INDUCTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE IN *NEUROSPORA CRASSA* BY ADDITION OF GLUTAMATE TO THE MEDIA

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[*Manuscript received October* 14, 1968]

Summary

There are two glutamate dehydrogenases (GDH) produced by wild-type strains of *N. crassa*, one of which is specific for the coenzyme NADP and the other for the coenzyme NAD. The latter enzyme (NAD-GDH) is induced if glutamate is used as the sole carbon and nitrogen source and is induced to a lesser extent if inorganic nitrogen is added. Addition of sucrose to the medium prevents uptake of glutamate and there is no induction of the enzyme.

NADP-specific GDH activity is drastically decreased if glutamate is used as the sole carbon and nitrogen source. Addition of inorganic nitrogen causes a smaller decrease of NADP-GDH.

If inorganic nitrogen is used as the sole nitrogen source together with sucrose as the carbon source, NADP-GDH activity is maintained for a longer period and NAD-GDH is not induced.

I. INTRODUCTION

Wild-type strains of *Neurospora crassa* produce two glutamate dehydrogenases, one of which is specific for the coenzyme NAD and the other for the coenzyme NADP (Sanwal and Lata 1961, 1962). Sanwal and Lata (1962) found that the NAD-specific dehydrogenase [L-glutamate : NAD oxidoreductase (deaminating), EC. 1.4.1.2] (NAD-GDH) of wild-type strains was not induced by the addition of 50 mM L-glutamate alone or by the addition of ammonium nitrate. However, the addition of both L-glutamate and ammonium nitrate or the addition of urea alone produced a marked induction of the NAD-GDH as measured by an increase in specific activity. Concurrently with the increase in specific activity of the NAD-GDH, there was a decrease in the specific activity of the NADP-specific glutamate dehydrogenase [L-glutamate : NADP oxidoreductase (deaminating), EC. 1.4.1.4.] (NADP-GDH). They also found that, in two mutants (arg-4 21502 and arg-5 27947) in which the conversion of glutamate to ornithine was blocked, NAD-GDH was not induced by growth on glutamate plus ammonium nitrate. From this evidence they maintained that a product of urea metabolism was the agent responsible for the induction of the NAD-GDH. The induction was said to be due to a metabolic product of urea rather than to urea itself because the NAD-GDH of a strain lacking urease (U^{25}) was not induced when grown on urea. Stachow and Sanwal (1967) indicated that it was "likely that the actual effector *in vivo* for the concurrent regulation of the NAD- and the NADP-specific glutamate dehydrogenases is ammonia". However, the method of assaying for urease was not stated.

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A plausible hypothesis is that one of the glutamate dehydrogenases is biosynthetic and that the other is degradative (Sanwal and Lata 1962). Since the specific activity of the NADP-GDH is initially high and later decreases, whilst the specific activity of the NAD-GDH is initially low and later increases, it seems possible that the NADP-GDH is biosynthetic and the NAD-GDH is degradative. However, regardless of the metabolic function of these two enzymes, it is unlikely that both glutamate and ammonium ions would be needed for induction since one compound must be a substrate and the other compound must be a product. The work reported in this paper attempted to elucidate the conditions under which induction of NAD-specific GDH takes place.

II. Materials and Methods

(*a*) *Chemicals*

NADH and NADPH (enzymatically reduced), α-ketoglutarate, and cycloheximide were obtained from Sigma Chemical Company, St. Louis, Missouri. [U-^{14}C]-L-Glutamate (> 180 mCi/m-mole) was obtained from New England Nuclear Corporation.

(*b*) *Strain of* Neurospora

All studies were done using wild-type strain 74-OR8-1a which was obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire.

(*c*) *Culture Media and Growth Conditions*

10^6 conidia of strain 74-OR8-1a were inoculated into 100 ml of Vogel's medium "N" (referred to in the remainder of this paper as medium N) in 250-ml Erlenmeyer flasks, and grown at 30°C on a rotary shaker at 160 r.p.m. for 48 hr (Vogel 1956). The pad was then harvested, washed under sterile conditions, and transferred to 100 ml of modified Vogel's medium "N" (referred to in the remainder of this paper as medium M) in 250-ml Erlenmeyer flasks. Medium M did not contain ammonium nitrate. It was diluted and supplemented with the desired concentration of organic or inorganic nitrogen or sucrose or both. Cycloheximide was sterilized by passage through a Millipore filter.

(*d*) *Enzyme Preparation and Assay*

Mycelial growth was harvested by filtration, washed with distilled water, and stored at −18°C. Crude enzyme extracts were prepared as follows: a frozen mycelial pad, # 16 glass beads (1 g per 2 g mycelium), and cold 100 mM sodium phosphate–potassium phosphate buffer, pH 8·0, containing 1mM 2-mercaptoethanol (5 ml buffer per 2 g mycelium) were added to a chilled mortar and ground for 5 min. The homogenate was centrifuged at 12,840 *g* for 20 min at 0°C. The resulting supernatant was assayed immediately for NAD-GDH and NADP-GDH activity. Assay systems measuring the reductive amination of α-ketoglutarate and based on the decrease in absorbance at 340 mμ attending the oxidation of NADH or NADPH were used. A unit of enzyme activity is defined as a decrease in optical density at 340 mμ of 0·02 per minute measured with a Cary-15 recording spectrophotometer. Specific activity is defined as units of activity per milligram of protein.

The assay mixture for NAD-GDH contained (in a volume of 2·7 ml) 130 μmoles Tris buffer (pH 8·4), 33·4 μmoles α-ketoglutarate, 150 μmoles NH_4Cl, 0·2 μmoles NADH, and 0·1 ml suitably diluted enzyme extract. Since there was some non-specific oxidation of NADH in the absence of substrate, enzyme activity was measured as the difference in optical density change in the presence and absence of substrate.

The assay mixture for the NADP-GDH contained (in a volume of 2·7 ml) 130 μmoles Tris buffer (pH 7·8), 33·4 μmoles α-ketoglutarate, 150 μmoles NH_4Cl, 0·17 μmoles NADPH, and 0·1 ml suitably diluted enzyme extract. Since the NADP-GDH is activated in the presence of α-ketoglutarate (West *et al.* 1967), all assay components except NADPH were incubated for 5 min before the reaction was initiated by addition of NADPH. Non-specific oxidation of NADPH was not detectable at the usual dilutions necessary for measurement of NADP-GDH.

Protein content of the extracts was determined by the colorimetric method of Lowry *et al.* (1951). Dialysis of the extracts decreased the apparent protein content by 5–10%. Extracts used in this study were not dialysed since this error in protein measurement would not invalidate the observed increases or decreases in specific activity.

III. Results

(*a*) *Uptake of* L-*Glutamate as a Function of the Sucrose Concentration*

Mycelial pads were produced by growth on medium N plus 58 mM sucrose for 48 hr. The pads were harvested, washed with distilled water, and transferred to fresh medium N (i.e. 25 mM NH_4NO_3) containing 10 or 50 mM [U-^{14}C]-L-glutamate and varying concentrations of sucrose. The uptake of L-glutamate was estimated by measuring the decrease in [U-^{14}C]-L-glutamate in the medium at various times.

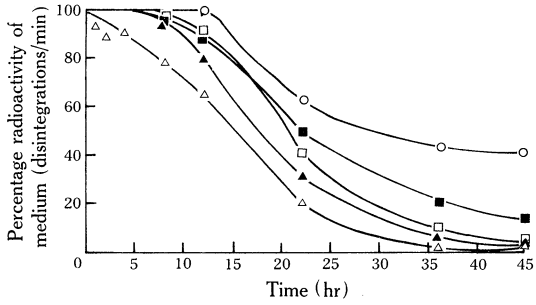


Fig. 1.—Uptake of [U-^{14}C]-L-glutamate by mycelial pads of *N. crassa* as a function of sucrose concentration. △ No sucrose. ▲ 15 mM sucrose. □ 29 mM sucrose. ■ 58 mM sucrose. ○ 146 mM sucrose.

Figure 1 shows the decrease in radioactivity in the medium in the presence of 10 mM L-glutamate. It can be seen that the uptake of L-glutamate has an inverse relationship to the concentration of sucrose. In the presence of 50 mM L-glutamate, but in the absence of sucrose, the decrease in radioactivity was proportionally less, being only 22% after 48 hr compared with a decrease of 96·8% in the medium containing 10 mM L-glutamate.

(*b*) *Induction of NAD-GDH in Mycelial Pads Grown on 50 mM* L-*Glutamate plus Variable Concentrations of Sucrose*

The mycelial pads obtained from growth for 48 hr on medium N were transferred to medium M (no ammonium nitrate) plus 50 mM L-glutamate without sucrose or with 58 or 146 mM sucrose. The mycelial pads were incubated for various times in these media, harvested, washed extensively in distilled water, and proteins extracted as described in Section II.

The specific activities of the NAD-GDH and NADP-GDH are given in Table 1. In the presence of 146 mM sucrose the specific activities do not change over a 48-hr period. If the concentration of sucrose is lowered to 58 mM (the usual concentration

used in previous investigations, e.g. Stachow and Sanwal 1967) there is a slight induction of the NAD-GDH and some decrease of the NADP-GDH activity. However, if sucrose is omitted from the medium, there is a strong induction of the NAD-GDH and the NADP-GDH is decreased to a level which may represent the basal

TABLE 1

SPECIFIC ACTIVITIES OF NAD-GDH AND NADP-GDH IN MYCELIAL PADS GROWN ON MEDIUM M PLUS 50 mM L-GLUTAMATE AND VARIOUS CONCENTRATIONS OF SUCROSE

Growth Period (hr)	Specific Activity with No Sucrose		Specific Activity with 58 mM Sucrose		Specific Activity with 146 mM Sucrose	
	NAD-GDH	NADP-GDH	NAD-GDH	NADP-GDH	NAD-GDH	NADP-GDH
0 (control)	13·7	111·1	16·5	107·5	14·1	108·3
2	—	—	12·9	80·0	16·1	90·5
4	21·9	52·5	8·3	75·7	15·9	84·4
8	25·6	23·3	14·8	89·6	16·6	97·3
12	33·3	4·7	10·6	77·9	11·8	86·4
24	40·0	1·2	11·0	81·9	12·1	73·9
36	60·0	1·2	27·4	34·7	13·1	84·2
48	100·3	1·1	23·8	44·7	12·7	109·7

specific activity. The metabolic status of the mycelial pads during the second 48-hr incubation period is indicated by the changes in dry weight (Table 2). Apparently L-glutamate can be metabolized, but growth is poor if it is the only carbon and nitrogen source (Tables 2 and 4).

TABLE 2

PERCENTAGE CHANGE IN DRY WEIGHTS OF MYCELIAL PADS GROWN ON MEDIUM M PLUS 50 mM L-GLUTAMATE AND VARIOUS CONCENTRATIONS OF SUCROSE

Growth Period (hr)	Percentage Change in Dry Weight		
	No Sucrose	58 mM Sucrose	146 mM Sucrose
2	−2	22	28
4	10	25	40
8	19	34	72
12	18	52	84
24	15	97	134
48	20	141	179

(c) *Lack of Induction of NAD-GDH in Mycelial Pads Grown on 58 mM Sucrose plus Various Concentrations of Ammonium Nitrate*

The mycelial pads obtained from growth for 48 hr on medium N plus 58 mM sucrose were transferred to medium M plus 58 mM sucrose plus either 25 or 250 mM NH_4NO_3. The mycelial pads were incubated for various times in these media, harvested, and washed extensively. Proteins were extracted and assayed for

glutamate dehydrogenases (Table 3). There was no increase in specific activity of the NAD-GDH but there was a decrease in specific activity of the NADP-GDH after growth for 24 hr.

TABLE 3

SPECIFIC ACTIVITIES OF NAD-GDH AND NADP-GDH IN MYCELIAL PADS GROWN ON MEDIUM M PLUS 58 mM SUCROSE AND VARIOUS CONCENTRATIONS OF AMMONIUM NITRATE

Growth Period (hr)	Specific Activity with 25 mM NH_4NO_3		Specific Activity with 250 mM NH_4NO_3	
	NAD-GDH	NADP-GDH	NAD-GDH	NADP-GDH
0 (control)	14·8	87·8	14·2	108·3
2	—	—	12·5	102·7
4	16·9	119·2	—	—
8	11·8	124·0	22·0	121·5
12	13·2	119·0	15·1	141·0
24	9·9	57·4	15·5	68·0
36	10·2	34·4	15·2	68·5
48	10·9	31·6	17·1	59·8

(d) Induction of NAD-GDH in Mycelial Pads Grown on Medium N without Sucrose but with Addition of Various Concentrations of L-*Glutamate*

Growth on medium N (which contained 25 mM NH_4NO_3), without sucrose but with 10 mM or 50 mM L-glutamate, produced an increased specific activity of NAD-GDH and a decreased specific activity of NADP-GDH (Table 4), but these changes were not nearly so pronounced as in the absence of all ammonium nitrate (Table 1). Furthermore, the maximum increase in specific activity of NAD-GDH in the presence of 25 mM NH_4NO_3 and 10 or 50 mM L-glutamate was reached after incubation for

TABLE 4

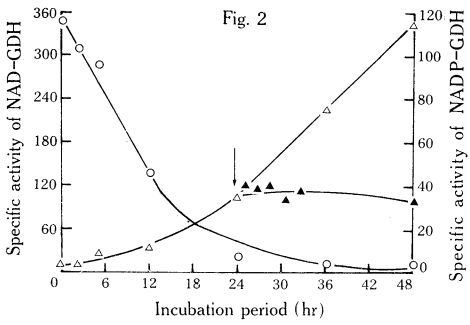
SPECIFIC ACTIVITIES OF NAD-GDH AND NADP-GDH IN MYCELIAL PADS GROWN ON MEDIUM N WITHOUT SUCROSE BUT WITH ADDITION OF VARIOUS CONCENTRATIONS OF L-GLUTAMATE

Growth Period (hr)	Specific Activity		Change in Dry Weight (%)	Specific Activity		Change in Dry Weight (%)
	NAD-GDH	NADP-GDH		NAD-GDH	NADP-GDH	
	10 mM L-glutamate			50 mM L-glutamate		
0 (control)	9·8	104·3	—	16·5	107·5	—
2	25·0	99·2	−19·5	—	—	21·9
4	38·6	76·4	−16·3	39·0	80·0	25·0
8	42·9	67·9	−16·0	50·0	80·9	34·3
12	28·1	70·8	−16·6	40·8	79·3	52·5
24	28·4	63·3	−6·5	42·0	55·1	97·5
36	24·4	59·0	−7·8	36·4	53·2	159·7
48	29·4	53·8	−15·3	46·4	63·4	146·7

8 hr. This maximal value was maintained on 50 mM L-glutamate but decreased on 10 mM L-glutamate. The specific activity of NADP-GDH was decreased but not nearly to the same extent as in the absence of all ammonium nitrate. The changes in dry weight indicate that 10 mM L-glutamate is insufficient for growth (Table 4).

(e) *Activation: Inactivation* v. *Induction: Repression*

In order to differentiate between activation of a previously formed enzyme and induction, the mycelial pads obtained from growth on medium N plus 58 mM sucrose were transferred to two types of medium for 24 hr, then cycloheximide (10 μg/ml) was added to each flask. Firstly, the mycelial pads were transferred to medium N plus 58 mM sucrose and 50 mM urea. This medium produces an enormous increase in specific activity (Fig. 2) in agreement with previously published results



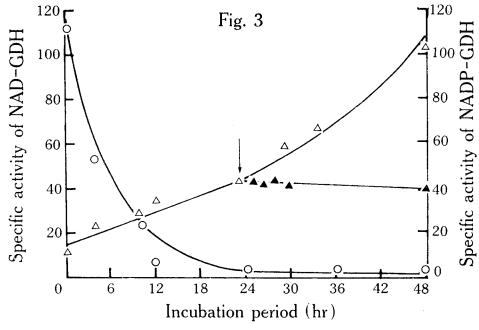


Fig. 2.—Specific activities of NAD-GDH and NADP-GDH of mycelial pads of *N. crassa* grown on medium N plus 58 mM sucrose and 50 mM urea. ○ NADP-GDH. △ NAD-GDH. ▲ NAD-GDH after addition of 10 μg of cycloheximide per millilitre of medium (indicated by arrow).

Fig. 3.—Specific activities of NAD-GDH and NADP-GDH of mycelial pads of *N. crassa* grown on medium M (no NH_4NO_3 or sucrose) plus 50 mM L-glutamate. ○ NADP-GDH. △ NAD-GDH. ▲ NAD-GDH after addition of 10 μg of cycloheximide per millilitre of medium (indicated by arrow).

(Sanwal and Lata 1962; Stachow and Sanwal 1967). The addition of cycloheximide at 24 hr halted the increase in specific activity of NAD-GDH. Secondly, the mycelial pads were transferred to medium M plus 50 mM L-glutamate. This medium produces a moderate increase in the specific activity of NAD-GDH but, again, the addition of cycloheximide at 24 hr halted the increase (Fig. 3).

IV. Discussion

The results reported here indicate that, if glutamate is used in the culture media of wild-type *N. crassa* as the sole carbon and nitrogen source, then the NAD-GDH is induced and the specific activity of the NADP-GDH decreases to a very low level. The induction of the NAD-GDH is prevented by the addition of sucrose to the culture medium. However, the uptake of glutamate is an inverse function of the sucrose concentration in the media, so the lack of induction of NAD-GDH may be due to the prevention of glutamate uptake. The decrease of NADP-GDH is correspondingly less at higher sucrose concentrations.

These conclusions are supported by competition experiments using rose tissue (Dougall 1965). It was found that, if [^{14}C]glucose was supplied, then exogenous alanine, glutamate, glutamine, or aspartate had no effect on the degree of labelling of these protein-bound amino acids. Joy and Folkes (1965) also found that if [^{14}C]sucrose was fed to barley embryos in the presence of unlabelled exogenous

glutamate, then the unlabelled amino acid had no effect on the degree of labelling of protein-bound glutamate. On the other hand, externally supplied lysine or leucine almost completely suppressed the incorporation of ^{14}C into residues of these amino acids in embryo protein. These experiments could also be interpreted as due to inhibition of amino acid uptake by the carbohydrate supplied.

Barratt (1963) showed that glutamate alone depressed NADP-GDH specific activity. However, he used 57 mM sucrose in the medium and therefore the decrease was not as large as might have been expected in the absence of sucrose.

Lé John and McCrea (1968) found two glutamate dehydrogenases in *Thiobacillus novellus*. One was specific for NADP and the other for NAD. The latter enzyme was fully induced if glutamate was supplied exogenously as the sole carbon source.

On the other hand, the activity of the NAD-GDH of *Saccharomyces cerevisiae* was high when growth occurred on glutamate or other amino acids as the sole nitrogen source (Hierholzer and Holzer 1963). Glutamate was not utilized as a carbon source and so glucose was essential for this increased level of NAD-GDH. It was also found (as in the present study on *N. crassa*; Tables 1 and 4) that addition of NH_4^+ to a medium in which glutamate was the sole nitrogen source hindered the increase of NAD-GDH activity.

Stachow and Sanwal (1967) regarded the findings of Barratt (1963) that ammonia repressed the NADP-GDH as support for their hypothesis that ammonia is the regulator of NAD- and NADP-GDH levels *in vivo*. However, Barratt found that ammonia disproportionately repressed the NADP-GDH only at high concentrations of ammonia and so this decrease was likely to be due to a general ammonia toxicity. In fact, Barratt found that at NH_4^+ concentrations up to the optimal for protein synthesis, NADP-GDH specific activity and protein synthesis were proportional. In the present study, specific activity of the NADP-GDH decreased after 24 hr regardless of the concentration of ammonium nitrate (Table 3). This decrease in specific activity may reflect the exhaustion of the 58 mM sucrose supplied (see Table 1).

If 25 or 250 mM NH_4NO_3 is used as the sole nitrogen source together with 58 mM sucrose as the carbon source, the NAD-GDH of *N. crassa* remains at a low level (Table 3). Westphal and Holzer (1963) also found that, in protoplasts of *Saccharomyces carlsbergensis*, the synthesis of NAD-GDH was repressed by NH_4^+.

It is still necessary to determine whether the increase in specific activity of NAD-GDH was due to activation of an already-synthesized enzyme as opposed to *de novo* synthesis. The hypothesis of *de novo* synthesis is suggested by the fact that the increase in specific activity of NAD-GDH produced by urea or L-glutamate was halted by the addition of 10 μg cycloheximide per millilitre of medium.

V. Acknowledgments

This investigation was supported by a grant GB-3101 from the National Science Foundation and by an Institutional Research Grant of the American Cancer Society.

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