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Kinetics of the Growth of Mycelial Pellets of *Aspergillus nidulans*

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Summary. 1. The radius of *Aspergillus nidulans* pellets grown singly (i.e. 1/flask) increased linearly with time for most or all of their growth; they grew at about half the rate of colonies of the mould upon solid medium. The final pellet diameter, the duration of the linear growth phase and the initial radial growth rate decreased as the pellet concentration was increased.

2. Single pellets initially increased in dry weight at an exponential rate; the specific growth rates at 20°, 25° and 30°C but not at 37°C were almost identical with the organism's maximum specific growth rates at these temperatures.

3. The cube root of the dry weight of pellets grown singly also increased linearly with time for most or all of their growth, but a smaller proportion of the total growth of cultures inoculated with several pellets was of this form. Cube root kinetics were only maintained as long as the pellets grew at a constant rate.

4. When *Aspergillus nidulans* cultures were inoculated with various conidial concentrations the ratio, conidia per flask: pellets formed per flask, varied between $2-6\times10^4$. Cultures only grew at the maximum specific growth rate when inoculated with at least 6×10^5 conidia/ml of medium.

Camici *et al.* (1952) found that the morphology of *Penicillium chrysogenum* in submerged culture varied between "pellet" (spherical compact masses of hyphae) and "filamentous" (hyphae homogeneously dispersed throughout the medium) forms, depending upon the number of germinating conidia in the medium; pellet formation occurred in cultures inoculated with fewer than $2-3\times10^5$ conidia/ml of medium whilst the mycelium grew in a filamentous form at higher conidial concentrations. Many moulds form mycelial pellets when grown in submerged, shake flask culture (Burkholder and Sinnott, 1945). The growth kinetics of *Neurospora* pellets were studied by Emerson (1950). He concluded that "There is a cube root phase in the growth of *Neurospora* in liquid cultures that corresponds to the logarithmic phase in the growth of unicellular organisms" and suggested that these kinetics resulted from pellets increasing in diameter at a constant rate. This cube root growth is described by the equation (Pirt, 1966)

$$M^{1/3} = k_c t + M_o^{1/3},$$

where M = mass of mycelium/cm^3 at time t, and k_c is a constant.

Marshall and Alexander (1960) also obtained cube root kinetics with several moulds in submerged culture and suggested that their growth could best be defined in terms of the rate of this phase. However, in a recent study Gillie (1968) was unable to detect a cube root growth phase in *Neurospora crassa*; instead growth could be divided into a short exponential phase, a phase of declining growth rate and a phase of constant growth rate (linear growth). These kinetics are identical with those of mould colonies on solid medium (Trinci, 1969) or of individual hyphae (Trinci and Banbury, 1967). The apparent contradiction between the results of Emerson and Gillie on the same organism may be due to a difference in the cultural conditions employed; Emerson's cultures were probably shaken (although he does not actually state this) whilst Gillie used static cultures. A heterogenous growth is produced in static cultures which includes surface colonies and submerged, loose textured mycelial aggregates, whilst compact pellets are formed in shaken cultures. Significantly, Pirt (1966) has pointed out that each of the reports of the validity of the cube root growth has associated it either implicitly or explicitly with the pellet form of growth. He suggested that the mycelial texture of pellets would have a considerable influence upon their growth kinetics, and envisaged two extreme conditions: (i) pellets composed of densely packed hyphae such that nutrients would only be able to enter by simple diffusion; (ii) pellets with a loose hyphal texture such that eddies caused by turbulence in the medium could penetrate and supply the inner mycelium with oxygen and other nutrients. He calculated that growth of the former kind of pellet would become oxygen limited when they exceeded a diameter of c. 154 μ with a result that pellets larger than this would consist of a thin outer rind (77 μ wide) of growing hyphae and an inner mass unable to grow because of lack of oxygen. Growth of the second kind of pellet would presumably be exponential until either lack of internal space or lack of nutrients limited growth. Significantly, Foster (1949) records, "When these spongy spherical colonies (pellets grown in submerged culture) are bisected the central portion has a strong alcoholic odour, indicating a lack of oxygen for the cells in the interior". More recently, Yoshida *et al.* (1968) found that the Q_{O_2} of the mycelium of *Lentinus edodes* pellets which had a diameter of 24 mm, decreased with distance from the circumference and fell to zero for the central 8 mm portion.

Pirt (1966) proposed that growth of cultures containing a number of pellets could be described by the equation,

$$M^{1/3} = \left(\frac{4\pi pn}{3}\right)^{1/3} \alpha w t + M_o^{1/3}$$

where M = the total masss, p = dry weight of the organism per unit volume of pellet, w = width of the peripheral growth zone of the pellets,

n = number of pellets in the culture and α = specific growth rate of the organism. He predicted that if this equation was an accurate description of pellet growth, the time coefficient (rate of cube root phase) would be directly proportional to $n^{1/3}$.

The present study analyses the growth kinetics of mycelial pellets in submerged shake flask culture.

Materials and Methods

The organisms were *Aspergillus nidulans* (BWB 224) and *Penicillium chrysogenum* (WIS 54-1255). The culture medium (DAN) and techniques employed were as described previously (Trinci, 1969) unless stated otherwise. Pellets for inocula were grown at the same temperature as that at which they were subsequently cultured. Flasks (250 ml) were either inoculated with 5 ml of a suspension of pellets which had been prepared by resuspending overnight cultures in fresh DAN medium or with single pellets dispensed with a sterile Pasteur pipette. The medium used to resuspend pellets was pre-warmed to the incubation temperature. The number of pellets used to inoculate flasks never varied by more than 10—20% within any given experiment. Flasks were incubated on rotary shakers at 220 rev/min. The dry weight of cultures was obtained by harvesting 3 or more flasks and measurements of pellet diameter were made using a Shadomaster (Buck & Hickman, Otterspool Way, Warford, Herts.). Pellet were fixed in formal-acet-alcohol (Righelato *et al.*, 1968) prior to measurement. *Penicillium chrysogenum* and *Aspergillus nidulans* were normally grown at 25° and 37° C respectively.

Results

Growth of Single Pellets of *Aspergillus nidulans*

Growth of *Aspergillus nidulans* pellets was studied over the temperature range 20—37° C. Each flask was inoculated with a single pellet; the mean diameter of the pellets at the time of inoculation varied between 0.7 mm for the 20 C° cultures to 1.1 mm for the 37 C° cultures. Any satellite pellets which had formed in the cultures after 24 hrs growth were removed with a sterile Pasteur pipette.

The pellets grew at a constant radial growth rate until they attained a diameter of 15—30 mm (Figs. 1 and 2). The radial growth rates (K_p) of the pellets and the growth rates (K_c) of *Aspergillus nidulans* colonies on solid media over the same temperature range are compared in Table 1; the ratio, colony radial growth rate:pellet radial growth (K_c:K_p) had a value of about 2 over the temperature range tested. The pellets initially increased in dry weight at an exponential rate (Figs. 1 and 2). The pellet specific growth rates during this exponential phase are compared in Table 2 with the specific growth rate of the mould in cultures inoculated with $1.5—2.5 \times 10^6$ conidia/ml of medium. The specific growth rate of the mould in the latter type of culture presumably represents its maximum specific growth rate for the conditions. The pellet

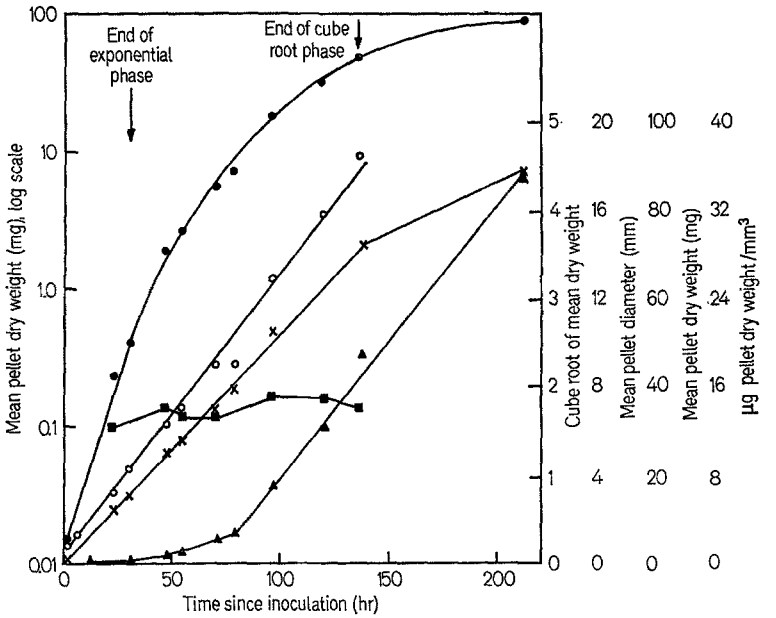

Fig. 1. Growth of *Aspergillus nidulans* pellets grown singly at 25°C. Dry weight of plotted on log (•), cube root (×) and arithmetic (▲) scales. The mean diameter of the pellets (○) and the dry weight per cubic millimeter of pellet (■) are also recorded. Each flask contained 50 ml of half strength DAN medium and was inoculated with a single pellet

Table 1. *Comparison between the radial growth rates of pellets grown singly in submerged culture and colonies of* Aspergillus nidulans *on solid medium. The cultures were grown on the same medium (DAN)*

Temperature (°C)	Radial growth rate (K_p, µ/h) of single pellets in submerged culture	Radial growth rate (K_c, µ/h) of colonies (from Trinci, 1969)	K_c/K_p
20	43	86	2.0
25	65	146	2.2
30	104	215	2.1
37	130	297	2.3

specific growth rates during the exponential phase only differed significantly from those of the mould in high spore inocula cultures at 37°C. The mean diameter and dry weight of the pellets at the end of the exponential phase are also shown in Table 2.

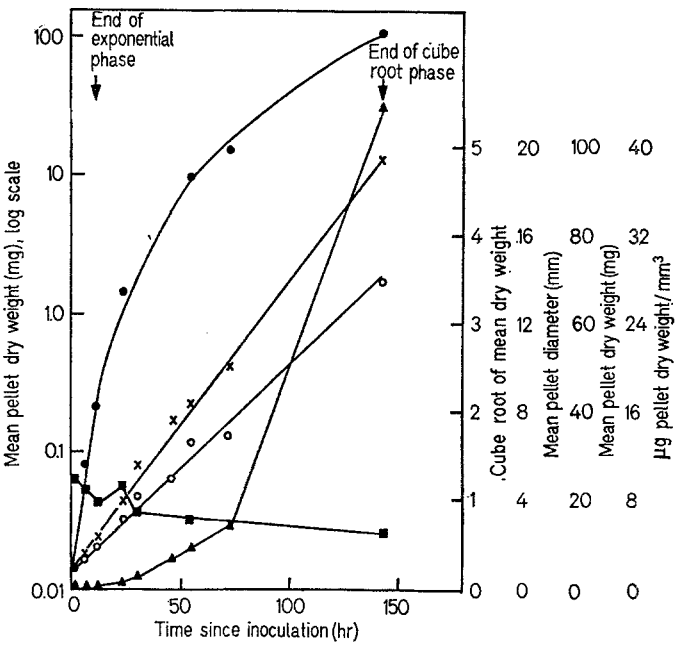

Fig. 2. Growth of *Aspergillus nidulans* pellets grown singly at 37°C. Dry weight of pellets plotted on log (•), cube root (×) and arithmetic (▲) scales. The mean diameter of the pellets (○) and the dry weight per cubic millimeter of pellets (■) are also recorded. Each flask contained 50 ml of half strength DAN medium and was inoculated with a single pellet

Table 2. *Comparison between the specific growth rates of single pellets of* Aspergillus nidulans *during the initial exponential phase and the specific growth rates of cultures inoculated with high spore densities (1.5—2.5 × 10⁶ conidia/ml of medium)*

Temperature (°C)	Pellet size at the end of the exponential phase of growth		Pellet growth		High spore density cultures (from Trinci, 1969)		α_p/α_h
	Mean diameter (mm)	Mean dry weight (µg)	specific growth rate (α_p, h^{-1})	doubling time (T_d, h)	specific growth rate (α_h, h^{-1})	doubling time (T_d, h)	
20	5.2	47	0.095	7.3	0.090	7.7	1.06
25	4.3	40	0.124	5.6	0.148	4.7	0.84
30	5.0	60	2.224	3.1	0.215	3.2	1.04
37	3.7	21	0.248	2.8	0.347	2.0	0.71

At each temperature tested the cube root of the mould's dry weight increased linearly with time for most or all of the growth period (Figs. 1 and 2). The dry weight per pellet unit volume remained fairly constant at 25C° (Fig. 1) but decreased in value at 37°C (Fig. 2).

The results obtained suggest that growth of *Aspergillus nidulans* pellets grown singly at 30C° and below was only nutrient (oxygen) limited when they exceeded a diameter of 4—5 mm. Pellets larger than this presumably consisted, as suggested by Pirt (1966), of a peripheral region of growing hyphae and a non-growing inner mass. The results (Table 2) suggest that the width of the growth zone (g) is of the order of 1.8 to 2.5 mm (Diameter/2). The proportion of g contributing to the radial extension of pellets may be calculated from the equation proposed by Pirt (1966) to describe pellet growth,

$$r = w\alpha t + r_o$$

where r is the radius of the pellet at time t, w is the width of the zone contributing to radial extension, and α is the specific growth rate of the organism. Thus,

$$w = \frac{r - r_o}{\alpha t} .$$

Values for w at different temperatures were calculated using this formula and are shown in Table 3. The specific growth rates (α) used in these calculations were those obtained from high spore inocula cultures (Trinci, 1969). Pirt (1966) calculated that if the extent of the growth zone was limited by the rate at which oxygen diffused into the pellet it would only be 77 μ wide. This value is clearly exceeded in the case of *Aspergillus nidulans* pellets. Clutterbuck and Roper (1966) found that the peripheral zone contributing to radial growth of *A. nidulans* colonies in solid medium was about 500—600 μ wide.

Table 3. *Values for the growth zone (g) and the peripheral portion of the growth zone (w) contributing to radial extension of* Aspergillus nidulans *pellets grown singly*

Temperature (°C)	Experimentally observed value for the width of the growth zone (g) i.e. diameter of pellet at end of exponential phase/2 (μ)	Calculated value for the width of growth zone (w) contributing to radial extension (μ)
20	2,600	478
25	2,150	439
30	2,500	482
37	?	375

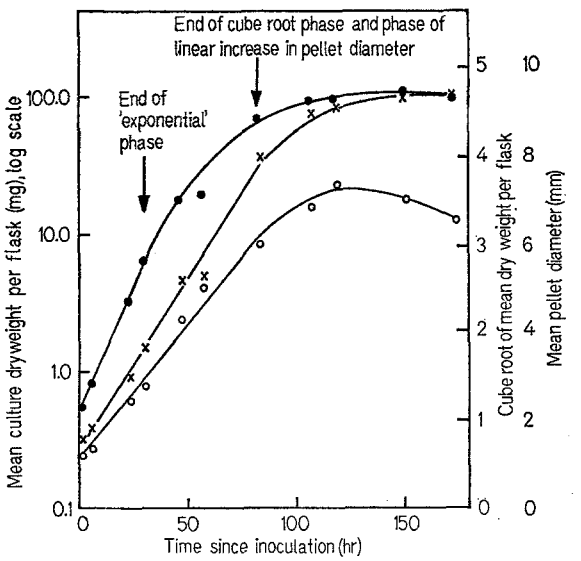

Fig. 3. Increase in dry weight and diameter of cultures of *Penicillium chrysogenum* pellets. Each flask contained 30 ml of DAN medium which was inoculated with 5 ml of a pellet suspension (mean number of pellets per flask, 13) and incubated at 25°C. Dry weight of pellets plotted on log (•) and cube root (×) scales. Mean pellet diameter (○)

Growth of *Penicillium chrysogenum* and *Aspergillus nidulans* Cultures Inoculated with Several Pellets

Growth of *Penicillium chrysogenum* and *Aspergillus nidulans* cultures containing *c.* 13 and *c.* 60 pellets per flask respectively are shown in Figs. 3 and 4. The pellets of both species initially increased in size at a constant radial growth rate, *Penicillium chrysogenum* at 29 μ/hr and *Aspergillus nidulans* at 108 μ/hr. These rates are respectively 56% and 36% of the radial growth rates of colonies of the moulds on solid media under the same conditions (Trinci, 1969). The linear growth phase was followed by a deceleration in radial growth rate and finally in *Penicillium chrysogenum* there was a reduction in pellet diameter. This latter phase was associated with a smoother appearance of the pellets, i.e. there had been a compaction of the hyphae at the pellet circumference.

The specific growth rates during the initial apparent exponential increase in dry weight (Figs. 3 and 4) were 0.082 hr^{-1} (doubling time, 8.5 hrs) for *Penicillium chrysogenum* and 0.154 hr^{-1} (doubling time, 4.5 hrs) for *Aspergillus nidulans*. These rates were respectively 66% and 43% of the specific growth rates of the organisms in cultures inoculated with $1.5-2.5 \times 10^6$ spores/ml of medium (Trinci, 1969).

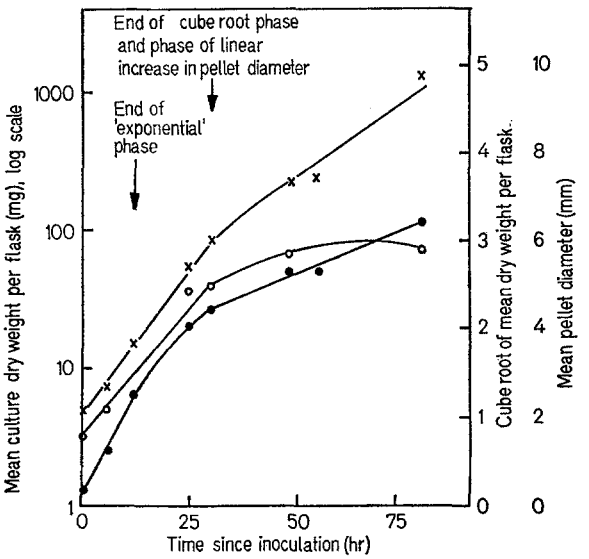

Fig. 4. Increase in dry weight and diameter of cultures of *Aspergillus nidulans* pellets. Each flask contained 30 ml of DAN medium which was inoculated with 5 ml of a pellet suspension (mean number pellets per flask, 60) and incubated at 37° C. Dry weight of pellets plotted on log (•) and cube root (×) scales. Mean pellet diameter (○)

The cube root of the dry weight of both moulds increased linearly with time for a considerable proportion of the growth period. Cube root growth and the phase of constant radial growth rate of the pellets terminated simultaneously.

Influence of Pellet Concentration on Final Pellet Size, Duration of the Linear Radial Growth Phase and the Growth Rates during the Exponential and Cube Root Phase

Influence on Final Pellet Size. An overnight suspension of *Aspergillus nidulans* pellets (mean diameter, 0.8 mm) was serially diluted in DAN medium warmed to 37° C. Flasks were inoculated with 5 ml volumes of the resulting suspensions and incubated at 37° C for 3 days. At the end of this period the pellets were fixed in FAA and their mean diameter (mean of 20 pellets where possible) was determined. The diameter of pellets in cultures inoculated with 40-1, 250 pellets per flask decreased linearly with the logarithm of pellet concentration (Fig. 5).

Influence on the Duration of the Linear Radial Growth Phase. The duration of the phase of constant radial growth rate (linear growth) of

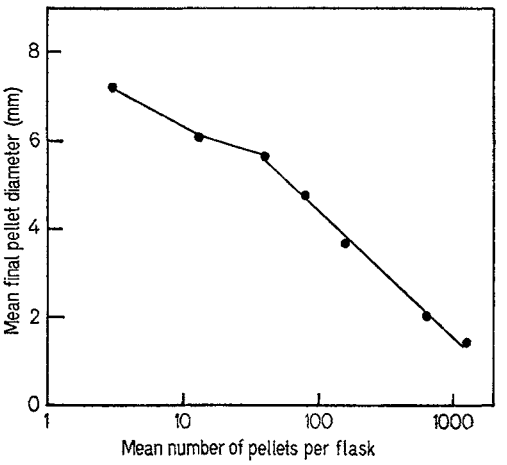

Fig. 5. Influence of pellet concentration on the mean final diameter of *Aspergillus nidulans* pellets after 3 days incubation at 37°C. Each flask contained 50 ml of half strength DAN medium and was inoculated with 5 ml of a pellet suspension

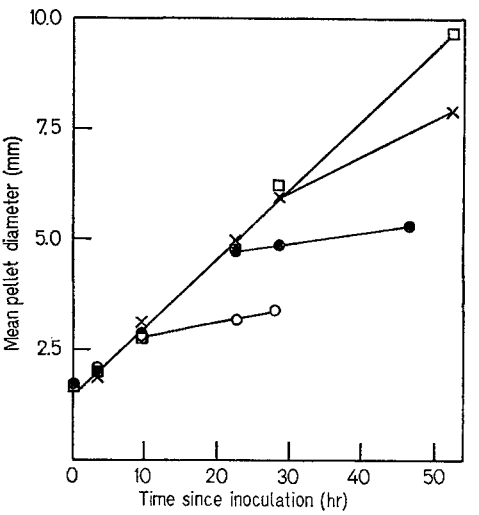

Fig. 6. Influence of pellet concentration on the radial growth of *Aspergillus nidulans* pellet at 37°C. Each flask contained 30 ml of DAN medium and was inoculated with 5 ml of a pellet suspension; □, 11 pellets/flask; ×, 44 pellets/flask; •, 176 pellets/flask; ○, 704 pellets/flask

Aspergillus nidulans pellets, and consequently the duration of the cube root phase, decreased with pellet concentration (Fig.6). Pellet concentration also influenced the initial radial growth rate of pellets; the linear

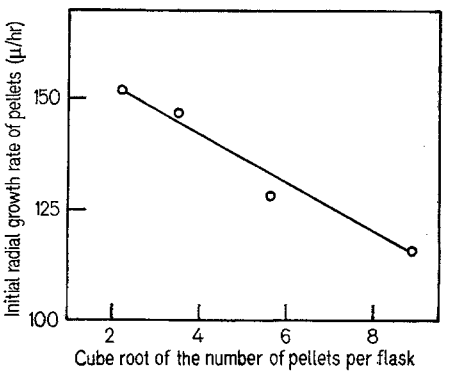

Fig. 7. Influence of the cube root of pellet concentration on the initial radial growth rate of *Aspergillus nidulans* pellets at 37°C. Each flask contained 30 ml of DAN medium and was inoculated with 5 ml volumes of a pellet suspension

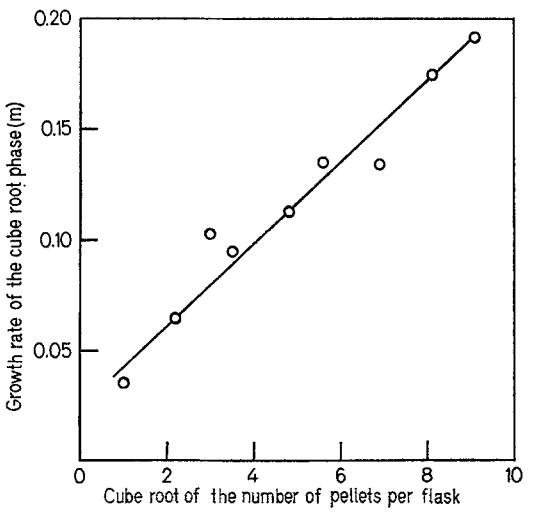

Fig. 8. Influence of the cube root of pellet concentration on the rate of the cube root phase of growth of *Aspergillus nidulans* pellets grown at 37°C

growth rate of pellets in cultures inoculated with *c.* 700 pellets per flask was *c.* 76% of the growth rate of pellets in cultures inoculated with *c.* 10 pellets per flask. The initial growth rate of pellets decreased linearly with the cube root of pellet concentration (Fig. 7).

Influence on Growth Rates during the Exponential and Cube Root Phases. Increase in the dry weight of cultures inoculated with between 1 and 704 *Aspergillus nidulans* pellets per flask was determined. The specific

growth rates during the initial "exponential" phase of these cultures varied between 52%—74% of the maximum specific growth rate of the mould (Trinci, 1969) and tended to decrease with pellet concentration. The fact that the pellets did not attain the mould's maximum of specific growth rate for the conditions (37° C) suggests that growth during this phase is probably not truly exponential.

The rate of increase (m) during the linear cube root phase of growth may be determined from the following.

$$m = \frac{Y_2^{1/3} - Y_1^{1/3}}{X_2 - X_1}$$

where Y_1 is the dry weight at time X_1 and Y_2 is the value at a later time X_2. The rate of cube root growth increased linearly with the cube root of the number of pellets in the culture (Fig. 8), thus confirming the validity of the equation suggested by Pirt (1966) to describe pellet growth during this phase.

Influence of Conidial Concentration on the Kinetics of Aspergillus nidulans *Growth.* Growth of cultures of *Aspergillus nidulans* inoculated with various conidial concentrations was determined. The majority of conidia in the inoculating suspensions were present as single spores but some formed chains of two or more spores; when each conidial chain was counted as a single unit the conidial concentration was only reduced by *c.* 25%. The viability of conidial suspensions always exceeded 95%. The specific growth rates during the exponential phase and the mean number of pellets formed per flask are shown in Table 4. The ratio, number of conidia per culture:number of pellets formed per culture varied between *c.* 21,000—58,500 over the range of conidial concentrations tested. Only the occasional spore was observed in culture filtrates after 24 hrs growth (24 hrs after inoculation, 10 ml of the culture medium was separated from the pellets centrifuged and the 0.25 ml at the

Table 4. *Influence of conidial concentration on the number of pellets formed and the specific growth rate of* Aspergillus nidulans *cultures*

Number of conidia used to inoculate each flask (C_f)	Number of conidia/ml of medium	Mean number of pellets formed per flask (P_f)	C_f/P_f	Growth during the "exponential" phase	
				specific growth rate (α, h^{-1})	doubling time (T_d, h)
2.8×10^5	9.3×10^3	13	21,154	0.165	4.2
1.1×10^6	3.7×10^4	26	42,308	0.195	3.6
4.4×10^6	1.5×10^5	75	58,667	0.158	4.4
1.8×10^7	6.0×10^5	352	50,000	0.365	1.9
7.0×10^7	2.3×10^6	2947	23,889	0.347	2.0

bottom of the tube examined microscopically). As a mycelial rind was not formed around the inside of the flask it seems unlikely that a substantial number of conidia were washed up and deposited on the walls of the flask. It would thus appear that most of the conidia in the inoculum were involved in some way in pellet formation.

Conidial concentration had a significant influence upon the maximum specific growth rate (Table 4).

Discussion

Since it has been shown that under favourable cultural conditions moulds grow exponentially (Zalokar, 1959; Pirt and Callow, 1960; Trinci, 1969) cube root growth indicates that parts of pellets are either not growing or growing under sub-optimal conditions. Pirt (1966) suggested that oxygen was most likely to be the growth limiting nutrient for pellets. He calculated that if oxygen could only enter by simple diffusion, growth of the pellet would be restricted to a peripheral zone 77 μ wide. This value is clearly exceeded in the case of single pellets of *Aspergillus nidulans*, suggesting that their texture is sufficient loose to permit some medium penetration by eddies and consequently mass transport of oxygen. Yoshida *et al.* (1967) using more direct methods have shown that oxygen uptake by *Lentinus edodes* pellets does in fact occur by bulk flow rather than simple diffusion. However, the fact that pellets do not continue to grow exponentially after they attain a certain diameter suggests that eventually a pellet is made up, as suggested by Pirt, of a peripheral growing zone and a non-growing inner mass which lacks oxygen.

Growth of pellets cultured singly at 30° C and below is initially exponential and subsequently follows cube root kinetics. When several pellets are used to inoculate cultures there is a decrease in the proportion of the growth period which follows cube root kinetics. The fact that at 37° C cultures inoculated with one or several pellets did not attain the maximum specific growth rate suggests that growth is not truly exponential; at this temperature exponential growth is probably restricted to pellets below *c.* 1.5 mm in diameter.

Emerson (1950) and Pirt (1966) suggested that maintenance of cube root kinetics was dependent upon pellets increasing in diameter at a constant rate. This prediction was confirmed by the present study. Pellets of *Aspergillus nidulans* and *Penicillium chrysogenum* increased in radius at much slower rates than colonies of these moulds on solid medium. This difference in growth rate may result from mycelium compaction caused by contact between the pellets and the sides of the culture vessel; hyphal density at the periphery of mould colonies has been shown

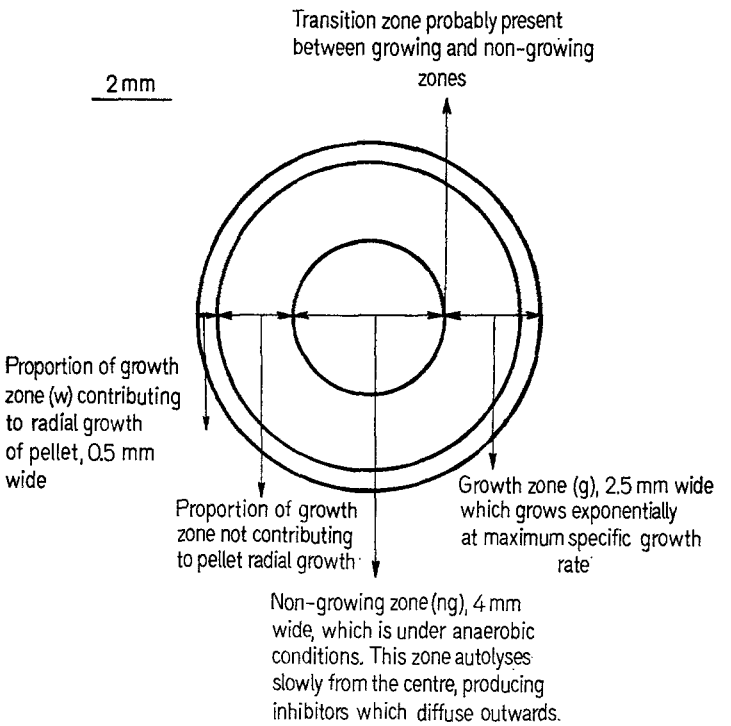

Fig. 9. Proposed zones of a hypothetical pellet of *Aspergillus nidulans* grown at 30° C and 9 mm in diameter

to influence their radial growth rate (Trinci, 1969). The eventual deceleration in the radial growth rate of pellets in cultures inoculated with several pellets (Figs. 3 and 4) may be due to lack of oxygen and/or accumulation of growth inhibitors in the medium (auto-inhibition). Production of inhibitors by moulds is often associated with growth under unfavourable conditions and one would expect them to be produced if the inner mycelium of pellets is starved of oxygen. Cultures of pellets may be a convenient source of the endogenously produced morphogens which have been implicated in mould differentiation (Park and Robinson, 1966).

Cytological observations indicate that the inner, non-growing component of pellets may undergo autolysis; several studies have shown that a space develops at the centre of large pellets (Camici *et al.*, 1952; Clark, 1962; Yanagita and Kogane, 1963). However, only at 37° C in *Aspergillus nidulans* was there a reduction in the dry weight per unit volume ratio of the pellets which would be consistent with substantial

internal autolysis. Although sporulation has been reported at the centre of some mould pellets (Yanagita and Kogane, 1963) it was not observed in the present study. The results obtained indicate that a pellet grown singly at 30° would probably be composed of the zones shown in Fig. 9.

In *Aspergillus nidulans*, unlike *Penicillium chrysogenum* (Camici *et al.*, 1952) pellet formation occurred at all conidial concentrations. Pellet formation is probably in part a consequence of an aggregation process similar to flocculation in yeasts (Morris, 1966). Conidial aggregation has been observed to precede germ tube formation in *Penicillium chrysogenum* (James and Trinci, unpublished results) but the basis of aggregation in filamentous fungi is not known (Galbraith and Smith, 1969). In addition, aggregation may occur between small mycelial clumps (aggregation between clumps 0.5—2 mm diameter has been observed) and spores may also become entangled in growing hyphae.

Exponential growth of moulds in submerged shake flask culture may be obtained by using a high concentration of spores in the inoculum ($1-2\times10^6$/ml of medium). In the case of *Penicillium chrysogenum* the high inoculum load will ensure that the organism grows in a homogeneous filamentous form and in *Aspergillus nidulans* only very small pellets (maximum diameter 1—1.5 mm) are formed. However, because of the presence of pellets in the latter species it is difficult to be certain that the specific growth rate attained in high spore inocula cultures truly represents the organism's maximum specific growth rate. When large pellets are formed by a mould, the mycelium will invariably be under heterogenous conditions and such cultures should not be used for biochemical and related studies.

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