Macromolecular Composition and Nuclear Division During Spore Germination in *Aspergillus nidulans*

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SUMMARY

Germ-tube emergence in *Aspergillus nidulans* exhibits partial synchrony. Germ tubes were first detected at 210 min. after inoculation of spores into a liquid medium. Almost all spores produced germ tubes within 360 min., 49.5 % of them doing so between 240 and 300 min. Increase in dry weight was first detected at 120 min. and various constituents at the following times: RNA (30 min.), Kjeldahl nitrogen (30 min.), protein (150 min.) and DNA (180 min.). Pauses in the increase of DNA, RNA and protein were observed. The DNA content per nucleus during the first replication cycle reached twice that of the resting spore, but at later stages of germination values only 10 % higher were observed. Spores containing 2, 4 and 8 nuclei were first detected at 270, 390 and 480 min. respectively.

INTRODUCTION

The spores of *Aspergillus nidulans* (Eidam) Winter are favourable material for spore germination studies (Shepherd, 1957; Kessel & Rosenberger, 1968). The spores (conidio-spores or conidia) are uninucleate and can be obtained in large numbers. In addition, studies have been made of nuclear division in *A. nidulans* spores (Weijer & Weisberg, 1966; Rosenberger & Kessel, 1967, 1968; Robinow & Caten, 1969). The present work was undertaken to study nuclear division and macromolecular synthesis during spore germination.

METHODS

Organisms. Strain BWB 50b11; w3 was used throughout this work. The strain, which has white spores and requires biotin, was originally obtained from Glasgow University.

Preparation of spore suspensions. Thirty ml. volumes of complete medium agar (MacKintosh & Pritchard, 1963) in 250 ml. conical flasks were inoculated with a spore suspension and incubated at 37° for 7 days. The spores were harvested by adding 20 ml. of 0.1 % (v/v) Tween 80 together with sterile glass beads or marbles. The flasks were agitated and the spore suspensions removed under aseptic conditions and pooled. Spore chains were broken with a Rotamixer (Hook & Tucker Ltd) and hyphal fragments removed by filtration through six layers of sterile muslin. The spores were washed twice and suspended in sterile distilled water. Spore counts were made with a Fuchs–Rosenthal haemocytometer slide, at least 300 spores being counted. Spores thus prepared had a viability of 95 to 99 % after storage at 4° for several days, although the experiments reported here were carried out within 24 h.

Cultural conditions and media. Spores were added to 400 ml. of liquid DAN medium containing 50 µg./l. biotin (Trinci, 1969) in a 2 l. conical flask to give a concentration of

2×10^7 spores per ml. Higher spore concentrations resulted in auto-inhibition of germination. Flasks were incubated at 37° on a Gallenkamp orbital shaker at 200 rev./min. Unless otherwise stated, all times indicated are minutes after inoculation of spores.

Assessment of germ-tube emergence. One ml. samples were added to 0.1 ml. Rose Bengal solution (Lingappa & Lingappa, 1965) which fixed and stained the spores. In each sample at least 200 spores were examined, and germ-tube emergence was recorded if the germ tube was equal to half the diameter of the spore (Manners, 1966). Some clumping occurred and separation was achieved by agitation of the suspensions with a finely drawn Pasteur pipette. Estimates of germ-tube emergence from replicate flasks gave standard deviations of 2 to 5 % so that a single sample was usually adequate. The standard deviation was greater at lower levels of spore germination and 400 to 500 spores were counted.

Chemical analyses. Samples were withdrawn, frozen quickly in an ethanol/dry ice mixture and stored at -20° .

Protein. Samples were acidified with 10N-perchloric acid (final concentration 0.2N) and kept at room temperature for 30 min. The supernatant was discarded and 2 ml. of distilled water and 1 g. of no. 2 Ballotini beads were added. This mixture was treated with a micro-probe on a 100 W MSE Ultrasonicator for 5 min. The tube was immersed in crushed ice during this process. This treatment resulted in 80 to 90 % disruption of conidia. Sodium hydroxide was added (final concentration 1N) and the tube was left overnight. The supernatant was assayed for protein (Lowry, Rosebrough, Farr & Randall, 1951) with crystalline bovine serum albumin as a standard.

Kjeldahl nitrogen was assayed by the micro-analytical method of Lang (1958). Intact spore samples were extracted with cold 0.2N-perchloric acid as above and assayed with Nessler's reagent after acid digestion.

RNA was assayed by the orcinol method (Schneider, 1957). Intact spore samples were extracted with 0.2N perchloric at 4° and samples were disrupted as above. Ribose was extracted from the disrupted conidia by treatment with 0.5N-perchloric acid for 30 min. at 70° . Two extractions were made and the extracts pooled. D-Ribose was used as a standard and the RNA present was calculated from the 'apparent' ribose assuming equimolar amounts of nucleotides in RNA.

DNA was assayed by the diphenylamine method of Burton (1956) as modified by Giles & Myers (1965). Deoxyribose was extracted by the method used for the estimation of RNA as above. 2-Deoxy-D-ribose was used as a standard and DNA was calculated from the 'apparent' deoxyribose assuming G-C content of 50 % (Pontecorvo, 1967).

Dry-weight determinations. Spore samples were washed twice with distilled water, collected in tared centrifuge tubes and dried to constant weight at 105° . Duplicate or triplicate samples were taken.

Determination of the number of nuclei per spore. Samples were fixed in 4 % (v/v) aqueous glutaraldehyde for 24 h. and suspended in 0.05M-sodium phosphate buffer pH 7.2 to which acridine orange was added, final concentration $50 \mu\text{g./ml.}$ (Clutterbuck & Roper, 1966). Spores were stained for 10 to 30 min. and spore clumps were separated with a finely drawn Pasteur pipette. The spores were suspended in phosphate buffer and viewed with a Zeiss photomicroscope with an ultraviolet light source (barrier filters 50 and 40, exciter filters I and/or II), the nuclei appearing green and the cytoplasm red. The nuclei present in at least 40 spores were usually counted. Estimates on replicate samples showed standard deviations of less than 5 %. A single estimate was made for most experiments but in the early stages of germination at least 200 spores were counted. Dividing nuclei were seen in only 1 in 50 spores, as was also found by Rosenberger & Kessel (1967), and were ignored.

RESULTS

Germ-tube emergence. Germ-tube emergence was detected at 210 min. and the maximum value of 95 to 98 % spores with germ tubes was reached at 360 min. (Table 1); 49.5 % of the spores produced germ tubes between 240 and 300 min. The percentage of spores which started to produce germ tubes in 30 min. intervals after inoculation showed a skewed distribution similar to that found by Vary & Halvorson (1965) for *Bacillus* spore germination. An analysis of the kinetics of germ-tube emergence using the method of McCormick (1964, 1965) will be published elsewhere (B. W. Bainbridge, in preparation).

Table 1. *Germ-tube emergence, dry weight and changes in macromolecules during spore germination*

All estimates, except for germ-tube emergence, are $\mu\text{g./}10^8$ spores.

Time (min.)	Germ-tube emergence (%)	Dry weight	RNA	Kjeldahl nitrogen $\times 6.25$	Protein	DNA
0	—	950	82.9	150	133	2.16
30	—	—	86.2	200	—	—
60	—	903	99.4	260	137.5	2.16
90	—	—	108	287	129	—
120	—	1101	106	486	—	2.2
150	—	—	188	537	171	—
180	—	1450	—	500	270.5	3.8
210	5.3	—	183	675	—	—
240	23.5	1740	238	975	316	4.5
270	57	—	309	1150	—	—
300	73	2680	635	—	346	4.4
330	88	—	—	1040	358	—
360	95	2710	822	1700	—	4.8
390	96	—	1095	—	790	—
420	98	2480	1265	1600	1000	5.8
450	—	—	—	—	1065	—
480	—	5720	1490	3200	1435	8.13
510	—	—	—	4300	1875	—
540	—	7580	1630	—	2165	—

Synthesis of macromolecules. Increases in RNA and Kjeldahl nitrogen were detected at 30 min. and dry weight and protein increases at 120 and 150 min. respectively (Table 1). Increase in DNA was detected at 180 min. There appeared to be pauses in the synthesis of macromolecules. Shoulders appeared between 150 to 210 min. for RNA, 240 to 330 min. for protein and 240 to 360 min. for DNA. The estimate of Kjeldahl nitrogen showed two shoulders at 120 to 180 and 240 to 330 min. The plateau for DNA content occurred when the DNA content per spore was almost exactly twice that present in the ungerminated spore (Table 1). Dry weight, nitrogen content and macromolecular synthesis all had a doubling time of approximately 90 min. (Fig. 1).

Numbers of nuclei per spore. Counts of the number of nuclei per spore showed that nuclear division first occurred at 270 min. (Table 2). The mean number of nuclei per spore had a doubling time of 90 min. (Fig. 1). Spores with 2, 4 and 8 nuclei appeared at 270, 390 and 480 min. respectively (Table 2). Between 120 and 240 h. the amount of DNA per nucleus doubled, presumably as a result of DNA replication in most nuclei. The DNA content per nucleus then decreased to a value only slightly greater than that of the ungerminated spore.

Fig. 1. Changes in mean number of nuclei per spore, DNA, protein, RNA, Kjeldahl nitrogen and dry weight during germination. Ordinates show $\mu\text{g.}/10^8$ spores except for protein which is $\mu\text{g.}/10^7$ spores and mean nuclear number which is numerical.

DISCUSSION

Partial synchrony of spore germination as judged by germ-tube emergence can be achieved in *Aspergillus nidulans* although lack of synchrony has been reported by Rosenberger & Kessel (1967). Conditions of incubation and strains used are important as high spore density and certain mutations reduce synchrony of germination (B. W. Bainbridge, unpublished results).

The partial synchrony of germ-tube emergence was also observed in nuclear division as at 360 min. 70 % of the spores had two nuclei. However, after this synchrony was lost spores containing 1, 2, 4 and 8 nuclei were observed at 480 min., as was found by Rosenberger & Kessel (1967). Partial synchrony of DNA replication was suggested by the occurrence of a pause in DNA increase at a stage when the DNA content per spore had almost exactly doubled, as was found in *Aspergillus niger* (Yanagita, 1957). An estimate can be made of the

times of the phases of nuclear replication and division. Eukaryotic nuclei exhibit a resting period after division (G₁), a period of DNA synthesis (S), a resting period after DNA synthesis (G₂) and mitosis during a division cycle. DNA replication was first detected at 120 to 180 min. and nuclear division occurred at 270 min. This means that the sequence of the phases S–G₂–mitosis must last for a period of 90 to 150 min. The S period has been estimated at 20 min. (Kessel & Rosenberger, 1968) whilst nuclear division takes only a few minutes. The phase G₂, by difference, must last therefore for a period of approximately 70 to 130 min. This conclusion is supported by the DNA content of the nuclei being twice that of the ungerminated spore nucleus. If the period G₂ was very short and mitosis therefore occurred almost immediately after the period S, then the amount of DNA per nucleus would be much lower.

Table 2. *Germ-tube emergence, nuclei per spore and mean DNA content per nucleus during spore germination*

Time (min.)	Germ-tube emergence (%)	% Spores with x nuclei per spore				Spores counted	Mean no. of nuclei/ spore	DNA/10 ⁸ spores	Relative DNA/ nucleus
		1	2	4	8				
0	—	100	—	—	—	100	1	2.16	1
30	—	—	—	—	—	—	—	—	—
60	—	—	—	—	—	—	—	2.16	1
90	—	—	—	—	—	—	—	—	—
120	—	—	—	—	—	—	—	2.2	1.02
150	—	—	—	—	—	—	—	—	—
180	—	100	—	—	—	110	1	3.8	1.76
210	5.3	100	—	—	—	100	1	—	—
240	23.5	100	—	—	—	106	1	4.5	2.08
270	57	98.12	1.88	—	—	106	1.02	—	—
300	73	92.3	7.7	—	—	104	1.12	4.4	1.92
330	88	52.1	47.9	—	—	92	1.46	—	—
360	95	28.3	71.7	—	—	81	1.72	4.8	1.29
390	96	24.7	70.8	4.5	—	89	1.85	—	—
420	98	13.6	64.4	22	—	59	2.37	5.8	1.13
450	—	4.1	58.9	37	—	73	2.56	—	—
480	—	0	30.5	67.8	1.7	59	3.46	8.13	1.09
510	—	2.13	12.8	63.8	21.27	47	4.67	—	—
540	—	0	8.6	57.1	34.3	35	5.2	—	—

However, estimates for the period G₂ of later division cycles differ. At 420 and 480 min. DNA replication in the spores is essentially asynchronous and the DNA per nucleus is approximately 110 % that of nuclei of ungerminated spores. An asynchronous culture of bacteria, in which DNA synthesis is continuous with one replication fork, would be expected to have a value of DNA per nucleus of 1.4 compared to an unreplicated nucleus (Pritchard, Barth & Collins, 1969). DNA synthesis in eukaryotes is, however, discontinuous and the DNA per nucleus will be affected by the ratio of the lengths of the phases G₁ and G₂. When phase G₂ is long and phase G₁ is short, the DNA content per nucleus in a population of spores might be nearly double that in ungerminated spores. A value only slightly greater than that of the resting spore would suggest that phase G₁ is long and that phase G₂ is short. This contrasts with a yeast system in which phase G₁ is short (Williamson, 1965). Kessel & Rosenberger (1968) found that the length of phases G₁ and G₂ in *Aspergillus nidulans* can be altered by changing growth conditions.

The synthesis of other macromolecules followed the expected pattern, RNA synthesis

preceding protein synthesis as found by van Etten (1969). However, the pauses in the synthesis of these other macromolecules were unexpected and may be associated with the initial nuclear division or with organizational changes in the spore during germination. A pause was also observed in dry weight increase at the time of germ-tube emergence. Possibly this is due to carbohydrate reserves being used for cell-wall synthesis or to the washing away of the outer layer of the spore wall which is sloughed off the spore at this stage (Border & Trinci, 1970).

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