Sporulation of *Aspergillus niger* in Submerged Liquid Culture

By JILLIAN C. GALBRAITH* AND J. E. SMITH

*Department of Applied Microbiology,
University of Strathclyde, Glasgow, Scotland*

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SUMMARY

Asexual sporulation of *Aspergillus niger* occurred in submerged culture in a liquid minimal medium without added nitrogen, in low ammonium-N concentrations, and in a wide range of nitrate-N concentrations. Ammonium salts containing more than 48 mg. atom N/l. were inhibitory to conidiation. Most amino acids overcame the ammonium inhibition of conidiation when added to an ammonium nitrate medium. Glyoxylate and several intermediates of the tricarboxylic acid (TCA) cycle also promoted conidiation in the presence of ammonium. Changes in the medium of conidiating and non-conidiating cultures were examined with respect to nitrogen and glucose concentrations, dry weight and pH value. The activities of two glutamate dehydrogenases, one requiring NAD and the other specific for NADP, and of aspartate and alanine amino transferases varied during growth as a function of the stage of the life cycle and of the growth medium. There was no clear correlation between the activities of these enzymes and conidiation of this fungus.

INTRODUCTION

Submerged conidiation of filamentous fungi occurs relatively rarely even in species which spore freely in static culture. This suppression of conidiation has been described as a feature characteristic of shake cultures (Cochrane, 1958). However, since the observations on submerged growth of fungi by Burkholder & Sinnott (1945) and Foster, McDaniel, Woodruff & Stokes (1945), there have been several studies on the induction of asexual spore formation of filamentous fungi in submerged culture by manipulation of the cultural conditions. Morton, England & Towler (1958), Morton, Dickerson & England (1960) and Morton (1961), who examined several *Penicillium* species, concluded that the most suitable condition for submerged conidiation was the absence or exhaustion of available nitrogen in the presence of an assimilable carbon supply. Some species spored freely in a wide range of conditions approaching nitrogen exhaustion, but *Penicillium griseofulvum* did not sporulate in a standard glucose ammonium-N medium even when the N-source was depleted. However, conidiation could be induced by increasing the concentration of sugar or by adding calcium, copper or crude glucose. The effect of crude glucose is now known to be due to traces of calcium and anhydroglucose (Armstrong, England, Morton & Webb, 1963). The importance of calcium for conidiation of *P. notatum* and *P. chrysogenum* has also been shown (Hadley & Harrold, 1958; Foster *et al.* 1945). The chemostat studies by Righelato, Trinci, Pirt & Peat (1968) showed that *P. chrysogenum* required a low

* Present address: Department of Biology, Paisley College of Technology, Paisley, Renfrewshire.

concentration of glucose to spore in submerged culture, but remained vegetative above a critical glucose concentration.

Vézina, Singh & Sehgal (1965) induced conidiation of several filamentous fungi in submerged culture, with spore production quantitatively comparable to surface culture. The medium found to be optimum for one species was not ideal for another, even when the organisms were closely related. In general, the carbon and nitrogen sources and the presence of trace elements were important in defined media; complex media containing corn-steep liquor and molasses, and for *Aspergillus ochraceus* a high salt concentration, afforded maximum conidiation. These workers stressed the balance necessary between the medium and the physico-chemical nature of the environment.

In spite of the various factors found to induce conidiation, an aeration stimulus remains the most potent factor in effecting conidiation of mycelium grown in submerged conditions (Morton, 1961). Stine & Clark (1966) induced conidiation in *Neurospora crassa* by transferring mycelium from shake culture to an empty Petri dish, but Turian (1966), who used a different medium, observed conidiation in a shaken well-aerated medium containing ammonium salts which did not support conidiation in static culture. Turian's study, and the observation that *Gibberella zeae* on a defined medium produced abundant typical macrospores only in shake culture (Cappellini & Peterson, 1965), are the only reports of submerged culture being more favourable to conidiation. The present study was undertaken to obtain submerged conidiation of *Aspergillus niger* and to investigate some of the biochemical changes that occur during this type of differentiation.

METHODS

Organism. Stock cultures of *Aspergillus niger* van Tieghem (IMI 41873) were maintained in potato glucose agar slopes in screw-cap bottles at 27°. Spores were harvested from 4-day slope cultures by covering the culture with sterile water and brushing gently with an inoculation loop. The spore suspension was filtered through a sintered glass funnel to remove hyphae or conidiophores and then washed by centrifuging for 10 min. at 3000 g. The spores were suspended in sterile water and shaken on a Griffin flask-shaker to break up clumps of spores. After counting spores in a haemocytometer, the suspension was used as the inoculum for liquid medium. During inoculation the spore suspension was constantly shaken to avoid variation in inoculum size due to sedimentation.

Cultural conditions. The basal (no added N source) culture medium contained in 1 l. distilled water: glucose, 10 g.; KH_2PO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; $\text{CuSO}_4 \cdot 8\text{H}_2\text{O}$, 0.234 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6.32 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1 mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3.5 mg.; CaCl_2 , 46.7 mg.; adjusted with HCl or NaOH to pH 4.5. The different nitrogenous compounds and organic acids were added to the basal medium in the concentrations indicated in the text. The medium was dispensed in 75 ml. volumes into 250 ml. Erlenmeyer flasks, plugged with non-absorbent cotton wool and autoclaved at 121° for 15 min. Glucose was autoclaved separately and added aseptically. Each flask was inoculated with 8×10^6 spores and incubated at 27° on a Gallenkamp orbital incubator at 150 rev./min.

Analytical methods. Mycelial dry weights were determined by suction filtration through previously dried and weighed Whatman No. 1 filter paper, drying the mycelial

mat for 4 hr at 125°, cooling in a desiccator and reweighing. Glucose was assayed by using a Schweizerhall glucose oxidase test kit (Unicam Instruments Ltd.). Ammonia was determined as mg. $\text{NH}_3\text{-N}/100$ ml. by microdiffusion (Conway, 1957) as was nitrate after reduction to ammonia with Devarda's alloy. α -Oxoglutarate was estimated with glutamic dehydrogenase (Wallenfels & Christian in Bergmeyer & Bernt, 1963*a*): the assay system contained 0.1 ml. test solution; 0.2 ml. 1.5 M- $(\text{NH}_4)_2\text{SO}_4$; 0.25 ml. 1×10^{-3} M-NADH; made up to 3 ml. with 0.1 M-phosphate buffer (pH 7.6). Extinction changes at 340 $m\mu$ were recorded on a Unicam SP800 equipped with a water jacket at 25°. Determinations of protein concentration were by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Preparation of cell-free extracts and enzyme assays. The mycelium was harvested by filtration and washed with ice-cold water until the washings were at pH 6.5. The mycelium was carefully pressed between cheese-cloth and filter paper to remove excess water and then 10 g. of this mycelium quickly weighed and used to prepare cell-free extracts by the method of Watson & Smith (1967). The cytoplasmic fraction was retained for the enzyme assays, since preliminary experiments had shown that the enzymes to be studied were concentrated in this fraction. For comparative enzyme studies equal weights of fresh mycelium were used, the conditions of extraction carefully standardized and the final cytoplasmic fractions made to equal volumes. Enzyme activities were measured in at least three, usually six, separate experiments.

Glutamate dehydrogenases were assayed by measuring either the oxidation or reduction of the appropriate nicotinamide adenine dinucleotide at 340 $m\mu$ in an SP800 spectrophotometer (Unicam Instruments Ltd.) with a 1 cm. light path and equipped with a water-jacketed chamber at 25°. All activities were assayed at optimum pH value and substrate concentration to assure enzyme saturation. The concentration of enzyme added was limited so that the initial rate of the reaction did not greatly exceed an extinction change of 0.01 per min. To measure glutamate formation (GDH-aminating) 3 ml. of reaction medium contained: enzyme preparation, 0.01 ml.; $(\text{NH}_4)_2\text{SO}_4$, 300 μmole ; potassium α -oxoglutarate, 30 μmole ; reduced nicotinamide adenine dinucleotide (NADH_2), 0.25 μmole ; phosphate buffer (pH 7.6), 215 μmole . To measure glutamate oxidation (GDH-deaminating) 3 ml. of reaction medium contained: enzyme preparation, 0.25 ml.; sodium glutamate, 100 μmole ; NAD or NADP, 0.25 μmole ; glycine + hydrazine buffer (pH 9.0), 150 μmole .

The rate of reaction of alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) were measured at 340 $m\mu$ by coupling their reaction products to NAD-linked dehydrogenases. GOT was determined by the action of malate dehydrogenase (MDH) on oxaloacetate, and GPT by the action of lactate dehydrogenase (LDH) on pyruvate. The indicator enzyme acts extremely rapidly on the reaction product of the first enzyme in the sequence, and the decrease in extinction due to the oxidation of NADH is an indication of the amount of reaction product formed with time. The reaction system for GPT contained in 3 ml.: enzyme preparation, 0.1 ml.; potassium α -oxoglutarate, 20 μmole ; L-alanine, 100 μmole ; NADH, 0.25 μmole ; lactate dehydrogenase, 0.025 ml. (activity: 36 units/ml.); phosphate buffer (7.4) 190 μmole . The reaction system for GOT contained in 3.0 ml.: enzyme preparation 0.1 ml.; potassium α -oxoglutarate, 20 μmole ; L-aspartic acid, 100 μmole ; NADH, 0.25 μmole ; phosphate buffer (pH 6.9), 145 μmole . The enzyme extract contained enough malate dehydrogenase to make it unnecessary to add this enzyme to the GOT system. In case of varia-

tions in the activity of endogenous malate dehydrogenase, a test was always made that the rate of reaction was limited by GOT. The assay systems are modifications of those of Smith (1962) and Bergmeyer & Bernt (1963*b, c*), respectively.

Evaluation of results. Enzyme activities were defined according to the International Unit; a unit is the amount of enzyme which converts 1 μ mole of substrate in 1 min. at 25°. An extinction coefficient of 6.22×10^6 cm.²/mole at 340 m μ for NADH and NADPH was used in the calculations (Horecker & Kornberg, 1948). Activity was related to the fresh weight of mycelium per flask, i.e. per culture, and expressed as units of activity/culture. Activity/culture was compared with specific activity (units of enzyme/mg. protein); cases in which the two methods of expressing the results showed a marked difference are indicated in the text. Unless otherwise stated, activity refers to activity/culture. Results are given for 24-hr periods of growth except where 3-hr sampling periods showed an additional feature of changing enzyme activity.

Special reagents. Glutamic dehydrogenase, α -oxoglutarate (sodium salt), pyridine nucleotides, L-glutamic acid (monosodium salt) and alanine were obtained from Sigma Chemical Company Ltd., London; lactate dehydrogenase was obtained from Boehringer & Soehne, Mannheim, Germany; α -aspartic acid from The British Drug Houses Ltd., Poole.

RESULTS

Induction of conidiation. In preliminary experiments conidiation occurred freely on basal medium supplemented with 31 mM-NH₄NO₃ in static conditions, but was almost totally suppressed with the same medium in shaken culture. The few conidiophores which did occur on prolonged incubation under submerged conditions were abnormally small and usually sterile. In the basal medium alone, i.e. in the absence of an added nitrogen supply other than contaminant traces, some mature conidiophores developed within 24 hr of germination in submerged conditions, although very little vegetative growth occurred. Since the importance of available exogenous nitrogen in fungal conidiation has frequently been shown (Turian, 1966; Morton, 1961; Cochrane, 1958, Hawker, 1957,) experiments were made to assess the influence on submerged conidiation of various sources and concentrations of available nitrogen.

The role of inorganic nitrogen sources

Effect of ammonium nitrate. Table 1 shows the effect on conidiation of adding ammonium nitrate to the basal medium. At the lowest N concentration (2 to 16 mg. atom N/l.) normal conidiation occurred, and as the concentration increased so the development of the conidiophore was delayed or inhibited. At 48 mg. atom N/l. the conidiophores were few in number and did not develop beyond the immature stage, and at 96 mg. atom N/l. the mycelium remained vegetative.

Effect of ammonium sulphate. Spore development was affected by different concentrations of ammonium sulphate in the basal medium in much the same way as different ammonium nitrate concentrations (Table 1). The only differences in the ammonium sulphate media were that phialides developed in 24 mg. atom/l. and vesicles in 48 mg. atom N/l., whereas these stages were not present in the same nitrogen concentration provided as ammonium nitrate. An aberrant form of conidiophore which was very much smaller than the normal conidiophore and had a poorly

developed vesicle and few phialides was found at concentrations above 24 mg. atom N/l. ammonium sulphate; these were most frequent in 48 mg. atom N/l., becoming fewer at higher concentrations. This form occasionally developed in the higher concentrations of ammonium nitrate.

Table 1. *Effect of nitrogen source and concentration on asexual sporulation of Aspergillus niger*

mg. atom N/l.	Source of Nitrogen							
	(NH ₄) ₂ SO ₄		NH ₄ NO ₃		NaNO ₃		Na glutamate	
	C	S	C	S	C	S	C	S
2	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+
24	+	±	+	+	+	+	+	+
48	±	—	±	—	+	+	+	+
60	—	—	—	—	+	+	+	+
96	—	—	—	—	+	—	+	±
120	—	—	—	—	±	—	—	—

C, conidiophore; S, conidiophore producing conidia. The maximum duration of each experiment was 96 hr.

Effect of sodium nitrate. Nitrogen supplied as nitrate showed a markedly different effect on conidiation (Table 1). Mature conidiophores were found over a nitrogen concentration range of 2 to 60 mg. atom N/l., the number and size of sporing heads increasing with increase in N concentration. The time of formation of conidiophores, vesicles, phialides and spores was not affected by concentration. The absence of spores at 96 mg. atom N/l. and of spores and phialides at 120 mg. atom N/l. suggests a slightly inhibitory effect of nitrate-N concentrations above 60 mg. atom N/l.

The role of organic nitrogen sources

Seventeen amino acids and asparagine were added separately at a concentration of 28 mM to the basal medium supplemented with 31 mM-ammonium nitrate and the effect on conidiation observed at 24 hr intervals after inoculation. Glycine and leucine induced conidiophores, vesicles and phialides in 48 hr and spore formation in 72 hr. Arginine, serine and proline also induced conidiation in 72 hr, but the cultures were purely vegetative at 48 hr. Development to the phialide stage occurred at 72 hr with glutamate, threonine and valine and normal conidiation at 96 hr. Cultures with ornithine, tryptophan, alanine, histidine, lysine or phenylalanine spored weakly by 96 hr, while cysteine, aspartate, methionine or asparagine did not induce conidiation.

The same amino acids and isoleucine, citrulline, and asparagine were supplied separately as the only nitrogen source in the basal medium at a concentration of 56 mg. atom N/l., i.e. a concentration at which ammonium-N inhibits conidiation. Normal conidiation occurred by 96 hr with glycine, leucine, isoleucine, serine, citrulline, proline, glutamate, threonine, ornithine, tryptophan, histidine, lysine, aspartate or asparagine. With arginine, valine or alanine development stopped at the phialide stage and with phenylalanine only immature conidiophores developed. Neither methionine or cysteine induced sporulation.

The addition of a range of concentrations (2 to 108 mM) of glutamate to the basal medium showed that all concentrations up to 60 mM-glutamate (60 mg. atom N/l.) gave normal conidiation by 96 hr. Mycelium in 84 mM-glutamate only developed a limited number of conidiophores and mycelium in 108 mM-glutamate remained vegetative (Table 1).

The role of tricarboxylic acid cycle intermediates

Since glutamate is readily converted to α -oxoglutarate by glutamic dehydrogenase, this α -keto acid and other intermediates of the TCA cycle and also glyoxylic acid were added at a concentration of 28 mM to the basal medium supplemented with 31 mM-NH₄NO₃ (62 mg. atom N/l.). In each case some degree of conidiation had occurred by 96 hr. Citrate, α -oxoglutarate, fumarate, malate and glyoxylate were the most effective inducers of conidiation, followed by pyruvate and oxaloacetate. Induction by acetate and succinate was least marked and the conidiophores were then abnormally small and sometimes sterile.

In a further experiment, basal medium supplemented with 31 mM-NH₄NO₃ was prepared, and to portions of this different amounts of α -oxoglutarate were added, covering the range from 2 to 108 mM. All concentrations induced at least the early stages of conidiation. At or above 12 mM- α -oxoglutarate, conidiation was complete by 96 hr; at lower concentrations development only occurred to the phialide stage.

Morphology of submerged sporulation

The conidiophores which developed in shaken submerged cultures were identical in appearance with those of static cultures. The first morphological sign of conidiation was a profusion of unbranched conidiophores which arose singly from somatic hyphae (Pl. 1, fig. 1). In the case of pellet mycelium, the conidiophores were on the surface or in the centre of the pellet. Each conidiophore had a characteristic foot cell at its base (Pl. 1, fig. 2) with a septum at either end of the foot cell delimiting the conidiophore from the vegetative mycelium. Apart from the presence of a foot cell the conidiophores could be easily distinguished from somatic hyphae by their greater width and more granular cytoplasm which stained deeply with cotton blue. Subsequent development was not completely synchronous, though the majority of the conidiophores developed into globular vesicles (Pl. 1, fig. 3) and a large number of two-celled phialides were produced over the surface of the vesicle. The tips of the phialides were constricted and abstricted spherical conidia (Pl. 1, fig. 4). Chains of spores so readily observed in aerial conidiophores were not seen in these submerged cultures. The absence of spore chains in submerged cultures of *Penicillium chrysogenum* has been considered to be due to the agitation of the culture (Righelato *et al.* 1968). The same situation may have existed in the present study, since unswollen spores were present in conidiating cultures, suggesting that the conidia were shed from the phialides.

Chemical changes in the medium

Changes in nitrogen and glucose concentration and mycelial dry weight were examined in conidiating and non-conidiating cultures. Changes in pH value of the media were recorded to ascertain whether the beneficial effect of α -oxoglutarate on conidiation was the result of physical buffering of the medium (Morton & MacMillan 1954). Vegetative mycelium was produced in basal medium supplemented with 31

mm-NH₄NO₃ (medium A), while conidiating mycelia were produced either in medium A + 14 mm- α -oxoglutarate (medium B), in basal medium + 62 mm-sodium nitrate (medium C) or in basal medium + 8 mm-NH₄NO₃ (medium D). As with *Penicillium griseofulvum* (Morton *et al.* 1958), the conidia did not germinate in the medium in which they were produced.

Dry weight. Dry weight changes in each medium showed the typical phases of lag phase, phase of rapid growth and autolysis (Fig. 1). Peak dry weight in medium A coincided with the time of glucose exhaustion, but in medium B there was a small increase in dry weight in the 24 hr following glucose depletion. The apparently slower rate of increase in dry weight in medium C was due to a longer lag phase. Nitrate reductase, an essential enzyme for growth on nitrate-N source, is not a constitutive enzyme, and its period of synthesis could explain a longer lag period. In general, the physiological age of mycelium from medium C may be regarded as 12 to 24 hr less than the age calculated from the time of inoculation. In medium D the lower dry weight yield was in accordance with the lower N concentration.

Table 2. *Changes in protein concentration in the mycelium of Aspergillus niger growing on different media*

Time of incubation (hr)	Growth medium		
	A	B	C
	mg. protein/g. fresh weight of mycelium		
22	6.56	6.92	6.0
46	3.87	4.26	5.12
70	4.80	5.49	5.92
96	3.23	3.48	2.58

Protein. The total protein per culture showed changes similar to the variations in dry weight with time of growth. However, the amount of protein/g. fresh weight mycelium was highest during the early stages of growth (Table 2).

Glucose. The patterns of both glucose and nitrogen metabolism were reflected in the dry weight changes. Thus, in each medium, the maximum dry weight was achieved at the time of glucose exhaustion. In medium B the rate of uptake was rapid and linear over the first 46 hr, whereas uptake in medium A was slightly slower in the first 22 hr, due probably to an initial delay in uptake (Fig. 2). The results of a closer analysis of glucose concentration in these two media showed that glucose was exhausted in medium B some 3 to 5 hr before depletion in medium A. The most rapid phase of glucose assimilation in medium C was from 46 to 70 hr; in medium D glucose was never completely utilised in the duration of the experiment.

Ammonium. The patterns of ammonia uptake in media A and B (Fig. 3) were relatively similar. Uptake occurred up to 46 hr, being more rapid in the first 22 hr. After 46 hr the concentration of ammonia in both media increased, indicating the onset of autolysis. In medium C, ammonia first appeared in the medium at 70 hr, presumably again associated with autolysis. In medium D, ammonia was completely exhausted by 40 hr and did not reappear, implying that autolysis did not occur in the duration of the experiment.

Nitrate. Approximately the same quantity of nitrate was assimilated in Media A and B (Fig. 4). However, uptake did not begin until 24 hr in medium A whereas uptake in medium B was almost linear over the first 46 hr. In medium A, uptake ceased at 46

Fig. 1

Fig. 2

Fig. 1. Changes in dry weight of *Aspergillus niger* with time in different growing media. Results are expressed as mg. dry weight/culture flask. ●—●, Medium A (basal medium + 31 mM-NH₄NO₃); ○—○, medium B (basal medium + 31 mM-NH₄NO₃ + 14 mM- α -oxoglutarate); ▲—▲, medium C (basal medium + 62 mM-NaNO₃), △—△, medium D (basal medium + 8 mM-NH₄NO₃)

Fig. 2. Rate of glucose uptake by *Aspergillus niger* from various media. Results are expressed as mg. glucose/100 ml. medium. Symbols as in Fig. 1.

Fig. 3

Fig. 4

Fig. 3. Rate of ammonium uptake by *Aspergillus niger* from various media. Results are expressed as mg. NH₃-N/100 ml. medium. Symbols as in Fig. 1.

Fig. 4. Rate of nitrate and α -oxoglutarate uptake by *Aspergillus niger* from various media. Results are expressed as mg. NO₃-N/100 ml. medium and as μ mole α -oxoglutarate/ml. Symbols as in Fig. 1, ×—×, α -oxoglutarate uptake from medium B.

hr and in medium B at 70 hr, in agreement with the dry weight changes. Assimilation took place throughout the 96 hr in medium C, being most rapid during the 22 to 46 hr stage before the rapid phase of glucose assimilation. Most of the nitrate was rapidly taken up in medium D though uptake was never complete.

α -Oxoglutarate. α -Oxoglutarate was taken up during the first 70 hr and remained present at a very low concentration during the final 24 hr (Fig. 4).

pH value. In medium A there was a steady decrease in pH value up to 72 hr, thereafter there was a slight increase. The decrease in pH value in medium B was less marked and may indicate that α -oxoglutarate did exert some buffering influence. The pH values in media C and D also showed increases reaching a value at 96 hr similar to medium B (Fig. 5).

Fig. 5. Changes in pH value of medium during growth of *Aspergillus niger*. Symbols as in Fig. 1.

Enzyme assays

Glutamate dehydrogenase (aminating). Both GDH-NADPH and GDH-NADH were identified in *Aspergillus niger*. Enzyme activity of GDH-NADPH per culture in media A, B and C rose to a peak at 46 hr and then decreased almost to zero at 96 hr (Table 3). However, activity in cultures from sporing media B and C was more than double that in A-grown cultures. Activity of GDH-NADH per culture in media A and B showed a similar pattern of change, reaching a maximum at 46 hr. Maximum activity in C was lower than in the other media, and was not reached until 70 hr. Expression of the results as a specific activity showed a different pattern of changing activity (Table 4).

In mycelium from both media A and B, GDH-NADPH showed a very high specific activity during the early stages of growth, decreasing rapidly almost to zero at 70 hr.

Table 3. *Changes in enzyme activity ($\mu\text{moles/min.}/\text{culture}$) during growth of *Aspergillus niger**

Growth medium	Enzyme	Medium A					Medium B				
		Time of incubation (hr)									
		22	46	70	96	Enzyme activity ($\mu\text{mole/min.}/\text{culture}$)	22	46	70	96	
Growth medium		0.193 (0.005)*	1.635 (0.252)	0.303 (0.018)	0	0.313 (0.025)	3.891 (0.582)	3.891 (0.582)	0.237 (0.058)	0	
	GDH-NADPH	0	4.958 (0.648)	2.411 (0.361)	0.380 (0.057)	0.025 (0.002)	5.664 (0.745)	5.664 (0.745)	0.952 (0.150)	0.403 (0.086)	
	GDH-NADH	0.029 (0.003)	0.495 (0.072)	0.555 (0.065)	0	0	1.239 (0.300)	1.239 (0.300)	0.076 (0.007)	0.056 (0.006)	
	GDH-NADP	0	0.589 (0.081)	0	0	0	0.389 (0.058)	0.389 (0.058)	0.047 (0.014)	0.014 (0.003)	
	GDH-NAD	0.136 (0.059)	5.226 (1.011)	1.358 (0.268)	1.071 (0.212)	0.103 (0.007)	7.781 (1.215)	7.781 (1.215)	2.375 (0.323)	1.814 (0.362)	
	GOT	0.675 (0.080)	10.726 (1.980)	4.389 (0.622)	1.071 (0.086)	0.641 (0.211)	14.163 (2.021)	14.163 (2.021)	4.265 (0.671)	2.336 (0.889)	
	GPT										
Medium C											
Growth medium	Enzyme	Time of incubation (hr)									
		22	46	70	96	Enzyme activity ($\mu\text{mole/min.}/\text{culture}$)					
	GDH-NADPH	0.026 (0.001)	4.050 (0.575)	0.616 (0.074)	0.055 (0.008)						
	GDH-NADH	0	0.095 (0.008)	2.559 (0.180)	0.088 (0.149)						
	GDH-NADP	0.016 (0.003)	0.202 (0.037)	0.410 (0.062)	0.353 (0.043)						
	GDH-NAD	0	0.244 (0.031)	0.519 (0.062)	0.349 (0.070)						
	GOT	0.015 (0.014)	1.597 (0.196)	7.143 (1.179)	4.416 (1.078)						
	GPT	0	5.582 (1.02)	3.467 (0.655)	3.292 (0.413)						

* Values in parentheses are standard deviations.

The highest maximum specific activity was recorded in mycelium from medium B. Specific activity of GDH-NADPH in C-grown mycelium was initially low, but at 30 hr reached a value comparable to that in medium A at 22 hr. The time of maximum specific activity of GDH-NADH did not coincide with that of GDH-NADPH, but occurred at 46 hr in media A and B, and at 60 hr in medium C (Table 4).

Glutamate dehydrogenase (deaminating). Activity of glutamate dehydrogenase in the direction of glutamate deamination was relatively low (Table 3). GDH-NADP activity in cultures in media A and C was essentially similar, but activity in medium B reached a higher maximum at an earlier time after inoculation. GDH-NAD activity rose from zero at 22 hr to a maximum at 46 hr in media A and B, or 70 hr in medium C, all of similar values.

Table 4. *Changes in specific activities of GDH-NADPH and GDH-NADH during growth of Aspergillus niger on different media.*

	GDH-NADPH growth medium			GDH-NADH growth medium		
	A	B	C	A	B	C
	Units of enzyme/mg. protein					
22	0.42 (0.05)*	0.66(0.07)	0.07	0	0.02	0.01
30	0.31 (0.04)	0.36(0.04)	0.46(0.05)	—	—	—
46	0.17 (0.02)	0.21(0.02)	0.25(0.03)	0.20(0.02)	0.28(0.03)	0.05
60	—	—	—	0.13(0.01)	0.14(0.01)	0.09
70	0.01	0.02	0.02	0.09	0.07	0.07
96	0	0.01	0.01	0.04	0.06	0.07

* Standard deviations in parentheses.

Aspartate aminotransferase. There were no marked differences in the activity of GOT which could be correlated with the sporing of the mycelium. Changes in media A and B were similar, increasing from a low value at 22 hr to a maximum at 46 hr and decreasing thereafter. Maximum activity in medium C was close to that in A, but was not reached until 70 hr (table 3).

Alanine aminotransferase. The pattern of GPT activity in extracts from media A and B resembled that of GOT in reaching a maximum at 46 hr and then falling off to almost zero at 96 hr (Table 3). Maximum activity in non-conidiating mycelium was intermediate between conidiating cultures in B and C.

DISCUSSION

The experimental data show that growth and production of conidia of *Aspergillus niger* were affected by the nitrogen status of the medium. The failure of conidiation to occur in media with high ammonium N-concentration, while conidiation occurred in similar concentrations of nitrate-N, implies that the repression of conidiation by ammonium-N compounds is more related to the presence of the ammonium ion than to the total nitrogen concentration. Repression of conidiation by ammonium-N also occurs in *Neurospora crassa* in conditions in which heavy conidiation would occur with nitrate as nitrogen source (Turian, 1966).

Conidiation of *Aspergillus niger* in 4 to 8 mg. atom N/l. supports the hypothesis

proposed by Morton (1961) that the most generally essential condition for conidiation is the absence of available nitrogen in the presence of an assimilable carbon supply. However, the studies with media B and C show that conidiation can also occur in the presence of excess ammonium-N or nitrate-N. In these media the carbon source is exhausted or is very low before the first signs of conidiation are present. Carbohydrate starvation can also induce conidiation in certain *Penicillium* strains (Jicinska, 1968). Righelato *et al.* (1968) have shown with *Penicillium chrysogenum* in chemostat culture that glucose is essential for conidiation and that maximum conidiation occurs at a growth rate between zero and the critical growth rate above which steady state vegetative growth occurs. A reorganization of cellular RNA and DNA concentrations accompanied the transition from the vegetative to the conidiating state and it was considered that a critical glucose ration was necessary to energise these processes. In the absence of glucose cell autolysis occurred without conidiation.

The inability of some fungi to grow and sporulate effectively on ammonium salts may be due in part to the accumulation of acidic ions in the medium (Cochrane, 1958). There are several reports that the utilization of ammonium-N was increased when the pH value of the medium was stabilized by the regular addition of alkali or by the addition of organic acids (Agnihotri, 1964, 1968; Agnihotri & Vaartaja, 1967; Morton & MacMillan, 1954). The results in Fig. 5 suggest that α -oxoglutarate did have a slightly buffering effect on the medium, but this did not appear to greatly influence the rate of uptake of ammonia (Fig. 3). However, the presence of α -oxoglutarate did stimulate nitrate and glucose assimilation in the early phase of growth.

The importance of amino acids in *Aspergillus niger* conidiation has been demonstrated by Behal & Eakin (1959*a, b*) while with *Neurospora crassa*, glycine can overcome the inhibition of conidiation by ammonium-N (Oulevey-Matikian & Turian 1968). Behal & Eakin (1959*a, b*) suggested that spore formation in *A. niger* is dependent on functionally complete glycolytic and TCA pathways. Inhibition of conidiation but not of growth, by 6-ethylthiopurine was interpreted as an interference with methionine metabolism causing a decrease in the activity of the condensing enzyme (Behal, 1959). The action of the TCA intermediates reported in the present study may be further evidence of the necessity of an active TCA cycle in the induction of conidiation. It is of interest to note that ammonia can interfere with the normal functioning of the TCA cycle in animal mitochondria (Katunuma & Okado, 1963, 1965; Katunuma, Okado & Nishii, 1965; Worcel & Erecinska, 1962).

There are no marked differences in the activities of either aspartate aminotransferase or alanine aminotransferase that can be specifically linked to the appearance of conidiophores. Specific activity and activity of GDH-NADPH and GDH-NADH per culture in mycelial extracts from media A and B suggests a more rapid rate of glutamate synthesis in pre-conidiating cultures than in cultures which remain vegetative. This is further substantiated for activity per culture for GDH-NADPH in medium C. However, high specific activity of GDH-NADPH is not correlated with conidiation in medium C, nor is GDH-NADH more active in terms of activity per culture or specific activity.

The available methods of measuring enzyme activity are not satisfactory for the comparison of activities in crude extracts taken from organisms at different stages of growth, and care must be taken in interpreting such results (Ursprung & Smith, 1965; Cantino, 1961). Before presenting these results, expressions of enzyme activity on a

basis of fresh weight or dry weight of mycelium, or per culture, or as specific activity were compared. The pattern of changes in activity at different stages of growth, or at the same age in different media was similar, regardless of the method of expression, except in the case of GDH-NADPH. This enzyme shows a high specific activity at an early stage of growth, but activity expressed on a culture basis is relatively low. High activity of any enzyme at a stage of growth when the ratio of protein: fresh weight is high will inevitably be missed by expression of the results per culture.

The presence of both NAD and NADP dependent GDH enzymes has also been detected in *Neurospora crassa* (Sanwal & Lata, 1961; Tuveson, West & Barratt, 1967), *Piricularia oryzae* (Kato *et al.* 1962), *Schizophyllum commune* (Dennen & Niederpruem, 1967) and *Fusarium oxysporum* (Sanwal, 1961), and, as in *Aspergillus niger*, the two enzymes do not show maximum specific activity simultaneously. The repression of GDH-NAD and the induction of GDH-NADP by ammonia in *Saccharomyces cerevisiae* (Holzer, 1966) and *N. crassa* (Sanwal & Lata, 1962) indicates that GDH-NADP is anabolic whereas GDH-NAD is catabolic. However, in a later paper Stachow & Sanwal (1964) proposed that GDH-NAD is important in the synthesis of glutamate for glutamine synthesis. In *A. niger*, GDH-NADPH specific activity decreases before the uptake of ammonia has ended. This implies either that nitrogen is incorporated in the later stages of growth by mechanisms other than GDH (which is generally considered to be the chief enzyme of ammonia incorporation in micro-organisms) or, more probably, that GDH-NADH can function synthetically in *A. niger*. Therefore, assuming a synthetic role of GDH-NADPH and GDH-NADH in *A. niger*, the higher specific activity of GDH-NADPH in conidiating medium B may indicate that at least part of the exogenous α -oxoglutarate is being utilised in amino acid synthesis. This does not eliminate the possibility of a role for α -oxoglutarate in sporulation through increasing the availability of α -oxoglutarate to the TCA cycle (Galbraith & Smith, 1969).

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EXPLANATION OF PLATE

Fig. 1. Immature conidiophores of *Aspergillus niger* in submerged culture, growing in medium B. pH 4.5. Phase; $\times 840$

Fig. 2. Conidiophore, vesicle and foot cell of submerged, sporing culture of *Aspergillus niger* in medium B. Phase; $\times 420$.

Fig. 3. Development of phialides on the vesicle of *Aspergillus niger* in submerged culture in medium B: $\times 1,260$.

Fig. 4. A mature sporing head of *Aspergillus niger* in submerged culture in medium B. Phase: $\times 630$.