Growth and Encystation of *Acanthamoeba castellanii*

By A. H. CHAGLA AND A. J. GRIFFITHS

Department of Microbiology, University College, Cardiff CF1 1XL

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SUMMARY

Complete encystment of *Acanthamoeba* occurred in peptone–yeast extract–glucose medium supplemented with 50 mM-magnesium chloride. There was an increase in the RNA, protein and DNA contents of the cells which coincided with an increase in the proportion of binucleate amoebae and commitment to encystation, suggesting that encystation could be a consequence of the inhibition of cell division when nutrient concentrations were limiting growth.

INTRODUCTION

Studies of the encystation of soil amoebae carried out in replacement medium (Griffiths & Hughes, 1969), a non-nutrient medium to which amoebae were transferred from growth medium, suggested that the conversion of amoebae to cysts was probably initiated by depletion of nutrients (Band, 1963; Neff, Ray, Benton & Wilborn, 1964; Griffiths & Hughes, 1968, 1969). It has not been possible, however, to obtain complete encystation of amoebae in growth medium at the end of logarithmic growth when lowered nutrient concentrations limit growth and encystment should thus be promoted. Depending on the composition of the growth medium and incubation conditions, the final level of encystment is usually between 6 and 70 % of the cell population present in the cultures at the cessation of growth (Neff *et al.* 1964; Byers, Rudick & Rudick, 1969). These observations suggest that either nutrient limitation is not an important initiator of encystation or the growth media routinely used for the culture of soil amoebae are somewhat deficient. This paper describes a modification of one such growth medium which allows the complete encystment of *Acanthamoeba*, and describes some of the cellular changes associated with the conversion of amoebae into cysts.

The availability of an alternative to the replacement technique for the experimental manipulation of encystation serves two purposes. Firstly, one of the main problems in the study of a process such as encystation, which involves a sequence of structural and physiological changes (Griffiths, 1970), is in establishing whether that sequence is unique to the differentiation (Mandelstam, 1969). It is only by observing the differentiation under a variety of conditions that the persistence of the sequence or its component parts can be determined. Secondly, recent studies have suggested that certain key events in the cell cycle, in particular the phase of DNA synthesis, are important in determining the point of transition between the mutually exclusive processes of growth and encystation (Byers *et al.* 1969; Neff & Neff, 1972). These studies suffered from certain disadvantages, being based on cultures giving only very low levels of encystment or necessitating the use of inhibitors to limit growth and initiate encystation. The method outlined here has overcome these disadvantages.

METHODS

The organism and its culture. *Acanthamoeba castellanii* (Protozoa: Sarcodina, Amoebida), originally obtained from Dr K. M. G. Adam, has been maintained in this laboratory since 1965. Cultures were routinely maintained and grown axenically in PGY medium containing (% w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Portions (5 ml) of a log-phase culture were used to inoculate 50 ml of medium and cultures were incubated in 250 ml conical flasks at 30 °C in a reciprocal shaking water bath (Griffiths & Hughes, 1968).

Cell counts. Counts of amoebae and cysts in suspensions diluted with either fresh growth medium or 50 mM-MgCl₂ were made in a Fuchs-Rosenthal haemocytometer (Werber & Sons, Lancing, Sussex).

Extraction and measurement of chemical components. Cellulose was estimated turbidimetrically as an alkali-insoluble residue (Griffiths & Hughes, 1969). Extraction and measurement of protein, DNA and RNA were as described by Byers *et al.* (1969). Total hexose was measured by the anthrone reaction (Scott & Melvin, 1953) on ethanol (80 %, w/v) extracts of amoebae and cysts. Colorimetric and turbidimetric measurements were made with a Unicam SP 600 spectrophotometer using 1 cm glass cuvettes.

Measurements of endogenous respiration rates. Cells were collected and washed with 50 mM-MgCl₂ in a bench centrifuge at 500 g for 5 to 10 min at laboratory temperature. Washed cell pellets were resuspended in 50 mM-MgCl₂ and their respiration rates measured polarigraphically (Lloyd & Griffiths, 1968).

Chemicals. All chemicals were of analytical grade.

RESULTS

Induction and time course of encystation in growth medium. In PGY medium the maximum population density, reached after 72 h incubation, was followed by a decline in cell numbers (Fig. 1*a*) and by an increase in the proportion of cysts (Fig. 1*b*) which finally reached 11 % of the maximum (72 h) population.

The only requirement for complete encystment of *A. castellanii* in replacement medium was 50 mM-MgCl₂, and it was found that addition of this concentration of MgCl₂ to PGY medium also resulted in complete encystment of the cultures (Fig. 1*b*). Encystation in magnesium-supplemented medium (PGYS) proceeded alongside growth and appeared to be initiated after about 48 h of growth when there was a decrease in the growth rate of the amoebae (Fig. 1*a*). Cellulose synthesis reached 50 % of its maximum value about 18 to 20 h after the initiation of encystation (Fig. 1*b*). Using morphological criteria, the mid-point of encystation, when 50 % of the population were identifiable as mature cysts, occurred about 40 h after initiation. Thus the formation of fully mature cysts required a further 20 h after cellulose synthesis had begun. In replacement medium the mid-point of cellulose synthesis also occurred after about 20 h but required a further 30 h for completion (Griffiths & Hughes, 1969). Encystation in PGYS was therefore more rapid than in the starvation conditions of the replacement medium.

Respiration during growth and encystation. Respiration rates of amoebae in PGY and in PGYS showed essentially similar trends except that in encysting cultures respiration declined to an almost immeasurable level by the time encystation was completed (Fig. 2). A dramatic increase in respiratory activity is therefore not necessarily associated with encystation as suggested by Griffiths, Lloyd, Roach & Hughes (1967) and Griffiths & Hughes (1969).

Fig. 1. (a) Growth of *Acanthamoeba* at 30 °C in PGYS (●) and PGY (○) measured turbidimetrically at 400 nm. (b) Encystation of *Acanthamoeba* in PGYS (●) and PGY (○), with cysts expressed as a percentage of the maximum cell numbers shown in (a); cellulose was also estimated as an alkali-insoluble residue in PGYS and expressed as a percentage of the maximum value at 168 h (□).

Fig. 2. Respiration rates of *Acanthamoeba* during growth and encystation in PGYS (●) and PGY (○). Measurements were made at 30 °C with an oxygen electrode using whole cells suspended in 50 mM-MgCl₂.

Decline of respiration to a very low, virtually immeasurable level, however, appears to be a consistent accompaniment of encystment.

Changes in the chemical composition of encysting cells. The levels of the major cell constituents showed quite striking differences in encysting and non-encysting amoebae. In encysting cultures in PGYS there was a more than twofold increase in the level of protein and a fourfold increase in RNA content during the period of cellulose synthesis (Fig. 3*a, b*); total hexose concentration also increased fourfold but reached its maximum only when encystation was complete (Fig. 3*c*). Unlike the total hexose content, the levels of protein and RNA had both declined to pre-encystment values by the time encystation was con-

Fig. 3. Measurements of protein (a), ribonucleic acid as pentose (b) and hexoses by the anthrone reaction (c) in *Acanthamoeba* during incubation in PGYS (●) and PGY (○).

Fig. 4. DNA content of *Acanthamoeba* in PGYS (●) and PGY (○) measured by the diphenylamine reaction, and the percentage of binucleate amoebae during incubation in PGYS (□).

cluded. In non-encysting PGY cultures neither protein nor RNA contents varied significantly over the whole period of incubation but the hexose content of the amoebae increased during the phase of decline in cell numbers (Fig. 3).

DNA contents and nuclei. There were marked differences in the DNA contents of encysting and non-encysting amoebae (Fig. 4). In encysting cultures the DNA increased consistently during the phase of cyst-wall synthesis and, even after a subsequent slight decrease, the DNA content of mature cysts was higher than that of amoebae in the pre-encystment phase. The average DNA content of non-encysting amoebae did not show this pattern, with only slight variations around a value of 2 pg deoxyribose/cell.

These measurements were consistent with observations of the nuclei of the amoebae during encystation. In the pre-encystation phase 16 to 17 % of the amoebae were binucleate and this proportion increased throughout encystation until, at 96 h, 26 % were binucleate.

Table 1. *Commitment to encystation by Acanthamoeba*

Time of transfer or MgCl ₂ addition (h)	Encystation (%)	
	Cells transferred from PGYS to PGY	MgCl ₂ added to cells in PGY
24	10.5	87.0
48	39.6	52.0
72	50.0	28.0
96	68.0	31.0
120	100.0	10.0

Amoebae were transferred from PGYS to PGY at the times shown, or MgCl₂ was added (to give a final concentration of 50 mM) to amoebae incubated in PGY. All cultures were then incubated for a total of 168 h, and the number of cysts was expressed as a percentage of the cells present at the time of transfer or at the time when MgCl₂ was added.

Table 2. *Competence of amoebae to form cysts*

Time of transfer from growth medium to replacement medium (h)	Cellulose content at time of transfer (mg/mg dry wt amoebae)	
	PGYS-grown cells	PGY-grown cells
24	0.13	0.16
48	0.15	0.14
72	0.27	0.10
96	0.19	0.02

Amoebae were grown in PGY and PGYS and transferred to a replacement medium at the times shown. Incubation was continued for a further 48 h. Encystment was assessed by measuring the cellulose produced during cyst wall formation.

Over the same incubation time the percentage of binucleate amoebae in PGY decreased from 16 to around 10 %.

Commitment to encystation. Although no cysts were present in PGYS cultures at 48 h, when cells were transferred to PGY at this time nearly 40 % eventually encysted (Table 1). Increasing proportions showed this commitment at 72 and 96 h. Thus, cells which had made the transition from growth to encystation in PGYS did not require the more favourable environment provided by that medium to complete cyst formation. Another series of experiments in which 50 mM-MgCl₂ was added to amoebae grown in PGY showed that the first 48 h was probably the most important period for cyst initiation, for if MgCl₂ was added after this time less than 50 % of the population completed encystation.

The amoebae also showed differences in their competence to form cysts when transferred to replacement medium from PGY or PGYS (Table 2), and the time at which transfers were made modified the encystment response. Amoebae grown in PGYS were more competent at 72 and 96 h than earlier, whereas amoebae from PGY were more competent earlier. Complete encystment was obtained with PGY-grown amoebae only when they were transferred after not more than 24 h growth, whereas PGYS-grown cells all showed complete encystment from 48 h onwards. Measurements of cellulose in cysts in both PGYS and replacement medium repeatedly showed that 100 % encystment, judged by microscope counts, was equivalent to a cellulose content of 0.15 mg/mg dry wt of amoebae. The higher cellulose levels in cells transferred at 72 and 96 h probably indicated the presence of thicker cyst walls.

DISCUSSION

There are both similarities and differences in the process of encystation as it occurs in PGYS and in the starvation conditions of replacement medium. The time courses of the early stages immediately following initiation appeared to be of about the same length. The period of wall synthesis was completed in a much shorter time in cells which encysted in PGYS, suggesting that these amoebae, although committed to encystment, were still influenced by the surrounding medium. This confirms earlier observations that, when $MgCl_2$ was added to amoebae growing in mycological peptone, the decline which normally followed logarithmic growth in this medium was inhibited but encystation, although clearly initiated, was not completed (Griffiths & Hughes, 1969). Thus the mere addition of magnesium ions to growth medium does not necessarily result in encystation, as initiation and completion of wall synthesis have different environmental requirements.

It is clear that increased respiration is not an absolute corollary of encystation and that the increases reported during encystation in replacement medium were probably associated with the starvation conditions obtaining in that medium or the ionic or osmotic changes to which the cells were subjected in the transfer from one medium to another.

The chemical changes occurring during encystation in PGYS were, in many ways, similar to those reported during encystation in replacement medium (Griffiths & Hughes, 1969), except that hexose increased after the completion of encystation in PGYS. This suggests that the sequence of changes involved in encystation is highly dependent on the nature of the surrounding medium. Hexoses are important precursors for cellulose synthesis and it appears that in PGYS the amoebae were less dependent on endogenous sources of these materials than they were in replacement medium. Byers *et al.* (1969) reported similar changes in chemical composition of amoebae at the end of logarithmic growth and suggested that they indicated that cyst initiation had occurred. However, one feature of encystation in PGYS during the phase of protein and RNA accumulation was the early commitment of the amoebae to encystation; this was not found by Byers and his co-authors, whose 'unbalanced' amoebae grew well when re-inoculated into fresh growth medium. There is thus a significant difference between stationary-phase amoebae and amoebae at the end of the growth phase when initiation and commitment have occurred, and accumulation of protein and RNA is not necessarily associated with encystation. That initiation and commitment are specific responses and not merely consequences of 'unbalanced growth' is suggested by some preliminary experiments in this laboratory which have shown that cycloheximide (20 $\mu g/ml$) and actinomycin D (50 $\mu g/ml$), which are inhibitors of protein and RNA synthesis respectively, both inhibit encystation if present in the medium up to the time of commitment but have little or no effect when added to PGYS later.

Neff & Neff (1972) have suggested that encystation is initiated by inhibition of DNA synthesis, a condition likely to occur in cultures at the end of logarithmic growth or on transfer of amoebae to a non-nutrient replacement medium. The increase in average DNA content and the large proportion of binucleate amoebae present during encystation in PGYS suggest that the mechanism of initiation is probably more complicated. The accumulation of binucleate amoebae indicates that not only had DNA synthesis been completed but that nuclear division had also been achieved in many of the cells. James & Byers (1967) and Byers *et al.* (1969) also reported multinuclearity in cultures of *Acanthamoeba* under conditions which limited growth. Furthermore, Mattar & Byers (1971) showed that cysts of *Acanthamoeba* contained enough DNA to allow several cell divisions in the absence of DNA synthesis during cyst germination, and Rastogi, Sagar, Kapoor & Agarwala (1972)

also demonstrated that cyst germination is insensitive to inhibition of DNA synthesis. It is likely that there is more than one transition point in the cell cycle when initiation of encystation can occur. The available evidence suggests that the phases of nuclear division and cell division are the most likely times. The use of asynchronous batch cultures imposes severe limitations on investigation of this problem and it is unlikely that much further progress will be made until it is possible to obtain synchronous populations of *Acanthamoeba*.

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