ENZYME ACTIVITIES DURING THE ASEXUAL CYCLE OF *NEUROSPORA CRASSA*

II. NAD- and NADP-Dependent Glutamic Dehydrogenases and Nicotinamide Adenine Dinucleotidase

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ABSTRACT

Three enzymes, (*a*) nicotinamide adenine diphosphate–dependent glutamic dehydrogenase (NAD enzyme), (*b*) nictoinamide adenine triphosphate–dependent glutamic dehydrogenase (NADP enzyme), and (*c*) nicotinamide–adenine dinucleotidase (NADase), were measured in separate extracts of *Neurospora crassa* grown in Vogel's medium N and medium N + glutamate. Specific activities and total units per culture of each enzyme were determined at nine separate intervals phased throughout the asexual cycle. The separate dehydrogenases were lowest in the conidia, increased slowly during germination, and increased rapidly during logarithmic mycelial growth. The amounts of these enzymes present during germination were small when compared with those found later during the production of the conidiophores. The NAD enzyme may be necessary for pregermination synthesis. The NADP-enzyme synthesis was associated with the appearance of the germ tube. Although higher levels of the dehydrogenases in the conidiophores resulted in more enzyme being found in the differentiated conidia, the rate of germination was uneffected. The greatest activity for the NADase enzyme was associated with the conidia, early phases of germination, and later production of new conidia. NADase decreased significantly with the onset of logarithmic growth, remained low during the differentiation of conidiophores, and increased considerably as the conidiophores aged.

INTRODUCTION

A variety of enzymes have been measured with respect to specific phases of development of the asexual cycle of *Neurospora crassa* (17–20). It has been reported (17) that there is little, if any, difference in the specific activity for succinic dehydrogenase found in the conidia and that found in the subsequent 10- or 15-hr mycelial cultures. These studies suggest that succinic dehydrogenase is present throughout germination and mycelial growth. A more recent report (13), however, indicates that succinic dehydrogenase was not necessary for germination; the enzyme was measurable in the ungerminated conidia, but not during the first 3 hr of conidial germination. It has also been reported that nicotinamide adenine dinucleotidase (NADase) was associated with the formation of conidia (3, 20). A study of this enzyme during the phasing of the complete asexual cycle (Stine, G. J. 1966. Data in preparation and herein.) shows that its appearance during germination and

mycelial growth was time-dependent; this suggests that it may be involved in the regulation of conidial germination and mycelial growth. The evaluation of the above studies emphasizes the necessity of looking at the entire life cycle; for example, if the different enzymes had been studied only in the ungerminated conidia and subsequent 10- and 15-hr-old mycelia (17) or 14-hr-old culture (20), it would have appeared that succinic dehydrogenase was present in and associated with conidial germination, and that NADase was associated only with the formation of conidia.

The method of phasing the asexual cycle during development which permits sampling of cells at any time (13) was used during this study. The total units per culture and specific activities of nicotinamide adenine diphosphate–dependent glutamic dehydrogenase (NAD enzyme), nicotinamide adenine triphosphate–dependent glutamic dehydrogenase (NADP enzyme), NADase, and soluble protein were determined throughout the asexual cell cycle, from germination of conidia, through growth of mycelia, production of conidiophores, and the conidiophores' differentiation into new conidia.

The NAD enzyme and/or NADP enzyme, widely distributed in a variety of organisms, are involved in the reversible deamination of L-glutamate to alpha ketoglutaric acid via a reduction or oxidation of NAD and/or NADP The reversibility of this oxidative deamination provides for the interconversion of alpha keto acids and amino acids coupled with the formation or utilization of ammonia. In *Neurospora* the reductive amination of alpha ketoglutarate is the only major path for the utilization of ammonia (11).

NADase from beef spleen, pig, and rat brain, and *Neurospora* are capable of splitting the oxidized pyridine nucleotides NAD and NADP into their corresponding nicotinamides (4, 6, 8). Since the pyridine nucleotides are essential participants in dehydrogenase-catalyzed, oxidation-reduction reactions (4), a loss of the cofactors to the cell, regardless of cause, ultimately results in cell death (2).

MATERIALS AND METHODS

Genetic Strain

Wild type STA 4 and 818a (an aconidial mutant) were obtained from the Fungal Genetic Stock Center, Dartmouth College, Hanover, N. H.

Minimal Medium

Neurospora was cultured first in Vogel's medium N (16), a standard synthetic *Neurospora* medium, for a determination of the levels of NAD enzyme, NADP enzyme, NADase, and soluble protein per culture at specific intervals throughout the asexual cycle. *Neurospora* was then grown in medium N altered by the addition of 0.05 M monosodium glutamate (11). The aconidial strain of *Neurospora* (sister strain to the wild type but lacking the ability to form conidia) was also cultured in unaltered medium N as an aid in determining the role of the separate dehydrogenases and NADase during morphological change.

Growth of Conidial Inoculum

The growth of conidial inoculum has been described previously (14).

Germination of Conidia and Growth of Mycelia for Assays and Production of Conidiophores and Conidia

Eight 1-liter Erlenmeyer flasks, each containing 200 ml of medium N, 2% sucrose, and 4 ml of a 1% solution of Tween 80 were inoculated with 3×10^9 conidia. Simultaneously, a control sample of 3×10^9 conidia was immediately disintegrated as stated below.

After 1, 3, and 7 hr of aeration, the contents of the designated liter flask were centrifuged at 3000 *g* for 15 min. The pellet was resuspended in 6 ml of 0.6% cysteine hydrochloride in 0.1 M $KHCO_3$ at pH 8.4 (20). This suspension was pipetted into a 20 ml stainless steel disintegration capsule which contained 2 g of Alundum 90 mesh. The sample was disintegrated for 2 min by using a water-cooled Nossal disintegrator (McDonald Engineering Company, Cleveland, O.). The disintegrated material was poured into a 15 ml centrifuge tube, and an additional 2 ml of the cysteine hydrochloride solution used to rinse the capsule were added to the centrifuge tube. The tube or tubes were centrifuged at 700 *g* for 20 min. The supernatant was decanted for protein and enzyme determinations. The pellet of Alundum and cellular debris was discarded.

After 24 or 48 hr of aeration, the mycelial suspensions were vacuum filtered on a Buchner funnel through sharkskin filter paper (Schleicher & Schuell Co., Keene, N.H.), washed with cold 0.1 M phosphate buffer at pH 7.0, and rinsed with cold, distilled deionized water. The mycelial mat was removed from the funnel, placed between sheets of Whatman No. 1 filter paper, and firmly pressed to extract residual moisture. This mycelial mat was referred to as a "pressed wet weight" mycelial mat. 1 g was taken for dry weight determination; 1 g was wrapped in Para-

film, placed at −20°C for 2 hr, broken into fine fragments, and disintegrated as described above.

The remaining flasks containing 48-hr mycelia were utilized as follows in producing conidiophores and conidia to complete the asexual life cycle. A Buchner funnel (inside diameter, 10 cm) was fitted to a sidearm flask, and the flask was connected to a manually controlled vacuum pump. A 9.5 cm piece of sharkskin filter paper which had been placed in the funnel was moistened with water. Sufficient mycelial suspension was added to the funnel to make a mycelial mat 2–3 mm thick after it had been filtered at 5 lbs/in^2. The mat was washed with 50 ml of cold, distilled, deionized water and filtered under a vacuum for an additional 1.5 min. Both mat and filter paper were placed in an empty Petri dish. A 2 ml solution of 0.1 M sodium phosphate buffer (pH 6.0) containing 400 U/ml of penicillin-streptomycin mixture (Baltimore Biological Supply Co., Baltimore, Md.) was distributed over the mat. The dishes containing mats of harvested mycelia were inverted and incubated at 25°C. Conidiophores appeared above the aging parent mycelia after 3 hr (51 hr after the initial inoculation); and at 8 hr (56 hr after inoculation), the conidiophores were differentiating into conidia. Following incubation (3 and 8 hr), a mycelial mat containing conidiophores was pressed between filter paper for pressed wet weight. 1 g of the pressed wet mycelia was taken for dry weight determination; and 1 g was wrapped in Parafilm, frozen (−20°C for 30 min), and disintegrated for protein and enzyme determinations. The remaining plates of incubating mycelia were left to form conidia for additional time to 96 hr. After 96 hr, the conidia were moistened with water and loosened with a glass rake; they were then filtered through two layers of absorbent cotton, centrifuged at 3000 *g* for 10 min, resuspended in water, and centrifuged again. The percentage germination was determined for these conidia as described (14), and 3×10^9 of these conidia were disintegrated for protein and enzyme determinations.

The procedure for growth and harvesting of the aconidial mutant was the same as that described above with the following modifications. The starter aconidial inoculum was grown for 24 hr in 200 ml of medium N. The 24 hr mycelial growth was divided into wet weight inoculum samples (approximately 0.2 g pressed wet weight per sample as determined by an equal control sample) and added to six 1-liter flasks containing 250 ml of medium N. A culture of the aconidial mycelia was harvested at 24 and 48 hr for dry weight, enzyme, and protein determinations. The remaining cultures of mycelia were harvested as described above and set to incubate even though the formation of conidia would not occur. Samples were taken after 3 and 8 hr of the parent mycelial incubation, and the enzyme and protein levels were determined. At 8 hr, the conidiophore growth was so dense that it was possible to completely separate the conidiophores, by using a rubber policeman, from the aging parent mycelia. Protein and enzyme determinations were made for both fractions, parent mycelia and conidiophores. At 96 hr, the conidiophores, from the aconidial mats, were again separated from the mycelia, and enzyme and protein levels were determined.

Enzyme Assay Procedures

NADP enzyme was measured by following the oxidation (decrease in absorbance) of reduced nicotinamide-adenine triphosphate ($NADPH_2$) by using a Beckman Model DU spectrophotometer at 340 mμ at room temperature (1).

NAD enzyme was measured by using the procedure described for the assay of NADP enzyme (1) with the following modifications. Activity was determined by following the oxidation (decrease in absorbance at 340 mμ) of reduced nicotinamide adenine diphosphate ($NADH_2$) at 15-sec intervals between 0 and 2 min in Tris–HCl buffer (pH of 8.3). Nonspecific $NADH_2$ oxidation was subtracted from the rate obtained after additions of alpha ketoglutarate. NADase was assayed according to Kaplan et al. (6).

Protein Determinations

Soluble proteins were measured by using the Folin-phenol (7) and the Biuret (15) techniques with bovine serum albumin as a standard.

RESULTS

Levels of NAD-Dependent Glutamic Dehydrogenase (NAD Enzyme)

NAD enzyme increased in the first hour in each of the separate cultures (Table I). Because the increase in enzyme was rather small, separate additional cultures were set up for enzyme determinations at 0 and 1 hr. The results of the additional tests invariably showed an increase in the level and specific activity of the NAD enzyme.

The NAD enzyme of the medium N culture increased slowly through 7 hr; it reached a plateau at 24 hr and declined slightly from 51 to 56 hr, a time of conidiophore elongation. The NAD-enzyme levels in the medium N plus glutamate culture, except for the threefold increase between 24 and 48 hr, were similar to those of the medium N culture. It can be seen that between 48 and 51 hr, a time of the mutant conidiophore formation (Table II), there was a fourfold increase in the NAD enzyme. Upon separation of the conidio-

TABLE I

NAD-Glutamic Dehydrogenase Activity During the Asexual Cycle of Wild Type Neurospora crassa

Medium		Time (*hr*)								
		0	1	3	7	24	48	51	56	96
N	TU*	80	98	80	198	1615	1700	1675	1450	75
	TP	4	4	7	15	111	100	130	120	5
	SPA	20	25	11	13	15	17	13	12	15
N + glutamate	TU	67	75	80	130	1445	4500	4130	3400	132
	TP	4	3	4	18	136	160	130	120	5
	SPA	17	25	20	7	11	28	32	28	26
Grams dry weight/culture		0.040‡				1.9§	2.4§			0.040‡
Percent germination		0	5	20	92	M‖	M	M + CP	M + CP some C	C

* TU, total units per culture; TP, total milligrams protein per culture; SPA, specific activity (units/milligram protein).

‡ Dry weight of 3×10^9 conidia used as inoculum, time 0 and at 96 hr.

§ Refers to medium N and medium N plus glutamate cultures.

‖ M, mycelia; CP, conidiophores; C, conidia produced on conidiophores of N and N plus glutamate cultures.

phores from the parent mycelia at 56 hr (a time when luxurious growth of the conidiophores has already occurred), there was approximately 21 times more of NAD enzyme in the conidiophores than in the mycelia from which they were differentiated. Further, the level of the NAD enzyme found at 56 hr persisted through 96 hr regardless of the fact that conidial differentiation did not occur (Table II).

The conidia from the medium N plus glutamate culture contained twice the amount of enzyme found in the conidia from the medium N culture, yet the percentages of germination were the same. The percentage of germination and enzyme content of the conidia from the medium N culture were similar to those of the initial conidial inoculum.

Levels of NADP-Dependent Glutamic Dehydrogenase (NADP Enzyme)

The total units of the NADP enzyme in the separate cultures decreased between 0 and 1 hr (Table III). The decrease in the level of the NADP enzyme and specific activity, although small, was a consistent finding (separate additional cultures were set up for enzyme determination at 0 and 1 hr). The conidia, if dependent on this enzyme for the initial processes of germination, 0–1 hr, may have to depend on the contribution of the NADP enzyme from the parent conidiophore since it does not appear to be actively synthesized until sometime between the first and third hours when 5 and 20% of the conidia, respectively, had already germinated.

The increase in the NADP enzyme between 48 and 51 hr from the medium N, medium N plus glutamate (Table III), and the aconidial cultures (Table II) was associated with the formation of conidiophores; the large drop in the NADP-enzyme level, which occurred between 51 and 56 hr, in the medium N and medium N plus glutamate cultures was associated with the time of initial conidial differentiation. When the conidiophores of the aconidial culture were separated from the parent mycelia at 56 hr, a time when the wild type cultures would begin differentiating conidia, the conidiophores contained about half the amount of the NADP enzyme found in the parent mycelia. At 96 hr the conidiophores contained about half the enzyme found at 56 hr in the conidiophores. These results are unlike those found for the NAD enzyme (Table II). The positive relationship existing between the increase in NADP enzyme and dry weight in the medium N culture between 3 and 24 hr, a time of logarithmic germination and mycelial growth, strongly suggests that this enzyme was essential for normal mycelial metab-

TABLE II

NAD- and NADP-Glutamic Dehydrogenases and NADase Determinations During Development of Neurospora

Strain	Medium	Enzyme	Time (*hr*) 24	48	51	56		96
			TU*	TU	TU	TU		TU
Wild type	N	NAD-	1615	1700	1675	1450		75
		NADP-	22610	10000	13000	4500		110
		NADase	0	0	260	7200		4150
		TP*	111	100	130	120		5
	N + glutamate	NAD-	1445	4500	4130	3400		132
		NADP-	14500	18000	19000	7900		132
		NADase	0	0	265	5100		4032
		TP	136	160	130	120		5
						Mycelia‡	Conidiophores	Conidiophores
						TU	TU	TU
Fluffy (aconidial)	N	NAD-	638	580	2400	160	3370	4790
		NADP-	13940	9000	11000	11000	4400	2820
		NADase	7225	22600	25000	17400	46700	49550
		TP	85	75	130	35	50	53

* TU, total units per culture; TP, total milligrams protein per culture.

‡ At 56 hr the conidiophores were separated from the parent mycelia; both fractions were assayed.

olism during this period of development. These data are in keeping with the fact that NADP-enzyme mutants grow very slowly in minimal medium; near normal, wild type growth occurs only if transaminable amino nitrogen is added to the medium (9).

Influence of Glutamate on the NAD- and NADP-Glutamic Dehydrogenases and Growth of Neurospora

Since the levels of the NAD enzyme in the medium N and medium N plus glutamate cultures were similar during the first 3 hr of aeration, it can be concluded that the added glutamate was not necessary for the presence and/or increase in the NAD enzyme during the initial or early germination synthesis, 0–1 hr of culture (Table I). However, the addition of glutamate had a pronounced effect on the synthesis of the NAD enzyme between 24 and 48 hr of culture. During this time, the NAD enzyme increased 2.5 times over the level of the NAD enzyme in the medium N culture. Sanwal and Lata (10) have reported a concurrent regulation of the NAD enzyme and NADP enzyme based on the presence of glutamate in Vogel's (herein called medium N) medium N. They state that in terms of specific activities the NADP enzyme was repressed between 24 and 48 hr in cultures containing 0.05 M glutamate, whereas the NAD enzyme was correspondingly derepressed. In terms of specific activities and levels of the respective enzymes in the medium N plus glutamate culture, glutamate did influence the greater synthesis of the NAD enzyme between 24 and 48 hr, but it did not correspondingly repress the synthesis of the NADP enzyme (Tables I and III). Actually, the NADP enzyme appeared to be repressed between 24 and 48 hr in the medium N culture and derepressed in the medium N plus glutamate culture.

Levels of Nicotinamide Adenine Dinucleotidase (NADase)

Initial aeration to 7 hr showed a rapid decrease in NADase activities in the medium N and medium N plus glutamate cultures (samples of aconidial mycelia were not taken during these time intervals), and at 24 and 48 hr NADase was not measurable. NADase activity was associated with the appearance of the conidiophores after 3 hr (51 hr, see Table IV) of the separate parent mycelial incubation. NADase continued to in-

TABLE III

NADP-Glutamic Dehydrogenase Activity During the Asexual Cycle of Wild Type Neurospora crassa

Medium		Time (*hr*) 0	1	3	7	24	48	51	56	96
N	TU*	225	180	1008	1900	22610	10000	13000	4500	110
	TP	4	4	7	15	111	100	130	120	5
	SPA	56	45	144	127	204	100	100	38	22
N + glutamate	TU	226	190	960	1729	14500	18000	19000	7900	132
	TP	4	4	4	18	136	160	130	120	5
	SPA	56	47	240	96	107	113	146	66	26
Grams dry weight/culture		0.040‡				1.9§	2.4§			0.040‡
Per cent germination		0	5	20	92	M‖	M	M + CP	M + CP some C	C

* TU, total units per culture; TP, total milligrams protein per culture; SPA, specific activity (units/milligram protein).

‡ Dry weight of 3×10^9 conidia used as inoculum, time 0 and at 96 hr.

§ Refers to medium N and medium N plus glutamate cultures.

‖ M, mycelia; CP, conidiophores; C, conidia produced on conidiophores of N and N plus glutamate cultures.

TABLE IV

NADase Determinations During Development of Neurospora

Medium		Time (*hr*) 0	1	3	7	24	48	51	56	96
N	TU*	4834	4704	3690	2160	0	0	260	7200	4150
	TP	4	4	7	15	111	100	130	120	5
	SPA	1208	1176	527	144	0	0	2	60	830
N + glutamate	TU	4750	4744	3688	1740	0	0	265	5100	4032
	TP	4	3	4	18	136	160	130	120	5
	SPA	1187	1581	922	96	0	0	2	43	806
Grams dry weight/culture		0.040‡				1.9§	2.4§			0.040‡
Per cent germination		0	5	20	92	M‖	M	M + CP	M + CP some C	C

* TU, total units per culture; TP, total milligrams protein per culture; SPA, specific activity (units per milligram protein).

‡ Dry weight of 3×10^9 conidia used as inoculum, time 0 and at 96 hr.

§ Refers to medium N and medium N plus glutamate cultures.

‖ M, mycelia; CP, conidiophores; C, conidia produced on conidiophores of N and N plus glutamate cultures.

crease between 51 and 56 hr, a time of conidiophore elongation. The conidia which were differentiated from the conidiophores, in turn, contained quantities of NADase similar to those found associated with the initial conidial inoculum. The lack of NADase activities in the 24- and 48-hr mycelia from medium N and medium N plus glutamate cultures can be readily explained. NADase was found to be very water soluble, with its production dependent on the age of the mycelia. It was discovered that the washing procedure on harvested mycelia at 24 and 48 hr reduced the NADase content to unmeasurable quantities (Stine, G. J. Manuscript in preparation.)

It can be seen, however, that washing of the aconidial mycelia when harvested at 24 and 48 hr did not result in a complete loss of NADase activity (Table II). The difference in measurable NADase activity after washing of the mycelia at 24 and 48 hr can be considered a distinguishing feature between wild type and the aconidial mutant mycelia.

During the 24- and 48-hr periods of aconidial mycelia aeration (Table II), the total units of activity increased better than threefold. During the first 3 hr of mycelial incubation (48–51 hr), old mycelia plus the new conidiophore NADase activity remained approximately constant with the 48-hr levels. At 56 hr (8 hr of incubation), the conidiophores were very long and were easily separated from the parent mycelia for protein and enzyme determinations. The 5-hr conidiophores contained 2.6 times the total units of NADase as that found in the mycelia from which they were produced. Considering that NADase is produced in increasing quantity as the mycelia age (Stine, G. J. Manuscript in preparation.), it can be stated that the conidiophores appear to age very quickly, i.e., they distinctly appear old with respect to production of NADase in time.

DISCUSSION

The percentages of germination for conidia derived from the medium N and that for conidia derived from the medium N plus glutamate cultures, regardless of their NAD-enzyme and NADP-enzyme content, were similar (Stine, G. J. Unpublished data.). There was no apparent synthesis of the NADP enzyme in the first hour of culture, during which time at least 5% of the conidia had germinated (Table III). Although there is a lag in germination of NADP enzyme–deficient conidia (*am* mutants), *they do germinate*. It is possible that there was some synthesis of the NAD enzyme during the first hour of culture (Table I).

Whether the NAD enzyme can be considered to be essential to germination processes will have to await the isolation of an NAD-enzyme mutant. Regardless of the number of attempts made so far, this has not been (satisfactorily) accomplished; perhaps this can be attributed to its requirement for germination.

The level and fluctuation of the NADP enzyme as measured in time, regardless of culture, were significantly greater than those of the NAD enzyme. Following the levels of the NAD and NADP enzymes (total units per culture and specific activity, Tables I and III) throughout the asexual life cycle implies that they are not concurrently regulated as has been suggested (10, 11). Also, and in contrast to the report of Sanwal and Lata (11), these investigations show that wild type conidia do contain the NAD–dependent glutamic dehydrogenase. In fact, it has been shown that as these conidia age the specific activity for the NAD enzyme increases (Stine, G. J. 1967. *Neurospora* Newsletter No. 11. 7.). The NAD enzyme was found to increase from the onset of logarithmic mycelial growth (3–24 hr) through 48 hr in the medium N and medium N plus glutamate cultures. The addition of glutamate to the medium N culture simply induced greater synthesis of both the NAD and NADP enzymes between 24 and 48 hr. In fact, a drop in the NADP enzyme (between 24 and 48 hr) occurred *only* in the medium N culture (absence of glutamate) while the NAD enzyme remained constant.

Why does *Neurospora* need the separate glutamic dehydrogenases, and why does the NAD enzyme persist throughout the asexual cycle, e.g. why does it rapidly increase during logarithmic mycelial growth (3–24 hr) as does the NADP enzyme? Since the reactions concerned with both glutamic dehydrogenases are reversible, could not the high levels of the NADP enzyme handle the vegetative needs? One can speculate that the NAD enzyme was necessary during some initial processes of germination and later formation of the conidiophore, since early synthesis of this enzyme occurred at both stages of development (Table I). Also, the persistence of NAD enzyme throughout the asexual cycle could be accounted for in light of the findings reported by Stachow and Sanwal (12). Their investigations showed that in vitro only the NAD–glutamic dehydrogenase was affected by the addition of various purine nucleotides. The NAD-glutamic dehydrogenase may produce the glutamate used as a precursor for glutamine, which in turn can give rise to purine nucleotides (5). Thus in *Neurospora*, as well as in other organisms possessing both glutamic dehydrogenases, the significant role of the NAD enzyme may be its association in the pathway to the formation of purine nucleotides. Thus, the NADP enzyme may act as the major enzymatic mechanism for the interconversion of alpha keto acids and amino acids coupled with the formation or utilization of ammonia.

The very interesting association of NADase with the asexual cycle will be reported in greater depth in the near future. As the specific activity or level of NADase decreased, the NAD enzyme, and NADP enzyme increased. This relationship of the amount of the pyridine-dependent dehydrogenases present to the level of NADase is very interesting since the dehydrogenases are cofactor-dependent and NADase splits either of the oxidized forms of NAD and NADP. However, it is not known whether the level of NADase actually alters the level of the cofactors or dehydrogenases herein.

It is very difficult to determine whether an enzyme is required for a certain morphological manifestation. At best it can be stated that there is an apparent association. The discussion of the results in this study was presented with this thought in mind.

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