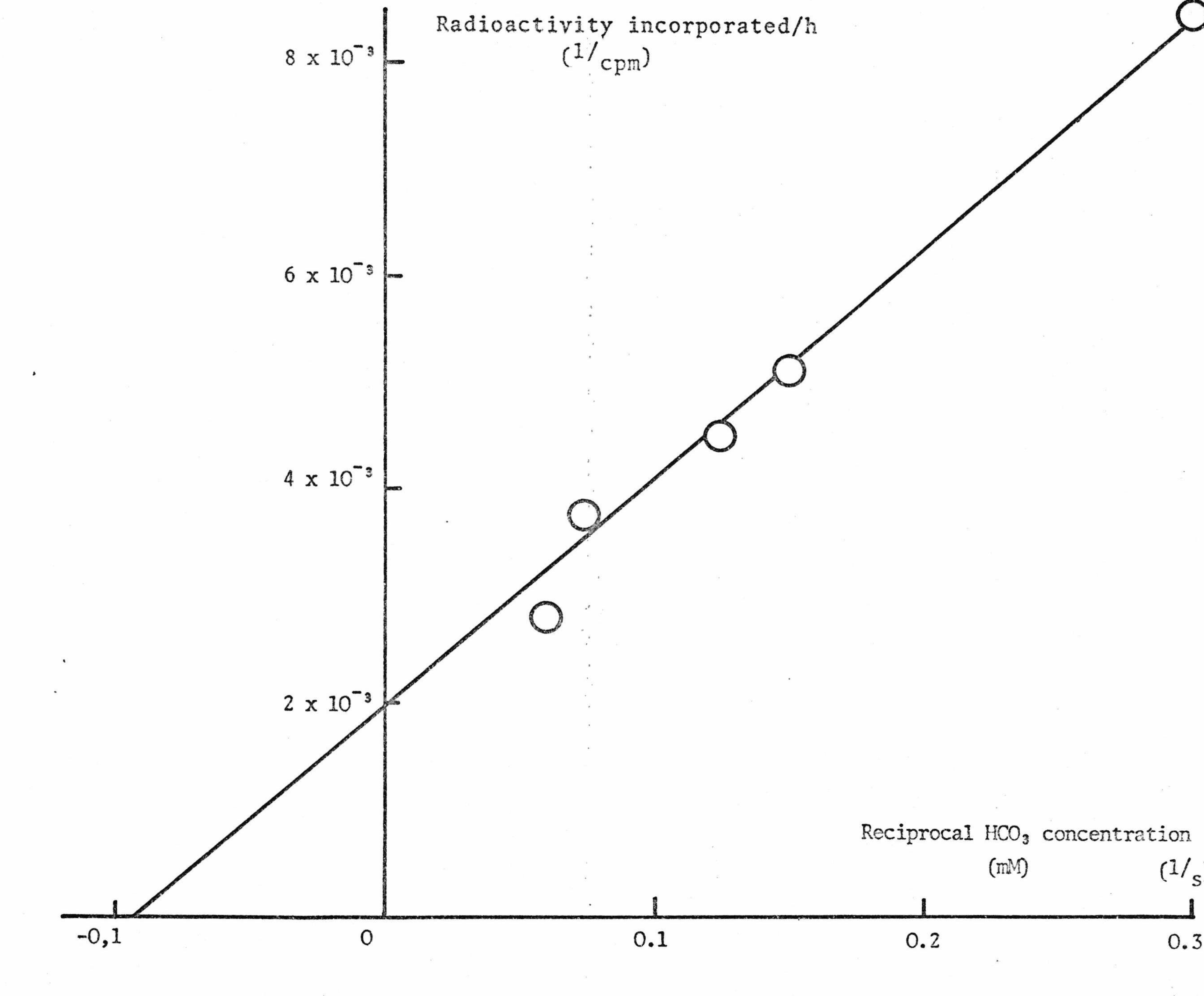
As the primary product of carboxylase-catalysed CO_2-fixation is protein, (see IV.3.g) the variation of total carboxylase activity with dilution rate (Figures 33) may reflect the variation of demand for anaplerotic metabolism as a response to variation in protein synthesis rates.

The growth rate-dependent change in predominance of the two

Figure 34.

Double reciprocal plot of pyruvate carboxylase activity against rate-limiting substrate (HCO_3) concentration.

Km = 10.86 mM

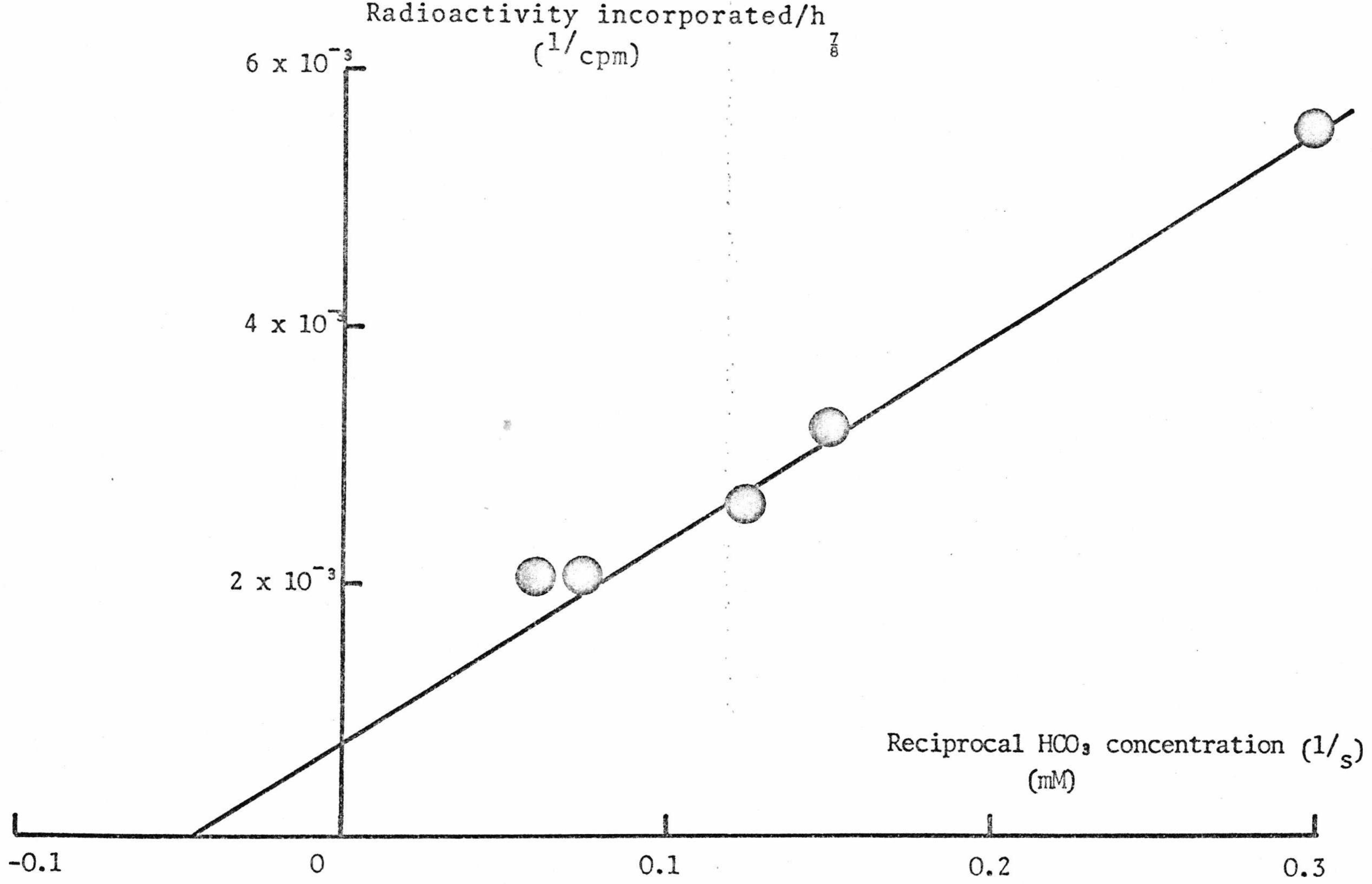


Radioactivity incorporated/h
(1/cpm)
8 x 10⁻³
6 x 10⁻³
4 x 10⁻³
2 x 10⁻³
Reciprocal HCO₃ concentration
(mM)
(1/s)
-0,1
0
0.1
0.2
0.3

Figure 35.

Double reciprocal plot of phosphenol pyruvate carboxylase activity against rate-limiting substrate (HCO_3) concentration.

Km = 21.7 mM



Radioactivity incorporated/h
($^1/$cpm)
6 x 10^{-3}
4 x 10^{-3}
2 x 10^{-3}
Reciprocal HCO_3 concentration ($1/_s$)
(mM)
-0.1
0
0.1
0.2
0.3

carboxylases may be related to their maximum reaction velocities. The Michaelis constants (Km) for PC (Figure 34) and PEPC (Figure 35) were determined from Lineweaver-Burk plots. The Km PEPC (21.7mM) is exactly twice that of PC (10.86 mM). As both sets of data produce linearity in the double reciprocal plot, the enzyme reaction rates may be assumed to follow ideal Michaelis-Menten kinetics. If this assumption is true, then the maximum rate of PEPC activity will be nearly three times that of PC (calculated from the y axis intercepts in figures 34 and 35). The change-over from pyruvate to PEP as the primary CO_2-acceptor molecule at higher growth rates could, perhaps, reflect the necessity for a higher rate of CO_2-fixation to keep pace with more rapid cell division rates. The preferential operation of PC at low growth rates is not explicable in this way, however. Although the conversion of PEP to pyruvate is energy yielding, the subsequent carboxylation is ATP-consuming. It is difficult, therefore, to conceive a teleological reason for the predominance of pyruvate carboxylation where enzyme capacity exists.

(d) The effect of dilution rate upon mycelial HCO_3-fixing activity.

(i) Replacement cultures.

Samples of mycelium growing at a dilution rate of 0.04 h^{-1} were treated as described in IV.2.b. Incorporation of ^{14}C-labelled bicarbonate was then followed for 40 min. The bicarbonate concentration in the replacement culture was made identical to that in the fermenter (2.5 mM) and the amount of ^{14}C-HCO_3 incorporated into the mycelium increased linearly with time (see Table 10). When the HCO_3 concentration in the replacement culture was halved, the initial rate of incorporation

fell by 34% and decreased to zero after 20 min. The addition of cycloheximide, which inhibits de novo enzyme synthesis, had no effect on the rate of incorporation, initially, but, after 30 min, no further increase in radioactivity with time could be detected. The addition of 1 mg ml^{-1} glucose caused a decrease of 39% in the rate of incorporation. When 5 mg ml^{-1} glucose was included in the replacement medium, however, the decrease in incorporation was 32% and inclusion of glucose at 10 mg ml^{-1} reduced the percentage decrease in activity to 18%.

The lack of effect of cycloheximide on the rate of HCO_3 incorporation indicates that HCO_3 incorporation measured over the first 34 min of incubation proceeded without additional enzyme synthesis and that no turnover of the enzymes responsible for fixation took place.

The rate of HCO_3 incorporation into intact mycelium decreased when the concentration of HCO_3 in the medium was lowered. This suggested that fixation rate could be limited by the concentration of environmental HCO_3.

The findings of the present study suggest an analogy between the control of the two carboxylase enzymes and the phenomenon of catabolite repression, observed in prokaryotes (Loomis & Magasnik, 1964). The observation that these enzymes are undetectable in acetate-grown batch cultures but detectable if glucose is the carbon source, suggests glucose induction of PC and PEPC. The reduction in CO_2 fixation when excess glucose was added to carbon limited cultures suggests a catabolic repression. Catabolite repression by glucose would, also, result in an increase in enzyme activity in carbon limited cultures over batch cultures. The maximum observed when PEPC activity is plotted

TABLE 10.

Incorporation of ^{14}C-labelled bicarbonate into *Aspergillus nidulans* mycelium taken from a glucose-limited chemostat (0.04 h^{-1}) with a culture medium bicarbonate concentration of 2.5 mM.

Replacement medium composition.	Incorporation rate ($\mu M HCO_3$ ml^{-1} h^{-1})
No additions	4.7×10^{-3}
1.25 mM HCO_3	3.1×10^{-3} (initial rate
Cycloheximide (1.5mg ml^{-1})	4.7×10^{-3}
Glucose (1 mg ml^{-1})	2.86×10^{-3}
Glucose (5 mg ml^{-1})	3.19×10^{-3}
Glucose (10 mg ml^{-1})	3.85×10^{-3}

as a function of dilution rate is also consistent with this motion. Clarke, Houldsworth & Lilly (1968) observed a maximum in the steady state values of the specific activity of an inducible amidase in carbon-limited *Pseudomonas aeruginosa* cultures. This was interpreted as a balance between induction and catabolic repression. Under carbon limitation, the residual concentration of the carbon substrate in the growth medium increases as a function of dilution rate; (II.3.f) catabolite repression and induction could, therefore, increase simultaneously (Clarke & Lilly, 1969).

The effect of glucose supplementation on HCO_3-fixation in replacement cultures requires an alternative explanation. (Circumstantial evidence has been obtained (see IV.3.f) for the activity of acetyl CoA carboxylase (EC.6.4.1.2) in replacement cultures in which mycelium growing under glucose limitation was removed to glucose-rich medium. The overall depression in the rate of HCO_3-fixation in glucose supplemented cultures is probably due to catabolite repression. The increase in incorporation with increasing glucose concentration, however, may reflect glucose-induced acetyl-CoA carboxylase activity, stimulated as a response to the "nutritional step-up" conditions in glucose-supplemented replacement cultures.

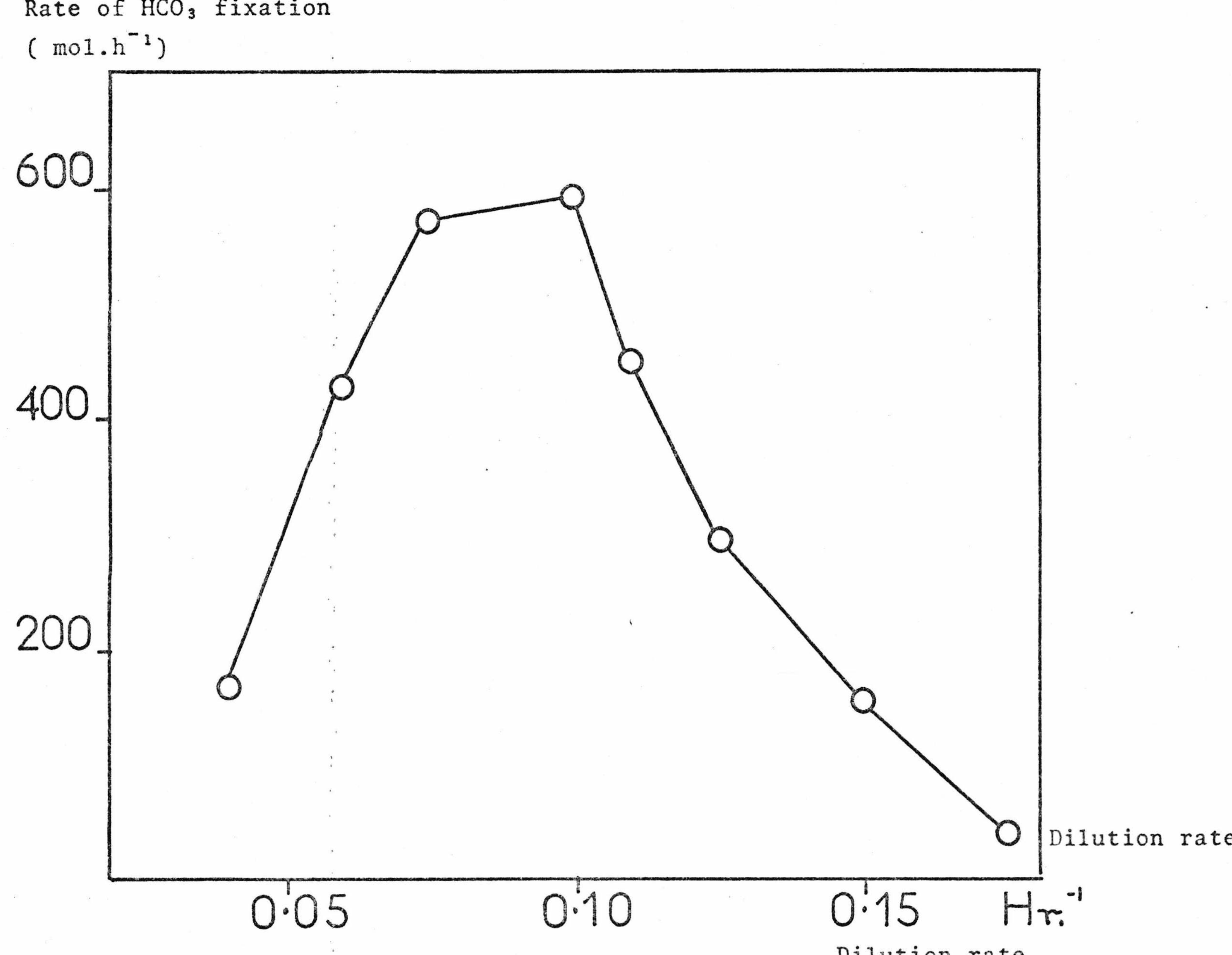
(ii) Rates of HCO_3-fixation at different steady-states.

Figure 36 shows the effect of dilution rate on the kinetics of incorporation of HCO_3 into whole mycelium. Incorporation was estimated using the procedure described, (IV.2.b) without supplementation of the incubation mixture. The levels of fixation observed were computed to be equivalent to the sum of the carboxylase activities measured. Also, incorporation rates

Figure 36.

Variation of rate of mycelial HCO_3 fixation (μmol HCO_3 fixed per hour) by 3.5 litre cultures of *Aspergillus nidulans* with dilution rate.

(pH 6.4; 3% ($v/_v$) CO_2 in the culture head-space).



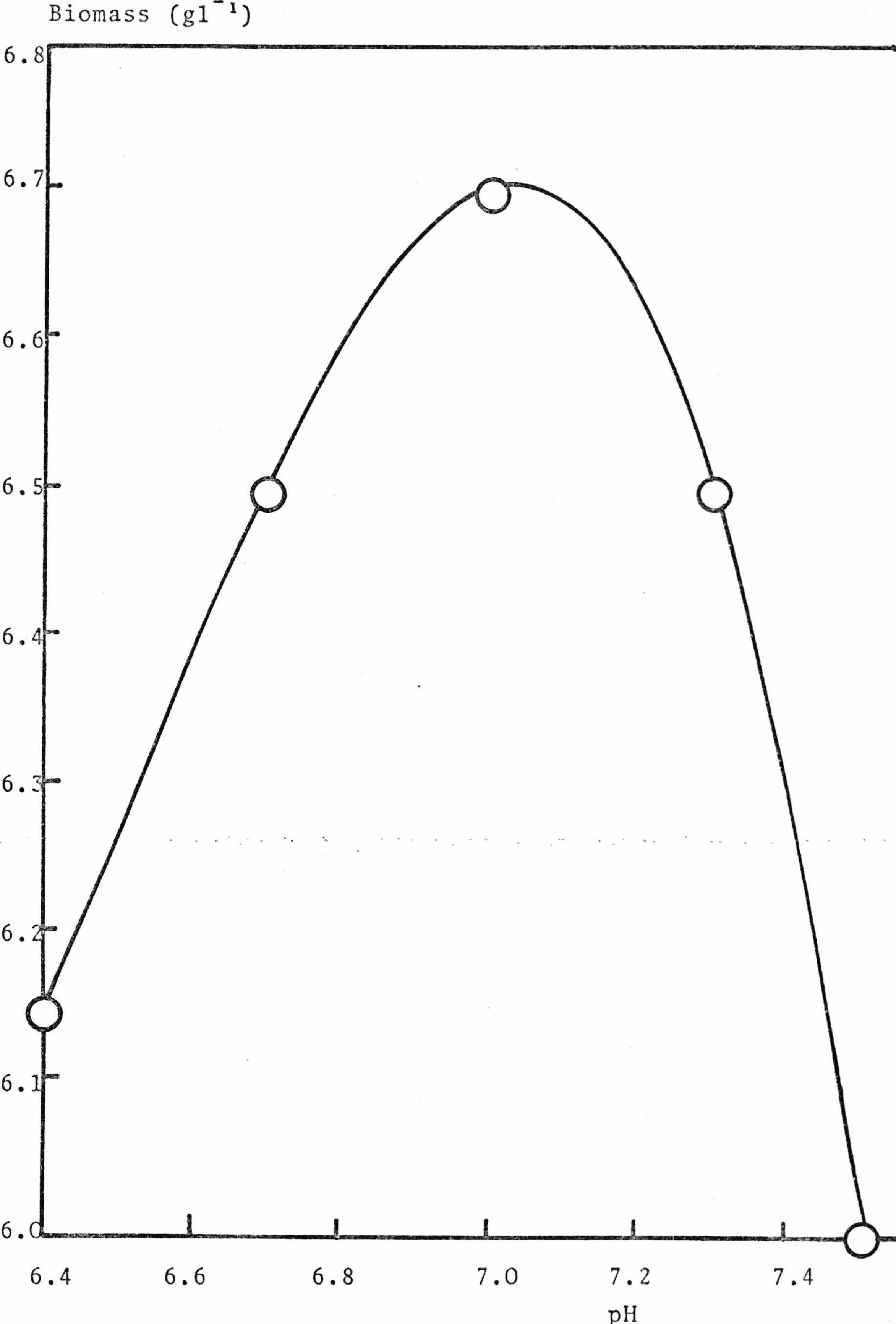
Rate of HCO_3 fixation
($mol.h^{-1}$)
600
400
200
Dilution rate
0·05
0·10
0·15
$Hr.^{-1}$
Dilution rate

reached a maximum at 0.11 h^{-1} which corresponded to the maximum observed enzyme activity. These results indicate that the most effective use of the carbon source for biomass production is being made at a dilution rate of 0.11 h^{-1}, in that less of it is "wasted" as effluent CO_2.

(e) <u>The effect of variation of the HCO_3 concentration of the medium upon HCO_3 incorporation.</u>

Figure 37 shows the effect of changing the culture pH on the steady state biomass at a dilution rate of 0.1 h^{-1}. Different pH values were attained by supplying the culture with medium containing KH_2/Na_2H PO_4 buffer with varying proportions of each phosphate salt. The effect of changing the pH of the culture whilst keeping the dissolved HCO_3 concentration (2.5mM) constant was to produce a symmetrical curve, passing through a maximum of 6.7 gl^{-1} at pH 7.05. To maintain a constant bicarbonate concentration, the rate of air supply to the fermenter was adjusted, thereby controlling the proportion of CO_2 in the head-space. The appropriate proportion of CO_2 was calculated using the Henderson-Hasselbalch equation (equation 6). The HCO_3 concentration then was checked using the probe, described in I.2.f.

The culture pH was also varied, keeping the gas-flow and, consequently, the proportion of CO_2 in the culture head-space, constant at 2%. This had the effect of varying the HCO_3 concentration. The steady-state biomass increased from 6.5 gl^{-1} (pH 6.4) to 8.5 gl^{-1} (pH 7.0), a difference of 30.8%. The biomass difference recorded when the pH varied but HCO_3 concentration was kept constant was 8%. Thus, the increase in biomass concentration obtained by optimising the HCO_3 concentration was approximately 23%.



Biomass (gl^{-1})
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
6.4
6.6
6.8
7.0
7.2
7.4
pH

Figure 37

Effect of culture pH on biomass concentration with dissolved HCO_3 concentration constant.

(Curve traced from computer optimisation out-put).

Figure 38 shows the change in HCO_3 concentration obtained when the pH was varied as described above. The corresponding change in rate of HCO_3 fixation are also plotted. Although the rate of incorporation increased slightly with pH at values above pH 7.0 (7×10^{-3}M HCO_3) no further increase in biomass was obtained, presumably due to the effects of suboptimal pH.

Figure 39 shows the effect of HCO_3 concentration on enzyme activity (PC and PEPC) as a function of culture pH. The variation in enzyme activity does not correlate with the in vitro pH optima of the enzymes (Figure 40) i.e. 7.55 and 8.1 for PC and PEPC respectively, but may reflect a partial regulation of PEPC activity by the concentration of HCO_3 available. Figure 39 indicates that PEPC activity increases as a function of increasing HCO_3 until a concentration of 7.5×10^{-3}M is obtained. The hypothesis tentatively suggested is that increasing the HCO_3 concentration in the medium increases the carboxylation of PEP accordingly, until a saturating concentration is reached (7.5mM HCO_3). Carboxylation of PEP would then result in a lower availability of PEP for pyruvate synthesis; the lower levels of pyruvate would then give rise to a smaller PC activity. The preferential utilisaiton of HCO_3 by PEPC is consistant with the relative magnitudes of the Km values of PC and PEPC.

(f) Transient States.

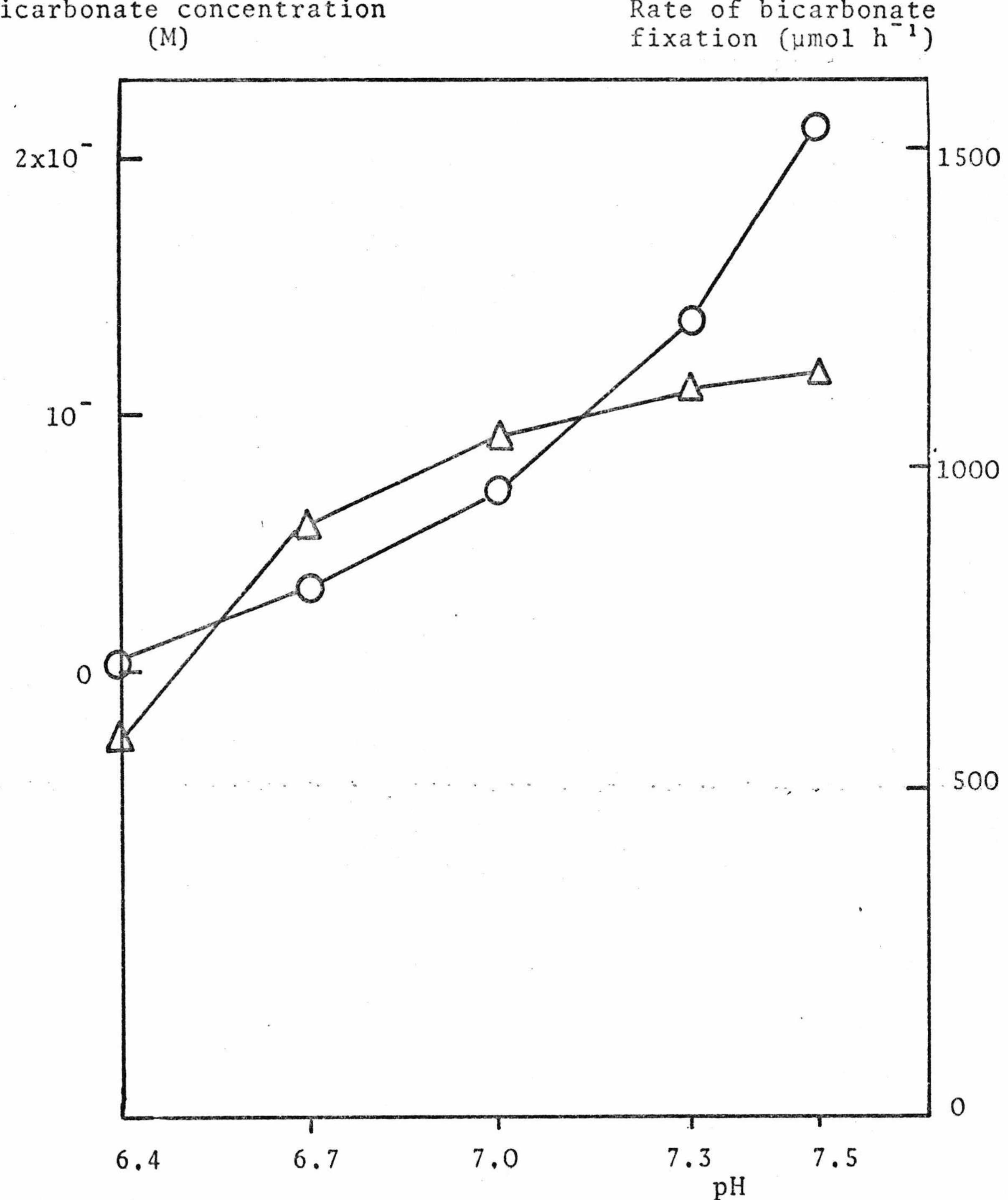
The fixation of HCO_3 by intact mycelium, the activities of PC and PEPC, and the rate of CO_2 production by glucose-limited continuous flow cultures of *Aspergillus nidulans* were measured in transient states following "step-up" ($D=0.125h^{-1}$ to $0.175h^{-1}$) and "step-down" ($D=0.175h^{-1}$ to $0.125h^{-1}$) changes of the dilution rate.

Figure 38.

Variation of HCO_3 concentration and HCO_3 fixation rate by 3.5 l cultures with pH

O HCO_3 concentration (M)

Δ rate of HCO_3 fixation (mol h^{-1})



Bicarbonate concentration (M)
Rate of bicarbonate fixation (µmol h⁻¹)
2x10⁻
10⁻
0
1500
1000
500
0
6.4
6.7
7.0
7.3
7.5
pH

Figure 39.

The effect of medium HCO_3 concentration on CO - fixation anaplerotic enzyme activity.

The variation in HCO_3concentration was effected by adjustment of culture pH.

O PEPC activity

▲ PC activity

Enzyme activity units; $mol.mg^{-1}h^{-1}$

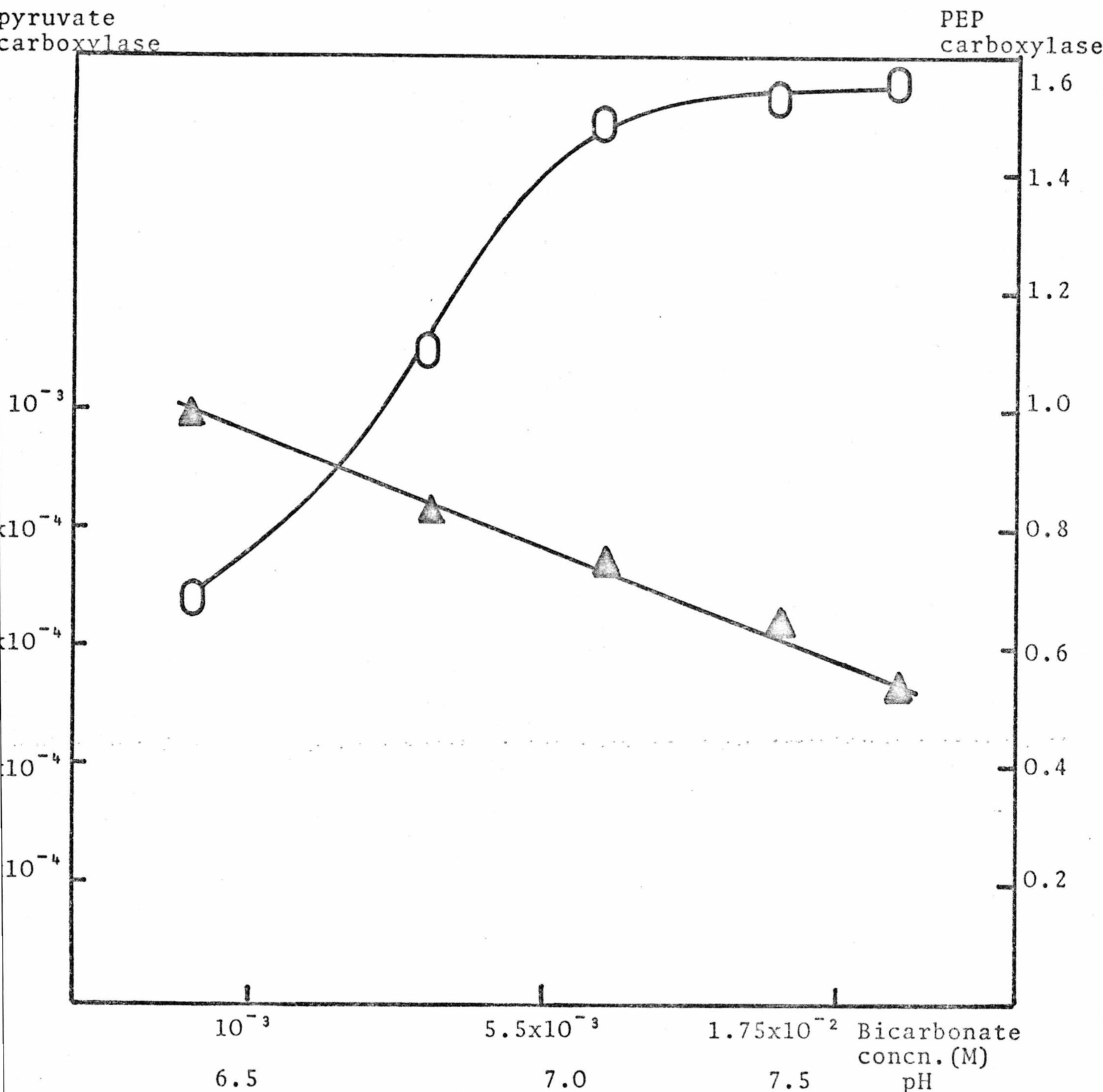


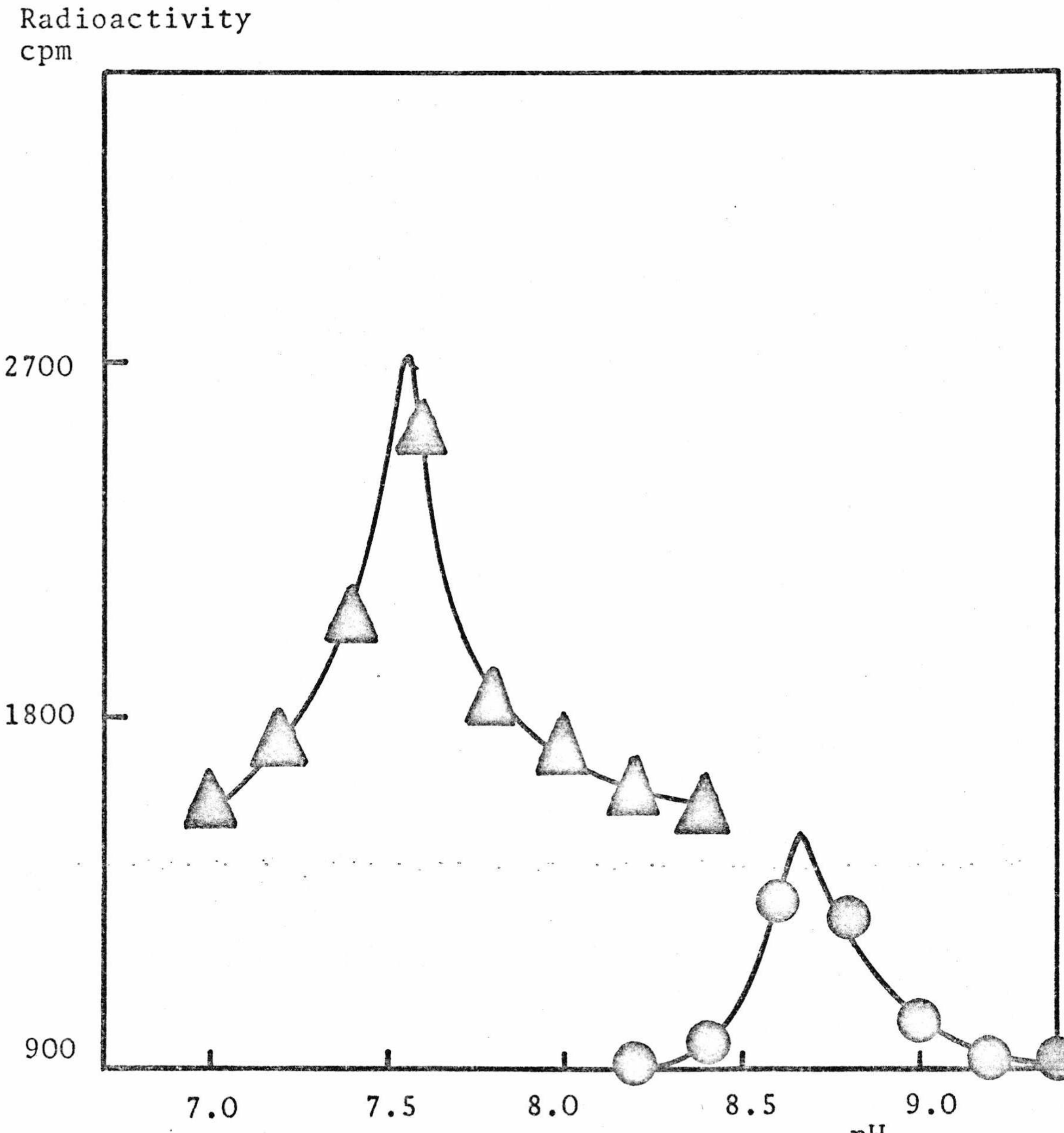
Figure 40

In vivo pH optima for PC (▲) and PEPC (O). Incorporation of ^{14}C-HCO into oxaloacetate is expressed as cpm.

pH optima:

PC ; 7.6

PEPC ; 8.7



Radioactivity
cpm
2700
1800
900
7.0
7.5
8.0
8.5
9.0
pH

Figure 41 shows the variation in the rate of HCO_3 fixation and the proportion of CO_2 in the culture head space with time in the step-down experiment. The HCO_3 fixation rate shows an immediate increase as a response to the new dilution rate, overshooting the $0.125h^{-1}$ steady state value. The proportion of CO_2 in the headspace shows a drop, followed by a rise which appears to correspond with the changes in HCO_3 fixation rate. The slight asynchrony between the two curves may reflect the rate limiting step between CO_2 solubility and HCO_3 formation.

Figure 42 shows the corresponding overshoot observed in enzyme activity in the transient state. The disimilarity between the curve showing total enzyme activity variation and the HCO_3 fixation in Figure 41 may be due to the fact that enzyme activities are expressed in terms of the protein content of the cell sap. It has already been established (III.3.b) that mycelial protein levels vary markedly in the transition state.

Figure 43 shows the variation in the CO_2 content of the jeadspace and the rate of mycelial HCO_3 fixation with time, in the step-up transient state. The HCO_3 fixation rate began oscillating 2 h after the dilution rate was stepped up to $0.175\ h^{-1}$, producing a peak in activity after 5 h. The variation in HCO_3 fixation rate did not appear to obviously affect the proportion of CO_2 in the head space, which increased gradually to the steady-state value. This observation may reflect a tendency for upward over shoot in CO_2 evolution as a response to step-up, which was damped down by the dramatic increase in HCO_3 fixation.

The variation of PC and PEPC activities in the transition state are shown in Figure 44. The peak in HCO_3 fixation at 5 h

Figure 41.

Variation in mycelial radioactivity due to $^{14}C-HCO_3$ incorporation (▲) and proportion of CO_2 in the culture headspace (O) with time in the transient state $D=0.175\ h^{-1}$ to $0.125\ h^{-1}$.

Radioactivity was measured in 10 ml aliquots of culture after 20 minutes incubation with $^{14}C-HCO_3$.

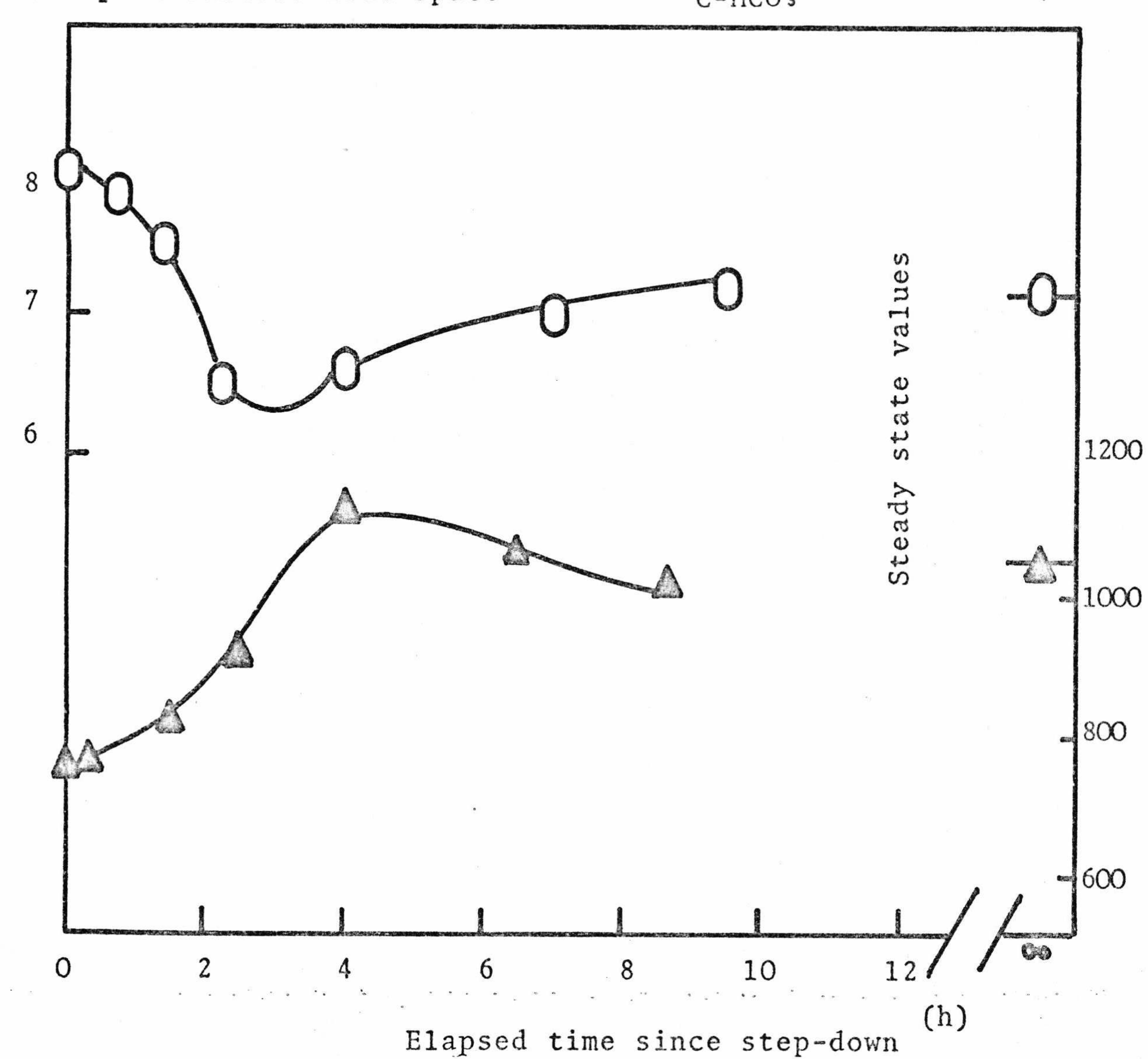
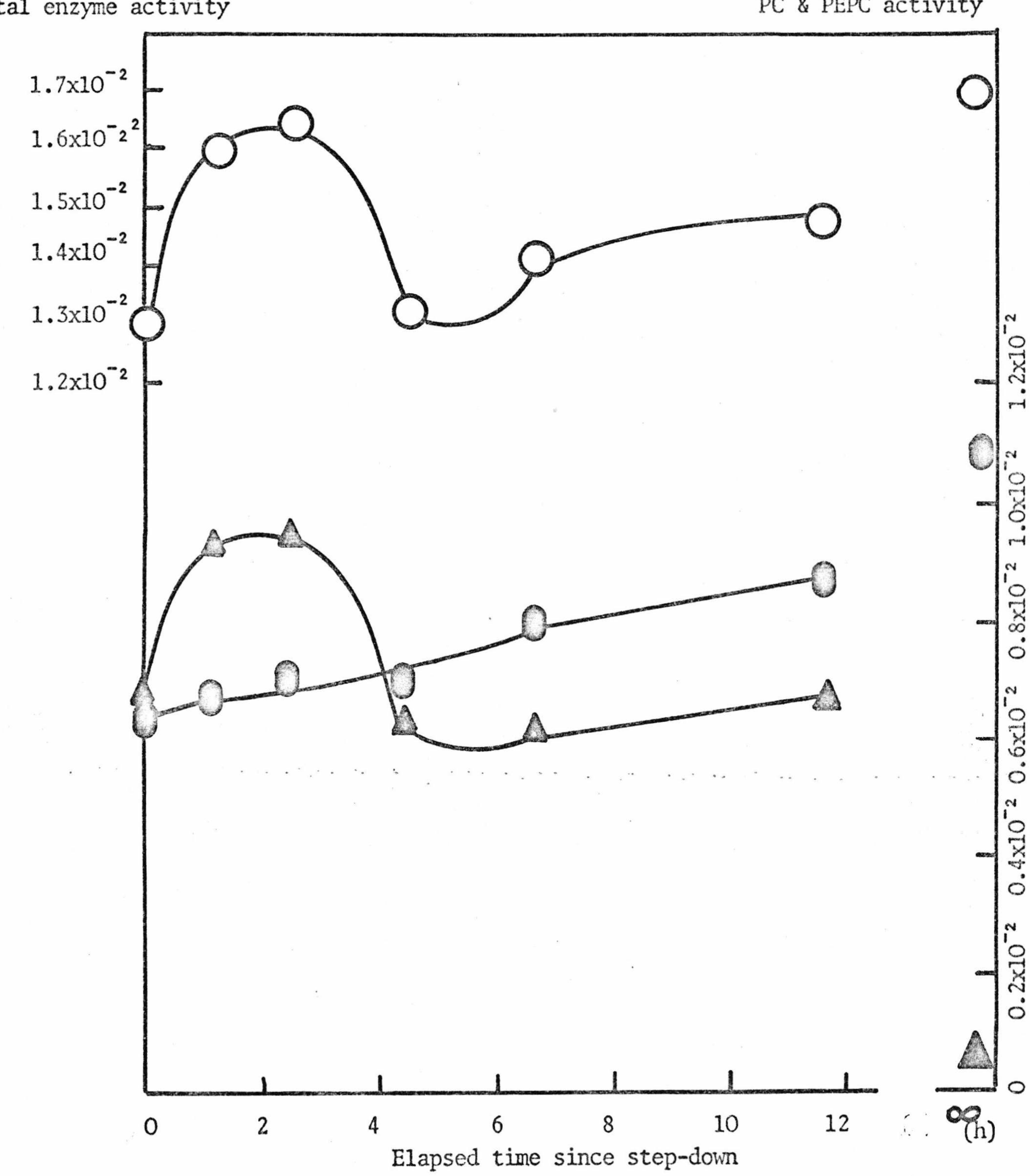

ʃ % CO_2 in culture head space
Mycelial radioactivity following exposure to ^{14}C-HCO_3
8
7
6
Steady state values
1200
1000
800
600
0
2
4
6
8
10
12
∞
(h)
Elapsed time since step-down

Figure 42.

Variation in PEPC (●); PC (▲) and total (O) enzyme activity with time in the transient state D = 0.175 h^{-1} to 0.125 h^{-1}.

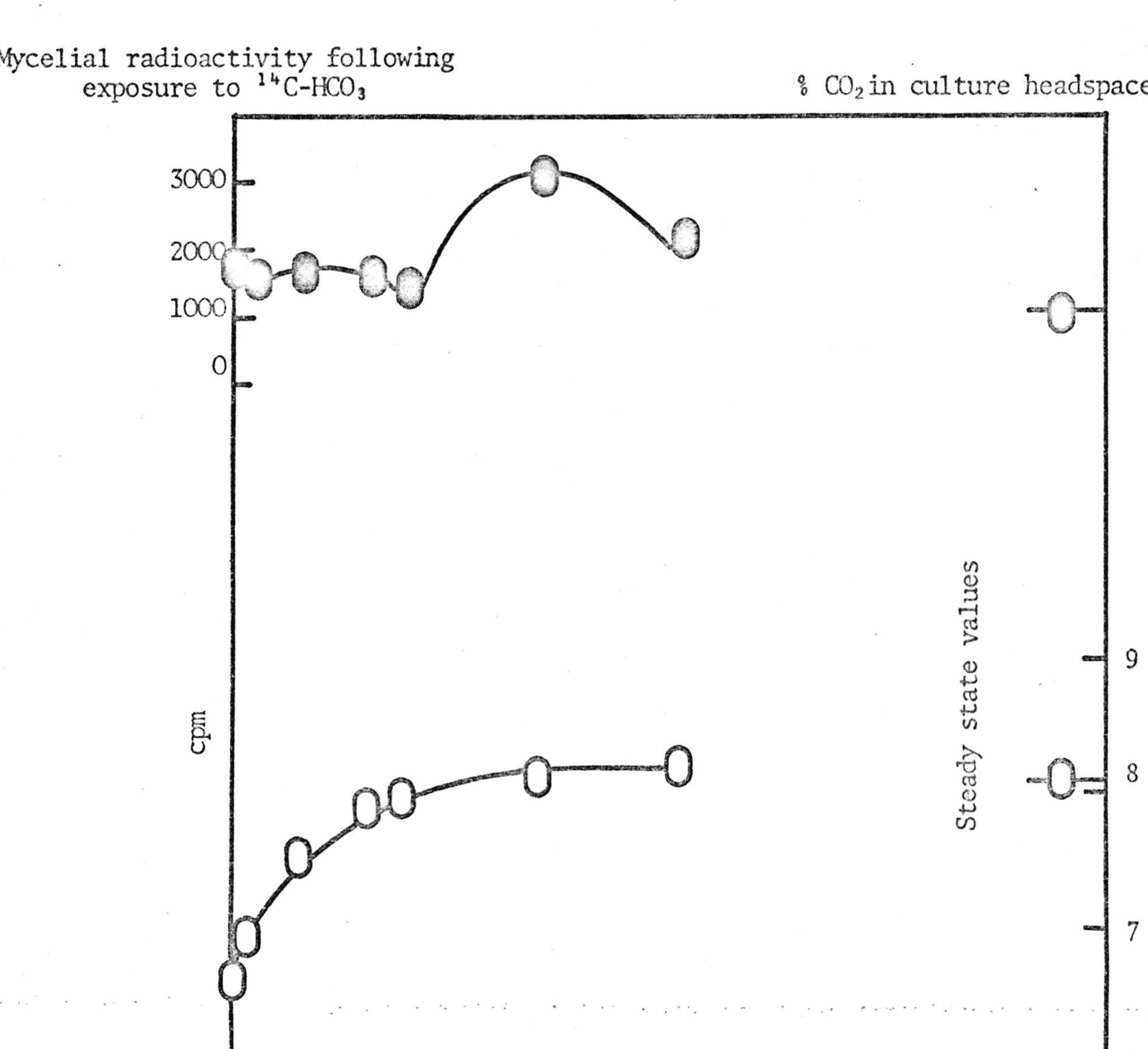


Enzyme activity (µmol mg⁻¹h⁻¹)
Total enzyme activity
PC & PEPC activity
1.7x10⁻²
1.6x10⁻²
1.5x10⁻²
1.4x10⁻²
1.3x10⁻²
1.2x10⁻²
1.2x10⁻²
1.0x10⁻²
0.8x10⁻²
0.6x10⁻²
0.4x10⁻²
0.2x10⁻²
0
0
2
4
6
8
10
12
∞
(h)
Elapsed time since step-down

Figure 43

Variation in mycelial radioactivity due to $^{14}C-HCO_3$ incorporation (●) and proportion of CO_2 in the culture headspace (O) with time in the transient state D = 0.125 h^{-1} to 0.175 h^{-1}.

Radioactivity was measured in 10ml aliquots of culture after 20 minutes incubation with $^{14}C-HCO_3$



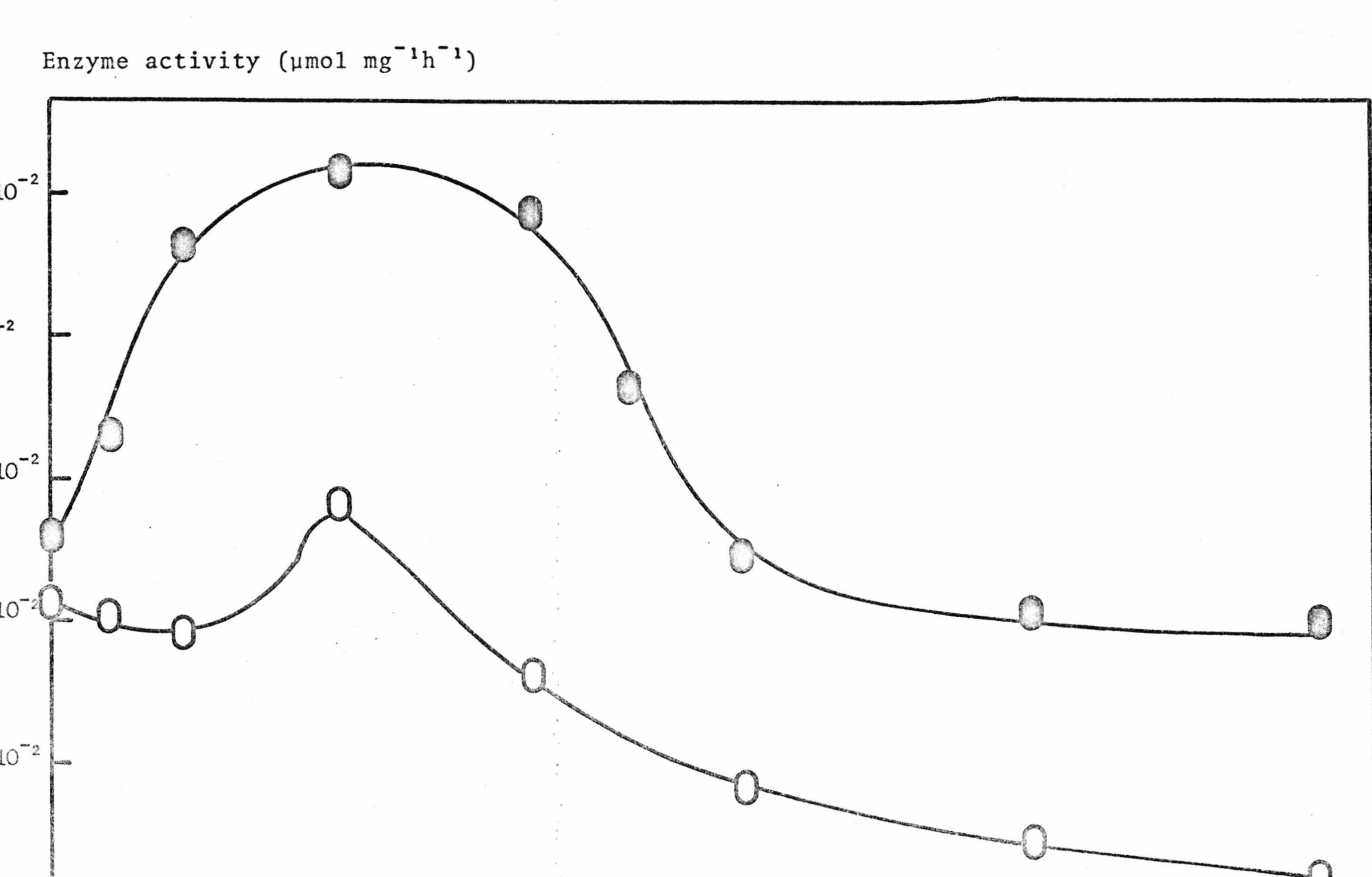
Mycelial radioactivity following exposure to ^{14}C-HCO_3
% CO_2 in culture headspace
3000
2000
1000
0
cpm
Steady state values
9
8
7
6
0
2
4
6
8
10
∞
(h)
Elapsed time since step-up

Figure 44.

Variation in PEPC (●) and PC (O) activity with time in the transient state

$$D = 0.125\ h^{-1} \text{ to } 0.175\ h^{-1}$$

The activities observed at 22 h were maintained throughout the steady state.



Enzyme activity (μmol mg⁻¹h⁻¹)
2.5x10⁻²
2x10⁻²
1.5x10⁻²
1.0x10⁻²
0.5x10⁻²
0
2
4
6
8
10
12
14
16
18
20
22(h)
Elapsed time since step-up

can be seen to be coincident with a peak in the activity of both enzymes.

Interpretation of these data in metabolic terms would require a more detailed knowledge of the enzyme control systems operating. It is interesting to note that, in each transient state, the carboxylase whose activity was predominant in the steady state shows the greatest initial response to changes in dilution rate. In the case of the step-down experiment, PC activity was greater than PEPC activity in the steady state. Changing the dilution rate to a lower value would have the immediate effect of slowing down growth rate. The present data may be interpreted to indicate that pyruvate formation continued initially at an unchanged rate, resulting in a large pool to act as a substrate for carboxylation.

In the case of the step-up experiment, the predominant carboxylase activity was that of PEPC, indicating a lower rate of conversion of PEP into pyruvate at 0.125 h^{-1} than was the case at 0.175 h^{-1}. The increase in PEP pool size as a response to increased dilution rate would be expected to take place at a faster rate than the increase in pyruvate pool size as another enzymic step would be required for the PEP → pyruvate conversion.

(g) Fractionation of mycelium.

To determine the role of fixed HCO_3 in hyphal composition, a chemical fractionation of mycelium, grown in the presence of ^{14}C-HCO_3 was performed (IV.2.c).

The radioactivity measured in each fraction is shown in Table 11.

The proportion of total radioactivity in each fraction appears to be relatively independent of incubation time over

the period observed. The distribution of radioactivity is consistent with an anaplerotic role for fixed HCO_3. The proportion of ^{14}C in low molecular weight compounds is small (4.4% - 7.2%) and this is predictable in anaplerotic metabolism as this fraction includes TCA cycle intermediates. Such substances would be short-lived, being used immediately for biosynthesis. The total lipid fraction (fractions 3 and 4) was relatively large (21% - 32.3%). This observation provides good circumstantial evidence for the fixation of HCO_3 by acetyl-CoA carboxylase (EC.6.4.1.2.) an enzyme whose activity in *Aspergillus nidulans* was postulated in IV.3.d(i).

It is unlikely, however, that this enzyme was sufficiently active to play a detectable role in overall mycelial CO_2 fixation in normal batch and continuous cultures. If so, its presence would, almost certainly, have been detected in PEPC reaction mixtures when acetyl-CoA was added (IV.3.a). Also, the similarity between total enzyme activities and overall CO_2 fixation suggest that anaplerotic CO_2 fixation accounts for virtually all the CO_2 fixation in normal batch and continuous cultures.

Table 11 shows that the majority of fixed bicarbonate appears in the residual protein fraction. This observation is consistent with anaplerotic function and such an effect would be advantageous in a commercial biomass fermentation. From this standpoint, maintenance of culture conditions in which anaplerotic CO_2 fixation was maximal, would have three main advantages:

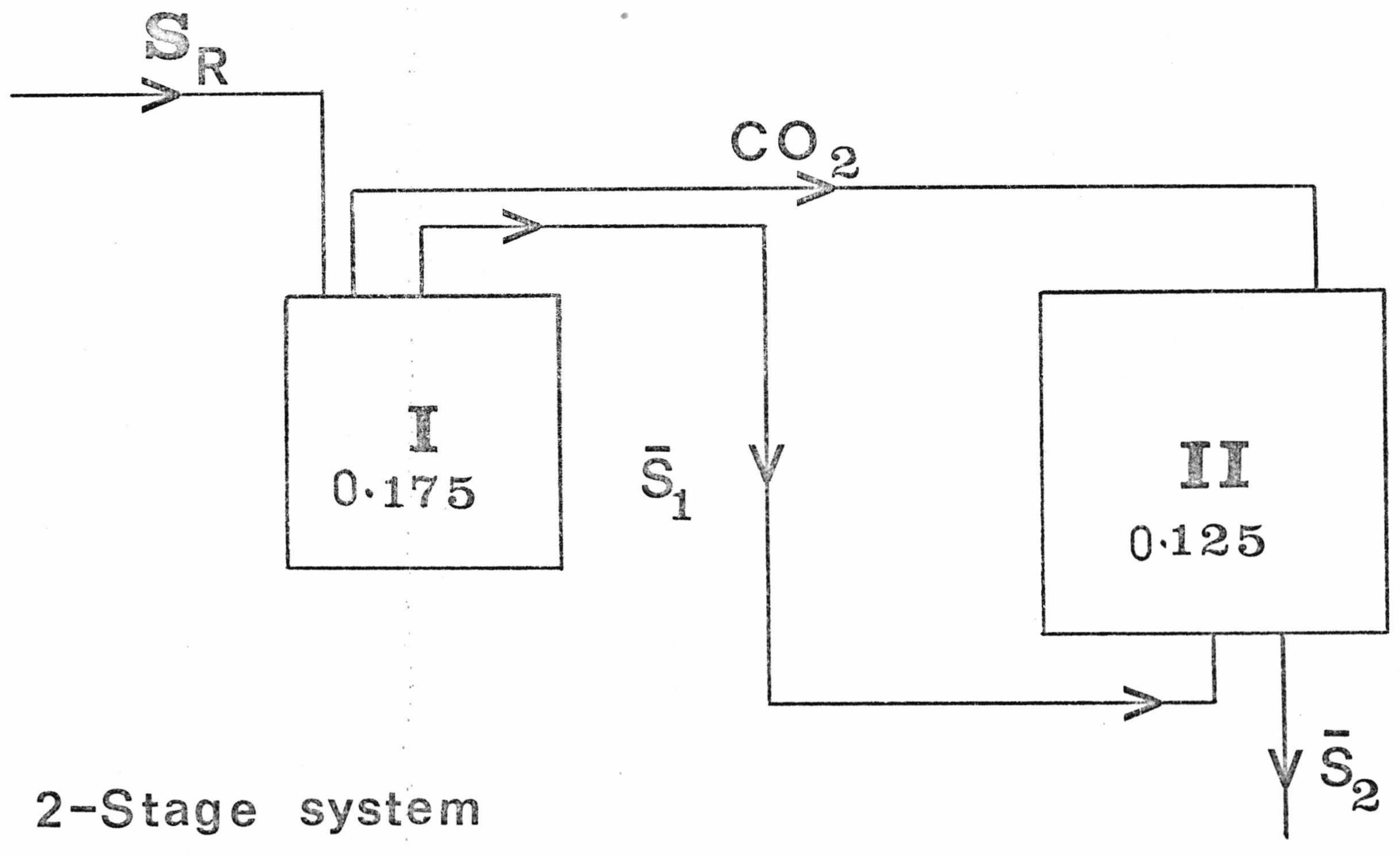
(1) Less of the carbon source would be "wasted" as respiratory CO_2;

TABLE 11

Distribution of ^{14}C in hyphal fractions of *Aspergillus nidulans* mycelium, exposed to ^{14}C-HCO_3

Radioactivity (d.p.m) and percentage of total ^{14}C fixed								
Fraction	Time of Harvesting							
	5 min		10 min		20 min		30 min	
	dpm	%	dpm	%	dpm	%	dpm	%
Total ^{14}C fixed	5878	100.0	4923	100.0	7377	100.0	9043	100.0
1. Extracellular products	0	0	0	0	0	0	0	0
2. Low molecular wt. (cold TCA-soluble)	325	5.5	355	7.2	350	4.8	400	4.4
3. Lipid (ethanol soluble)	595	10.1	640	13.0	1190	16.1	1475	16.3
4. Lipid (ethanol-ether soluble)	678	11.5	948	19.3	927	12.6	968	10.7
5. Nucleic acid (hot TCA soluble)	0	0	0	0	0	0	0	0
6. Protein (residual)	4280	72.8	2940	60.5	4910	66.6	6200	68.6

Total ^{14}C-HCO_3 added to each flask had a specific activity of 5.5×10^6 dpm



2-Stage system

FIG 45

(2) The protein content of the "biomass" would be increased (see next section):

(3) Fixed CO_2 does not appear to be used for RNA synthesis (Table 11).

Point(3) is relevant to the recommendations of the World Health Organisation protein advisory group, who have recommended that biomass RNA levels should be strictly controlled, following a report (Edozien, Udo, Young & Scrimshaw, 1970) on the effects of RNA intake upon uric acid metabolism.

(h) Two-stage experiments.

The objective of the 2-stage chemostat studies was to exploit anaplerotic CO_2 fixation in the enhancement of biomass production from *Aspergillus nidulans* on a continuous basis.

Figure 45 is a diagramatic representation of the experimental system. The 3.5 L fermenter unit described previously was used as a first stage (stage I) and the 1 L unit (IV.2.d) as a second stage (stage II). Mycelium growing at a dilution rate of 0.175 h^{-1} in stage I was pumped into stage II at a flow rate equivalent to a dilution rate of 0.125 h^{-1}. Stage II was provided with a "feed" of CO_2 in the form of the effluent gas stream (containing respiratory CO_2) from stage I. At a biomass of 6 gl^{-1} (15 gl^{-1}glucose), incomplete mixing was observed in the second stage. A glucose concentration of 7 gl^{-1} was used therefore in 2-stage studies. The rate of mycelial CO_2 fixation, the biomass concentration and the proportion of CO_2 in the gas stream entering and leaving the fermenter vessel were measured in stage I and stage II. Biomass measurements were also made at other dilution rates (Table 12).

TABLE 12

Biomass production in a 2-stage chemostat culture of *Aspergillus nidulans*.*

	Stage I	Stage II
Dilution rate (D)	0.125 h^{-1}	0.175 h^{-1}
Biomass conc^{n} ($\bar{x}$)	2.496 gl^{-1}	2.276 gl^{-1}
D	0.15 h^{-1}	0.10 h^{-1}
$\bar{x}$	2.48 gl^{-1}	2.39 gl^{-1}
D	0.175 h^{-1}	0.125 h^{-1}
$\bar{x}$	2.085 gl^{-}	2.570 gl^{-}

* Culture pH was 6.4

Table 13 shows the effect of pH upon biomass, mycelial HCO_3 fixation and the proportion of CO_2 leaving the fermenter in stages I and II.

When the experiment was performed with the dilution rate in stage I at 0.125 h^{-1} and that in stage II at 0.175 h^{-1} a lower biomass concentration was observed in II. The decrease (8.8%) observed is probably due to turnover of hyphal energy reserve materials, as a response to the higher dilution rate in II. Similarly, dilution rates of 0.15 h^{-1} (I) and 0.175 h^{-1} (II) resulted in a 3.6% decrease in biomass concentration in II. Some hyphal lysis was observed. The biomass decrease and cytological effects observed, are possibly a reflection of the long residence time (10h) at this dilution rate, which represents, essentially, a period of glucose starvation.

An increase in biomass concentration of 0.485 gl^{-1} was observed in the second stage with dilution rates of 0.175 h^{-1} (I) and 0.125 h^{-1} (II). Analysis of the gas stream entering and leaving II under these conditions (Table 13a) indicated a drop in the proportion of CO_2 (5.0% CO_2 entering; 4.1% leaving). Rates of HCO_3 incorporation in each stage were compared and an increase of 23% observed in the second stage. The above studies were all carried out at pH 6.4. Recalling the increase in dissolved HCO_3 concentration, rate of HCO_3 fixation and biomass; concentration, obtained by raising the culture pH from 6.4 to 7.0 (IV.3.e), the experiment was repeated using medium with a pH value of 7.0. An even greater increase in biomass (1.4 gl^{-1}) was observed at the higher pH value, although the increase in the rate of HCO_3 fixation (23%) was the same as that observed at pH 6.4. The proportion of CO_2 leaving II was lower than that

entering it, dropping from 3.6% to 2% of the total gas stream volume.

The increase in biomass, drop in gaseous CO_2 content and rate of HCO_3 incorporation in II were all calculated in comparable units (Table 13b) as productivity values, as follows:

(a) The biomass increase ($\delta\bar{x}$) was expressed as the number of μmol of HCO_3 to produce a biomass increase of $\delta\bar{x}$g. This value, multiplied by the dilution rate, gave the number of μmol of HCO_3 per unit time required for the biomass increase observed.

(b) The difference between the percentage of CO_2 in the gas stream entering and leaving the second stage was expressed as μmol HCO_3, removed from the gaseous environment, per unit time.

(c) The rate of incorporation of ^{14}C-HCO_3 in II was measured directly.

When the equivalent productivities, expressed as μmol.ml culture^{-1}h^{-1} are compared, it can be seen that they are within the same order of magnitude at each pH value. This provides circumstantial evidence that the drop in CO_2 content in the gas stream, entering and leaving II is due to HCO_3 fixation. The rate of HCO_3 fixation measured would account for the observed biomass increases if all the fixed material were used to manufacture biomass.

The two-stage system was constructed to provide a "perpetual transition state", thereby achieving a situation in which the upward overshoot in HCO_3 fixation, observed following step-down (Figure 41) was operating continuously.

TABLE 13.

(a) Biomass concentration, gas analysis and HCO_3 incorporation rates in the 2-stage system.

	Stage I ($D=0.175h^{-1}$)		Stage II ($D=0.125h^{-1}$)	
	pH 6.4	pH 7.0	pH 6.4	pH 7.0
Biomass	$2.085gl^{-1}$	$3.047gl^{-1}$	$2.570gl^{-1}$	$4.447gl^{-1}$
% CO_2	5.0	3.6	4.1	2
incorporation rate ($dpm.ml^{-1}$ in 25 min)	14941	30232	18443	37318

(b)

	Productivity values ($\mu mol\ HCO_3\ ml^{-1}\ min^{-1}$)	
	pH 6.4	pH 7.0
Biomass	1.656×10^{-2}	2.8675×10^{-2}
% CO_2	3.323×10^{-2}	6.724×10^{-2}
incorporation rate	9.051×10^{-2}	12.0625×10^{-2}

Because the steady-state glucose concentration at a dilution rate of 0.175 h^{-1} is high (2.265 gl^{-1}) it was possible to limit growth of the culture in the second stage with residual glucose from the first stage, $\bar{s}_1$ (Figure 45). The steady state glucose concentration in the second stage (S_2) became zero. This situation produced an enhanced CO_2-fixing capability in the second stage. The culture mean residence time in II was 8 h. Any age distribution amongst the hyphae in the culture would therefore result in a proportion of the culture existing in a transition state. This system would be economically advantageous in a commercial biomass production process as no external CO_2 is supplied to the culture. The resultant increased appetite for CO_2 in the second stage is satisfied by a feed of respiratory CO_2 from the first.

Fixation of CO_2 is a process which consumes energy and reductive power. As the glucose supply to the second stage is completely utilised by the culture, and the biomass increase may be attributed to the mass of HCO_3 fixed, it may be assumed that glucose is completely re pired. Assuming three sites of oxidative phosphorylation and two substrate level phosphorylation steps in glycolysis, (i.e. 38 mol ATP per mol glucose oxidised) the supply of glucose to the second stage ($\bar{s}_1$) would provide 13.285 mol ATP (or equivalent) for each mol of CO_2 fixed. This would appear to be ample energy and reductive power for CO_2 fixation and macromolecular synthesis in the second stage.

The observation that the majority of fixed HCO_3 is used for synthesis (TABLE 11) is consistent with the results obtained when the protein content of hyphae in the first and second stage were compared. The protein content of hyphae from stage III was

57.54% in the first stage.

In concluding this section, it may be stated that carbon dioxide transfer and assimilation parameters form important control criteria which may have particular relevance to biomass fermentations.

APPENDIX.

The observation that the growth kinetics of *Aspergillus nidulans* may take two alternative forms (II.3.d) i.e. Dcrit may coincide with μmax or occur at a growth rate approximately half of this value, is the subject of this appendix. The following are offered as models which could form the basis of experiments to elucidate the phenomenon of premature washout from continuous cultures of microfungi; these models are based on hyphal morphology parameters.

(a) The Spatial model.

This model assumes that the rate of biomass increase in filamentous fungi is exactly equivalent to the rate of hyphal extension, i.e.

$$l = lo\ e^{\mu t}$$

where lo is the initial length of a hyphal filament and l is its length after time t during exponential growth.

Growth by simple hyphal extension is considered as a doubling in hyphal length after each "generation time" and growth by branching is considered as the formation of two new lateral branches, each equal in length to the "parent" hyphal strand, during each generation.

In the model, a recently emerged germ tube of length 4a units is illustrated after each generation when growing in the branched and the non-branched form. The total hyphal length has been doubled during each generation time period.

NON-BRANCHING			BRANCHED		
Distance (1) between tip and origin	Length of (2) hyphal strand / No. of growing tips	Mycelial configuration	Distance (1) between tip and origin	Length of (2) hyphal strand / No. of growing tips	Mycelial configuration
2a	2a	4a	2a	2a	4a
4a	4a	4a, 4a	3a	2a	a, 4a, a, a, a
8a	8a	4a, 4a, 4a, 4a	4a	2a	4a, a ...
16a	16a	4a, 4a, 4a, 4a ...	5a	2a	4a, a ...

In the Table, columns (1) contain the total length of hyphae between the growing tip and the origin (the centre of the original strand). Columns (2) list the ratio of total length of the fragment to the total number of tips. This latter parameter is an expression describing the maximum length of hyphae available for absorption of nutrients for further growth, assuming that all growing tips have access to an "equal share" of the total length of the mycelium. If the entire hyphal fragment is available for nutrient absorption, then the values in column (1) represents the maximum length over which nutrients must be translocated to the growing tip.

In each case, the maximum length of hyphae available for nutrient absorption, as well as the maximum length for translocation of nutrients, increases in direct proportion to the length of newly-synthesised hyphae for each generation.

The values in column (2) are equivalent to the hyphal growth unit (II.1.c) of the mould. In the case of non-branched growth, this value increases with each generation. This prediction conflicts with experimental observations (Trinci, 1973; Righelato, 1974) that the hyphal growth unit is a constant parameter for any one growth rate. A fixed rate of hyphal fragmentation which balances growth must be postulated, therefore, in submerged cultures of unbranched mycelium.

Microscopic examination of hyphal fragments growing in both morphological forms would enable the measurement of the parameters in columns (1) and (2) for each form. The hypothesis that the branched form reaches a "critical configuration"

wherein the translocation rate balances the growth rate could then be tested by varying the rate of hyphal fragmentation and testing the maximum growth rate obtainable for each fragment size.

(b) The Substrate Limitation model.

This model is based on the concept that growth via the formation of two new branches in each generation would involve the consumption of more energy substrate than growth by simple hyphal elongation. Writing a balance equation for substrate utilization:

Rate of change in substrate concentration	=	Rate of substrate supply	-	Rate of substrate removal	-	Rate of utilization for growth	+	Rate of utilization as energy to form new branches
$^{ds}/_{dt}$	=	DS_R	-	Ds	-	$\frac{\mu x}{Y}$	+	b

where b is the rate of uptake of energy substrate, specifically for branch formation (units, $gl^{-1}h^{-1}$).

In a steady state:

$$D(S_R - \bar{s}) = \frac{\mu\bar{x}}{Y} + b$$

$$S_R - \bar{s} = \frac{\bar{x}}{Y} + \frac{b}{D}$$

$$Y(S_R - \bar{s}) = \bar{x} + \frac{bY}{D}$$

$$b = D\{S_R - \bar{s}) + \bar{x}/_Y\}$$

If the saturation constant (K_s) is the same for branched and non-branched forms, then the rate of glucose uptake will be the same

in each case. At low dilution rates, where uptake is maximal ($\bar{s}$ is minimal, tending towards zero) then the glucose uptake rate will be sufficient to accommodate branching as well as growth. At higher dilution rates, however, $\bar{s}$ becomes large and the glucose uptake rate is low. If the uptake rate were to fall below a value equal to $\frac{D\bar{x}}{Y}$ + b, then washout would occur. To test this hypothesis, cultures grown in the branched and non-branched forms could be compared with respect to values of $\bar{s}$, $\bar{x}$ and Y to determine whether b has a positive value.

These models represent alternative approaches to the metabolic explanations given in the text of this thesis.

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