Glucose and Penicillin Concentrations in Agar Medium Below Fungal Colonies

By GEOFFREY D. ROBSON,¹* STEPHEN D. BELL,¹ PAUL J. KUHN²
AND ANTHONY P. J. TRINCI¹

¹ Microbiology Group, Department of Cell and Structural Biology, School of Biological Sciences,
Williamson Building, University of Manchester, Manchester M13 9PL, UK

² Shell Research Limited, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, UK

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The growth of colonies of *Rhizoctonia cerealis* and *Penicillium chrysogenum* on solid media in plate cultures was studied. When grown on defined media containing 10–50 mM-glucose, *R. cerealis* did not cause a significant reduction in the glucose concentration of the medium in advance of colonization, but did cause the formation of a steep glucose concentration gradient in the substrate below the colony; the medium directly below the centre of a 7 cm diameter colony of *R. cerealis* was exhausted of glucose even when the fungus was grown on medium containing 50 mM-glucose. Penicillin produced by colonies of *P. chrysogenum* accumulated in the medium in advance of fungal colonization. For a period up to about 18 d after inoculation, the concentration of penicillin in the medium throughout the plate increased with colony development and thereafter, except at the margins of the plate, decreased.

INTRODUCTION

Pirt (1967) considered the formation of a microbial colony on nutrient agar from a small inoculum and suggested that (1) cells would grow at the maximum exponential rate as long as the nutrient concentrations remained much above the saturation constants (K_s values) and no condition inhibitory to growth was developed; (2) nutrient concentration gradients would be formed beneath the colony; (3) eventually the concentration of some 'growth-limiting' nutrient would fall virtually to zero in the medium under the centre of the colony and that therefore the central cells would stop growing; (4) growth of the colony would eventually be restricted to a peripheral annulus, the width of which would remain constant and would be determined by the balance between nutrient consumption and nutrient diffusion into the growing zone; and (5) after the establishment of a peripheral growing zone of constant width, the colony would expand in radius at a linear rate.

The initial exponential phase of growth predicted by Pirt has been confirmed experimentally for unicellular (Wimpenny & Lewis, 1977; Wimpenny, 1979) and filamentous (Schuhmann & Bergter, 1976) bacteria, and for moulds (Trinci, 1974). Also, Yanagita & Kogané (1963) showed that the specific rate of [³²P]orthophosphate uptake (= growth rate) at the centre of a colony of *Aspergillus niger* occurred at only 3% of the rate in the peripheral growth zone. Slow growth of cells has also been observed at the centre of bacterial colonies and has been attributed to oxygen limitation (Reyrolle & Letellier, 1979; Wimpenny & Parr, 1979).

As predicted by Pirt, colonies of most fungi and some bacteria (Burchard, 1974) increase in radius at a linear rate until they have completely colonized the plate. Trinci (1971) showed for a number of fungi that this linear growth rate (K_r) was a function of the width of a peripheral annulus (w , the peripheral growth zone) and the organism's specific growth rate (μ), thus $K_r = w\mu$. However, colonies of most unicellular micro-organisms (Palumbo *et al.*, 1971; Rieck *et al.*, 1973; Gray & Kirwan, 1974) and some fungal colonies, e.g. those of *Penicillium* spp., initially

grow at a linear rate but subsequently colony expansion slows and may eventually stop. Cooper *et al.* (1968) found that during this period of deceleration in growth rate, the area of a bacterial colony increased linearly with time, indicating a decrease in peripheral growth zone width. Rieck *et al.* (1973) showed that the deceleration phase of *Pseudomonas fluorescens* colonies was correlated with a decrease in glucose concentration at the colony margin. However, in other cases this deceleration in colony radial growth rate may be caused by inhibition of growth in the peripheral growth zone by the formation of secondary metabolites or autolytic products at the centre of the colony and their diffusion to the growing margin (Bull & Trinci, 1977). For example, oxygen deprivation of an obligate aerobe eventually results in cell death and consequently in the production of autolytic products inhibitory to growth.

The purpose of this study was to verify Pirt's hypothesis that nutrient concentration gradients develop in the medium below microbial colonies, and to assess if it was possible for secondary metabolites or autolytic products formed at the centre of a colony to influence growth in the peripheral growth zone. Glucose concentration gradients below colonies of *Rhizoctonia cerealis* were determined because this organism is sterile and therefore does not produce satellite colonies. *Penicillium chrysogenum* was used to study secondary metabolite production as penicillin assays can be easily made by simple techniques.

METHODS

Organisms and media. *Rhizoctonia cerealis* Van der Hoeven (strain R80/101) was obtained from the Plant Breeding Institute, Cambridge, UK, via Mr A. Goulds. *Penicillium chrysogenum* Thom (Wisconsin Q176) was obtained from King's College, University of London, UK (King's College culture collection number, P16) and *Bacillus subtilis* (Ehrenberg) Cohn was obtained from Dr M. Whalley of Manchester Polytechnic. The organisms were stored at 4 °C on malt extract (fungi) or nutrient (bacterium) agar slopes.

R. cerealis was grown on Vogel's (1956) medium with glucose instead of sucrose as the carbon source and *P. chrysogenum* was grown on defined medium (DM: Trinci, 1971) which contained 50 mM-glucose and was buffered at pH 6.8 with 0.1 M-phosphate buffer. Both media were gelled with 15 g agar l⁻¹ (Taiyo Technical Grade, Davis Gelatine) and, except for the glucose solutions, were prepared according to Vogel (1956) and Trinci (1971). Membrane (0.2 µm pore diameter) sterilized glucose solution was added to the other molten (50 °C) ingredients of the media to which distilled water had been added to replace the water lost during autoclaving (121 °C for 15 min). In Vogel's and DM media (Inch *et al.*, 1986), glucose is the nutrient which first becomes exhausted, i.e. it is the 'limiting' nutrient. All chemicals were of Analar grade and, unless stated otherwise, were obtained from BDH.

Inoculation and cultural conditions. The fungi were grown in 9 cm diameter Petri dishes containing 20 ml medium. The plates were poured and allowed to set whilst on a level surface, to ensure that each plate contained an even depth of medium (about 3.2 mm). The medium was sometimes overlaid with circles of cellophane (Rayophane, British Cellophane) about 8.5 cm in diameter; the cellophane was boiled twice (for 10 min each time) in distilled water to remove the plasticizers, and was then autoclaved in water at 121 °C for 15 min. Cellophane-overlaid plates were dried and then inoculated with 4.5 mm diameter plugs of *R. cerealis* taken from the margin of 3-4-d-old colonies (colonies in the linear phase of growth) grown on Vogel's medium lacking glucose. Plates of cellophane-overlaid DM were inoculated with 4.5 mm diameter plugs taken from a DM plate which had been spread with spores of *P. chrysogenum*, dried but not incubated. All cultures were incubated at 25 °C, and plates of *P. chrysogenum* were maintained at a high relative humidity to prevent them drying out during the long (up to 29 d) incubation periods.

Measurement of colony diameter. Colony diameters were measured with a Shadowmaster (Baty & Co., Burgess Hill, Sussex, UK), using a ×10 objective. Colony dry weights were obtained by washing mycelia off the cellophane onto predried, weighed filter papers (Whatman no. 1). The biomass was washed with 100 ml distilled water and dried at 60 °C to constant weight.

Measurement of ATP concentrations. Mycelial discs (3 mm diameter) were cut across a colony of *R. cerealis* (45 mm diameter) starting either side of the inoculum plug; the colony was grown on Vogel's medium containing 50 mM-glucose, overlaid with cellophane. ATP was extracted for 90 s in 5 ml boiling Tris/HCl buffer (20 mM, pH 7.75) containing 2 mM-EDTA, and ATP was estimated using the firefly luciferase bioluminescence assay (Lundin & Thore, 1975). The ATP contents of mycelial discs equidistant from the colony centre were combined as replicates. Reagents for the ATP assay were obtained from LKB.

Glucose assays. The cellophane and *R. cerealis* colony were stripped from a plate, and a 5 mm wide strip of agar medium which passed under the centre of the colony was removed using fixed pair of parallel razor blades. This strip was then cut into 5 mm lengths. Each block of agar medium (about 5 × 5 mm) was added to 1.5 ml distilled water in a pre-weighed, screw-capped bottle; the bottles were weighed again to determine the volume of the blocks

and then placed in a boiling water bath for 3 min to melt the agar prior to making glucose assays. Blocks equidistant from the colony centre were used as replicates. The glucose concentrations of blocks of media were determined using the oxidase/peroxidase assay described by Bergmeyer & Bernt (1974), using 2,2'-azino-bis(3-ethylbenzthiazoline)sulphonic acid as the chromogen. Enzymes and chromogen were obtained from Sigma. The glucose concentrations of agar surfaces were measured using the Reflocheck system (Boehringer Mannheim). Test strips were moistened with distilled water and placed onto the agar surface for 60 s, removed, and assayed for glucose after a further 60 s.

Penicillin production and assays. The cellophane and *P. chrysogenum* colony were stripped from a plate, and successive pairs of agar plugs (5 mm diameter) were cut with a cork borer from the medium across the diameter of the plate, starting on either side of the site originally occupied by the colony centre. The penicillin concentrations in pairs of plugs were determined using a penicillin bioassay system; the plugs were placed onto the surface of nutrient agar pour plates of *B. subtilis* which were then incubated at 37 °C for 24–36 h prior to measuring the diameter of the inhibition zones. Penicillin standards prepared in agar medium were treated in the same way as the test plugs.

RESULTS

Colony radial growth rates. At 25 °C, *R. cerealis* had a colony radial growth rate of about 270 $\mu\text{m h}^{-1}$ on Vogel's medium containing 10, 25 or 50 mM-glucose, and *P. chrysogenum* had a colony radial growth rate of 52 $\mu\text{m h}^{-1}$ on DM containing 50 mM-glucose.

Dry weight and ATP content of colonies of *R. cerealis* grown on media containing 10, 25 or 50 mM-glucose. Fig. 1 shows the relationship between the radius and dry weight of colonies of *R. cerealis* grown on Vogel's medium containing 10, 25 or 50 mM-glucose. Colonies which were about 15 mm in radius and which had been grown on 10, 25 or 50 mM-glucose had similar biomass densities (Fig. 1), suggesting that their growth was not limited by the availability of glucose. However, with further increase in colony radius, appreciable differences in biomass densities were observed between colonies of similar radius grown on media containing different concentrations of glucose: colonies about 32.5 mm in radius had average biomass densities of 62.5, 130.3 and 180.6 $\mu\text{g cm}^{-2}$ when grown on media containing 10, 25 and 50 mM-glucose respectively. This result suggests that, at least for the lower two glucose concentrations, increase in colony biomass was limited by availability of glucose.

Fig. 2 shows the ATP content of 3 mm diameter mycelial discs taken from points across a colony of *R. cerealis* grown on 50 mM-glucose Vogel's medium overlaid with cellophane. The relationships observed between ATP concentration and colony diameter suggest that, for colonies up to about 22.5 mm in radius, biomass increases exponentially throughout the colony.

Glucose concentrations at the margins of colonies of *R. cerealis* grown on media containing 10, 25 or 50 mM-glucose. Measurements were made of glucose concentrations in 5 mm diameter plugs of medium taken from just beyond the margin of colonies of *R. cerealis* cultured on Vogel's medium containing various concentrations of glucose; these colonies were cultured directly on the agar surface, not supported by cellophane. No significant difference (at the 5% level) was observed between the glucose concentrations of uninoculated control plates and of plugs taken from just beyond the colony margin (Table 1).

Glucose concentrations in the medium below colonies of *R. cerealis* grown on media containing 10, 25 or 50 mM-glucose. Fig. 3 shows the concentrations of glucose in the medium below colonies of *R. cerealis* grown for 3, 5 or 6 d on cellophane on Vogel's medium containing 10, 25 or 50 mM-glucose. For each glucose concentration, the extent of glucose depletion in the medium beneath the colony increased with increase in colony diameter. The shapes of the glucose concentration profiles which developed below colonies were remarkably similar for each glucose concentration tested (Fig. 3).

Six days after inoculation, when approximately half the plate had been colonized, the medium below the centre of colonies grown on all the media, even that which initially contained 50 mM-glucose, had become exhausted of glucose. During colony development, the glucose concentration profile in the medium became progressively steeper and the larger colonies even appeared to deplete the glucose in the medium in advance of colonization (but see above).

Fig. 1

Fig. 2

Fig. 1. Relationship between the radius and dry weight of colonies of *R. cerealis* grown at 25 °C on cellophane on Vogel's medium containing 10 (○), 25 (□) or 50 (△) mM-glucose.

Fig. 2. ATP content across a colony of *R. cerealis* grown at 25 °C on cellophane on Vogel's medium containing 50 mM-glucose, plotted on arithmetic (○) and log (●) scales (left and right, respectively). The vertical bars represent standard errors.

Table 1. Glucose concentrations in 5 mm diameter plugs taken from uninoculated plates and from just beyond the margin of colonies of *R. cerealis* grown for 3, 4, 5 or 6 d at 25 °C on media nominally containing 10, 25 or 50 mM-glucose

Plugs of uncolonized medium taken from:	Mean colony radius (mm)*	Glucose concn (mM) in plugs taken from uninoculated plates or from just beyond the margin of fungal colonies†		
		Nominal glucose concn (mM):		
		10	25	50
Uninoculated plates		9.5 ± 0.8	25.9 ± 0.2	53.3 ± 1.7
Margin of 3-d-old colonies	18.2 ± 0.7	9.5 ± 1.3	25.7 ± 0.8	52.1 ± 3.1
Margin of 4-d-old colonies	21.7 ± 0.3	8.2 ± 1.1	26.4 ± 0.7	51.2 ± 2.1
Margin of 5-d-old colonies	27.7 ± 0.2	9.2 ± 0.3	26.2 ± 1.4	52.2 ± 4.2
Margin of 6-d-old colonies	33.0 ± 0.4	9.6 ± 0.7	26.0 ± 0.8	54.6 ± 3.8

* Each result is the mean of five colonies ± SEM.

† Each result is the mean of 15 plugs taken from three plates (five plugs per plate) ± SEM.

Plugs of medium were taken from below the centre of colonies grown on cellophane on Vogel's medium containing 25 mM-glucose, and the glucose concentrations at the upper and lower surfaces (with respect to the colony) of the plugs were measured, as was the glucose concentration of the whole plug (Table 2). No significant difference (at the 5% level) was observed between the glucose concentrations in the whole plug and at its upper or lower surfaces (Table 2). Thus, although a marked 'horizontal' (from the centre to the margin of the plate) glucose concentration gradient was formed in the medium below a colony (Fig. 3*b*), there was apparently no significant development of a 'vertical' (from medium at the top to medium at the bottom of the plate) glucose concentration gradient. Hence, the glucose concentrations shown in Fig. 3 would be approximately the same as those to which the lower part of the colony was actually exposed, i.e. the glucose concentration at the surface of the medium.

Fig. 3. Glucose concentration of medium in uninoculated plates (○), and of medium below colonies of *R. cerealis* grown at 25 °C on Vogel's medium containing (a) 10 mM-glucose, (b) 25 mM-glucose or (c) 50 mM-glucose for 3 (□), 5 (△) and 6 (▽) d. Each point is the mean of six replicates taken from three plates; the standard error was usually 5%, and never greater than 10%, of the mean. The arrows indicate the position of the colony margin 3, 5 and 6 d after inoculation.

Table 2. Glucose concentrations of plugs of medium cut from below the centre of colonies of *R. cerealis* grown at 25 °C on cellophane on Vogel's medium nominally containing 25 mM-glucose

Age of colony (d)	Colony radius (mm)*	Glucose concn (mM)*		
		In whole plug†	At upper surface (nearest to colony) of plug‡	At lower surface (furthest from colony) of plug‡
2	11.4 ± 0.2	27.4 ± 1.4	24.4 ± 0.2	> 25.0
3	19.0 ± 0.5	11.9 ± 0.8	10.4 ± 0.9	12.3 ± 1.0
4	24.2 ± 0.5	6.8 ± 0.5	6.6 ± 0.3	6.9 ± 0.4
5	31.2 ± 1.6	0.4 ± 0.1	< 1.1	< 1.1

* Each result is the mean of five replicate plates ± SEM.

† Glucose measured using the oxidase/peroxidase assay system.

‡ Glucose measured using the Reflocheck assay system.

Penicillin concentrations in the medium below colonies of P. chrysogenum. The concentrations of penicillin in plugs of medium cut from below the surface of colonies of *P. chrysogenum* grown on DM plates overlaid with cellophane are shown in Fig. 4. The highest concentration of penicillin detected in a plate was always found in the medium below the centre of the colony. However, for all colonies studied (12–29-d-old colonies), penicillin was detected throughout the plate, including uncolonized medium at the edge of the plate. Fig. 5 shows the change with time of the penicillin concentrations in plugs of medium taken from various fixed points below colonies of *Penicillium chrysogenum*. Up to about 18 d after inoculation, the concentration of penicillin in the medium throughout the plate increased with colony development and thereafter, except at the margins of the plate, it decreased.

Fig. 4

Fig. 4. Penicillin concentration in the medium beneath colonies of *P. chrysogenum* grown at 25 °C on cellophane on DM containing 50 mM-glucose for 12 (○), 15 (□) and 19 (△) d; each point represents the mean of six replicates taken from three plates and is given with the standard error. The arrows indicate the position of the colony margin 12, 15 and 19 d after inoculation.

Fig. 5

Fig. 5. Change in penicillin concentration with time at fixed points [2.5 (○), 7.5 (□), 12.5 (△), 17.5 (▽), 22.5 (●), 27.5 (■), 32.5 (▲) and 37.5 (▼) mm from the colony centre] in the medium below colonies of *P. chrysogenum* grown at 25 °C on cellophane on DM containing 50 mM-glucose.

DISCUSSION

Inch *et al.* (1986) showed that ATP provided a sensitive and reliable indicator of the biomass of *Paecilomyces fumosoroseus*, and it was for this reason that measurements of the ATP concentration within a colony of *R. cerealis* were made (Fig. 2). When *R. cerealis* was grown on Vogel's medium containing 50 mM-glucose, growth apparently proceeded at an exponential rate throughout the biomass of colonies up to 22.5 mm in radius (Fig. 2). However, growth of similarly sized colonies cultured on 10 or 25 mM-glucose was apparently eventually limited by glucose availability (Fig. 1); glucose is the first nutrient in Vogel's medium which becomes exhausted.

Fig. 3 and Table 2 confirm Pirt's (1967) hypothesis that nutrient gradients are formed beneath microbial colonies cultured on solid media and support his suggestion that growth at the colony centre eventually ceases because of nutrient exhaustion, although nutrients are still present in medium remote from the centre of the plate. In the case of *R. cerealis*, glucose exhaustion of the medium below the colony centre occurred when colonies had attained a radius of about 35 mm and, over the range 10–50 mM-glucose, the onset of nutrient exhaustion below the centre of the colony appeared to be independent of the initial glucose concentration (Fig. 3). The glucose concentration gradients established below colonies of *R. cerealis* grown on Vogel's medium initially containing 10, 25 or 50 mM-glucose were remarkably similar and presumably reflect a balance between glucose consumption by the fungus and glucose diffusion from uncolonized parts of the plate.

Colonies of *R. cerealis*, unlike those of *Pseudomonas fluorescens* (Rieck *et al.*, 1973), do not apparently deplete the glucose in the medium in advance of colonization (Table 2) even when they are cultured on medium containing only 10 mM-glucose. However, it should be noted that *Ps. fluorescens* was grown on media which contained only about 0.56–5.6 mM-glucose. Unlike the *Ps. fluorescens* colonies grown on 0.5–5.6 mM-glucose, the radial growth rate of colonies of *R. cerealis* was not affected by glucose concentration (over the range 10–50 mM). Thus, when *R.*

cerealis is grown on Vogel's medium containing 10 mM-glucose, hyphae at the colony margin extend continually into medium which has a composition very similar or identical to that of the original, uninoculated medium. However, Trinci (1969) showed that, when cultured on medium containing less than about 0.5 mM-glucose, colonies of *Aspergillus nidulans* behave like those of *Ps. fluorescens*, i.e. colony growth rate decreases with decrease in glucose concentration.

Secondary metabolites and products of autolysis formed at the centre of a microbial colony are likely to inhibit growth of the producer organism, and these products will also inhibit colony radial growth rate if they diffuse through the medium and accumulate below the peripheral growth zone (Bull & Trinci, 1977). Fig. 4 shows that penicillin produced by *P. chrysogenum* colonies accumulated in the medium in advance of fungal colonization. This result suggests that expansion of a microbial colony which has a slow radial growth rate may decelerate and stop because of the accumulation of growth-inhibitory substances in the uncolonized medium surrounding the colony; thus growth of some microbial colonies may cease because of auto-inhibition rather than because of nutrient exhaustion.

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