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REGULATION, PURIFICATION, AND SOME PROPERTIES OF THE NAD-SPECIFIC GLUTAMATE DEHYDROGENASE OF NEUROSPORA*

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SUMMARY

1. The NAD-specific glutamate dehydrogenase (L-glutamate:NAD oxidoreductase (deaminating), EC 1.4.1.2) was purified 300–600 fold and characterized.

2. Electrophoresis of *Neurospora crassa* extracts resolved the NAD-specific enzyme into four isozymes whereas the NADP-specific glutamate dehydrogenase yielded a single band.

3. Comparison of kinetic parameters, heat sensitivity and antigenic relationships of the NAD-specific enzyme revealed that the uninduced and induced enzymes are identical.

4. A genetic model is postulated for the mechanism of concurrent regulation of the two glutamate dehydrogenases from Neurospora.

INTRODUCTION

It has been demonstrated that yeast[1–3], *Neurospora crassa*[4], and some other organisms[5,6] possess two glutamate dehydrogenases, one specific for NAD, the other for NADP. In Neurospora these two enzymes have been shown to be concurrently regulated by a repression–depression type of mechanism. Thus in the presence of glutamate or its nitrogenous precursors (urea, ammonia, alanine, aspartate, *etc.*) the NADP-specific glutamate dehydrogenase is repressed and the NAD-specific enzyme is simultaneously depressed[7–9]. An important aspect of the concurrent regulation of these two enzymes is the apparent inverse relationship that exists between the amount of the two enzymes at any one time during growth either in the absence or presence of the various inducers[7,8]. Thus, under conditions where high levels of the NADP-specific enzyme are present the levels of the NAD-specific enzyme are invariably low and *vice versa*.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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These observations on regulation of the two glutamate dehydrogenases are reminiscent of the strikingly similar compensatory regulation of α and β chains of hemoglobin-F which as the principal component of foetal blood is gradually replaced by hemaglobin-A during late gestation[12], and at any time during this period there is a strict inverse relationship between the quantities of α and β chains[11].

As with hemoglobin, the problem of concurrent regulation of the two glutamate dehydrogenases of Neurospora poses certain important questions (which demand an answer). Some of the specific questions pertaining to the mechanism of concurrent regulation which we have attempted to answer in this communication are:

(1) Is there a structural similarity between the NAD- and the NADP-specific glutamate dehydrogenases such that one enzyme could be converted to the other during growth or under the influence of an inducer[2]?

(2) Out of a number of nitrogenous precursors of glutamate that can regulate the synthesis of the two enzymes[8], which one is the actual regulatory agent *in vivo*?

Since the structural and physical properties of the NADP-specific enzyme of *N. crassa* have already been analyzed[9,13,14] in great detail, we have started out by extensively purifying the NAD-specific glutamate dehydrogenase (L-glutamate: NAD oxidoreductase (deaminating), EC 1.4.1.2) and studying some of the properties of the enzyme isolated under both uninduced and induced conditions. Also included in this communication are the results of some experiments which were specifically designed to answer the second question raised above.

MATERIALS AND METHODS

Genetic strains

The strains of *N. crassa* used here were, STA4 (wild type), Am[3] and U[25]. The first two strains were obtained from the culture collection of the Fungal Genetics Stock Center located at Dartmouth College, New Hampshire. The wild type possessed both the NADP- and NAD-specific glutamate dehydrogenases. Am[3] was an amination-deficient mutant which lacked normal NADP-specific enzyme and required amino nitrogen for growth. U[25] was a mutant lacking urease and was derived in this laboratory from strain 497 (stock No. of the Fungal Genetics Center) by the inositol-less death method[15]. All of the strains mentioned above possessed a functional and active NAD-specific glutamate dehydrogenase.

Growth conditions

The minimal medium N described by VOGEL[16] was used throughout. It was supplemented with the required additives and 2% sucrose before use. Conidia from stock cultures were washed off the agar with sterile, deionized water, passed aseptically through four layers of gauze to remove hyphal fragments and washed 3 times by alternate centrifugation and suspension in water before being used as inoculum.

For small-scale induction experiments, erlenmeyer flasks of 500 ml capacity containing 100 ml medium N were seeded with approx. $5 \cdot 10^8$ conidia and grown at 28° for the required length of time with vigorous aeration. The resulting cells were harvested by filtration through two layers of cheese-cloth and repeatedly washed with distilled water. The cells were pressed dry between paper towels and used for the preparation of cell-free extracts as described earlier[8,9].

Large quantities of mycelia for enzyme purification were obtained by inoculating conidia into 15 l carboys containing 10 l of medium N supplemented with 0.05 M L-glutamate or L-alanine (inducers). Aeration was achieved by forcing sterile air through the medium. Carboys were incubated at 28° for 24–60 h. The mycelial mass was harvested in the usual manner, washed and lyophilized. The powder so obtained could be stored for at least 6 months at −20° without loss of NAD-specific glutamate dehydrogenase activity.

Extraction procedure

When working with small quantities of mycelia (1–10 g), the cells were disrupted by grinding with alumina as described earlier[4]. For the extraction of large quantities of NAD-specific glutamate dehydrogenase the lyophilized powder was suspended in 10 times its weight of 0.05 M Tris buffer (pH 7.5) containing 10^{-3} M GSH. This suspension was mechanically stirred at 4° for 30 min. The extract was filtered through cheese-cloth and centrifuged at 12 000 × *g* for 15 min. The supernatant constituted the crude enzyme extract.

Assay procedure

NAD-specific glutamate dehydrogenase was assayed in a mixture containing 20 μmoles α-ketoglutarate, 120 μmoles $(NH_4)_2SO_4$,0.50 μmole reduced NAD, enzyme preparation, and 273 μmoles Tris buffer (pH 9.0). The final volume in silica cuvettes of 1-cm light path was 3.0 ml. Velocity was measured by the decrease in absorbance at 340 mμ in a Gilford Model 2000 recording spectrophotometer. One unit of enzyme activity is defined as a change in absorbance at 340 mμ of 0.10 per min. Specific activity is defined as units per mg protein. NADP-specific enzyme was measured as outlined earlier[4,8].

Analytical procedures

Protein was determined according to the method of LOWRY *et al.*[17]. L-Glutamate was extracted from mycelia in boiling water and assayed by using L-glutamate decarboxylase[18]. Vertical starch-gel electrophoresis was performed according to SMITHIES[19]. The gel was prepared in 0.02 M borate buffer (pH 8.3). The buffer used in the electrode compartment was 0.15 M borate (pH 8.0). Electrophoresis was carried out at 150 V (20 mA) at 4° for 12 h. Individual dehydrogenases were localized by the specific neotetrazolium method of MARKERT AND MOLLER[20]. Disc electrophoresis was performed as described by ORNSTEIN AND DAVIS (reprint available from Distillation Products Industry, Eastman Kodak, Rochester, N.Y.), using Tris–glycine buffer (pH 8.3). The method of MARTIN AND AMES[21] was used for zone centrifugation of enzymes in sucrose density gradients. The 5% and 20% (w/v) sucrose solution also contained 0.10 M Tris–HCl buffer (pH 7.5). Fluorescence measurements were made with an Aminco–Bowman spectrofluorometer using a 416–992 xenon arc lamp and a flat-bed x-y recorder.

Immunological techniques

Antiserum against NAD-specific glutamate dehydrogenase was prepared by injecting adult albino rabbits subcutaneously with a highly purified enzyme preparation emulsified with Freund's adjuvant[22]. The rabbits were injected weekly with

4 mg of protein for a period of 5 weeks. One week after the last injection, when titres were high enough, the rabbits were exsanguinated and the serum obtained was clarified by centrifugation. The resulting supernatant solution was dispensed in portions in small tubes and stored frozen at −20°. All precipitation reactions were performed according to the standard serological techniques described by COHN[22].

Procedure for obtaining ^{14}C-labelled enzymes

Wild-type (STA 4) conidia of *N. crassa* were inoculated into 1 l of medium N containing 0.005 M L-[1-^{14}C]leucine ($1 \cdot 10^6$ counts) in a 2-l flask. They were allowed to grow with vigorous agitation at 28° for 18 h. The resulting mycelial mat was asceptically collected by filtration and washed with 2 l of sterile water. The washed mycelial mat was then sucked nearly dry by vacuum, and maintained for 6 h at 28° under aerated conditions in medium N *minus* a nitrogen source. After this period, 2% NH_4NO_3 (w/v) and 0.05 M L-alanine (inducer) were added to the medium and the cells were allowed to grow for an additional 18 h. The second 18-h incubation was necessary for the induction of the NAD-specific glutamate dehydrogenase. The cells were then collected by filtration, thoroughly washed, divided into two equal lots and used for the purification of NAD- and NADP-specific glutamate dehydrogenases. The NADP-specific enzyme was purified as outlined earlier[9] and the NAD-specific enzyme was described below. Each enzyme fraction was subjected separately to starch-gel electrophoresis, localized by a specific method[20] and eluted from the gel with 1.0 ml of 0.1 M phosphate buffer (pH 7.5). Radioactivity was measured in each eluate with a Nuclear Chicago scintillation counter. Samples were counted for a period of 100 min.

RESULTS

Purification of NAD-specific glutamate dehydrogenase

All steps described below were carried out at 4°.

To a crude enzyme extract from induced, lyophilized cells solid $(NH_4)_2SO_4$ was added to 0.3 satn. The precipitate which resulted after 30 min stirring was removed by centrifugation at 12 000 × *g* for 15 min. To the supernatant solution, solid $(NH_4)_2SO_4$ was again added to 0.4 satn. After stirring for 30 min the precipitate was separated by centrifugation at 20 000 × *g* (20 min) and dissolved in one-tenth the original volume of 0.1 M Tris–HCl buffer (pH 7.5) containing 10^{-3} M GSH. To this protein solution Cγ aluminum hydroxide gel was added at a concentration of 5 mg gel/mg protein. The suspension was gently stirred over a period of 20 min. The gel was recovered by centrifugation at 10 000 × *g* for 10 min and washed twice with small quantities of 0.1 M KH_2PO_4–NaOH buffer (pH 7.5), containing 10^{-3} M GSH. The adsorbed enzyme was eluted from the gel with 0.5 M KH_2PO_4–NaOH buffer (pH 6.5) containing 10^{-3} M GSH. Gel adsorption and elution was repeated 5 times and the eluates having the highest specific activity were pooled. The enzyme preparation obtained in this way was dialyzed against 0.01 M phosphate buffer (pH 6.5) for at least 6 h. The enzyme was then subjected to column chromatography on DEAE-cellulose. A chromatographic column (2.5 cm × 43 cm) was packed to a height of 30 cm and equilibrated with 0.01 M phosphate buffer (pH 6.5) containing 10^{-3} M GSH. About 20–50 mg protein from the alumina Cγ step was applied to the

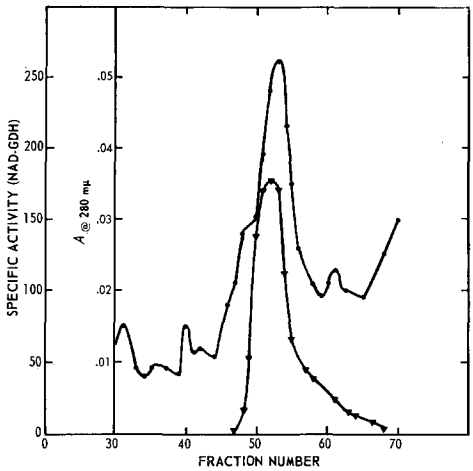


Fig. 1. Elution pattern of NAD-specific glutamate dehydrogenase (NAD-GDH) from DEAE-cellulose column. ●—●, extinction coefficient at 280 mμ; ▲—▲, specific activity with α-keto-glutarate.

top of the column. The enzyme was eluted with a linear gradient of 0.01 –0.5 M phosphate buffer (all buffers containing 10^{-3} M GSH) (pH 6.5). Fractions of approx. 3.5 ml were collected. Fig. 1 shows a typical elution pattern of the enzyme. Fractions 50–55 (Fig. 1) were pooled and used for all experiments reported later. Table I gives

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR NAD-SPECIFIC GLUTAMATE DEHYDROGENASE FROM *Neurospora crassa*

Procedure	*Volume (ml)*	*Protein (mg)*	*Total units/ml*	*Specific activity*	*Purification*
(1) Crude extract	60	882	600	40	
(2) $(NH_4)_2SO_4$ 0–0.30 satn. (supernatant)	60	714	1120	94	2.3
(3) $(NH_4)_2SO_4$ 0.30–0.40 satn. (supernatant)	20	204	1720	193	4.8
(4) Pooled Cγ eluates	20	57	1360	544	13.6
(5) DEAE-cellulose column	5	0.75	180	1200	30.0

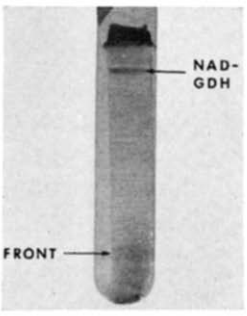
a summary of the purification procedure. This procedure yields a 30-fold purified enzyme. When it is considered, however, that the induced extracts have a specific activity about 20-fold higher[8] than that of the normal uninduced cells, our preparations represent an overall purification of about 500–600-fold.

Purity of the enzyme

Disc electrophoresis of about 100 μg of the purified enzyme yields only one major band and one or two very faint bands associated with the major one. Only the major band is visible in Fig. 2.

Isoenzymes of NAD-specific glutamate dehydrogenase

It was considered possible that the faint protein bands described above may actually be isoenzymes of glutamate dehydrogenase. Starch-gel electrophoresis of an induced extract of Neurospora resolved the NAD-specific enzyme into at least 4 distinct bands (Fig. 3); NADP-specific enzyme yielded only a single band.



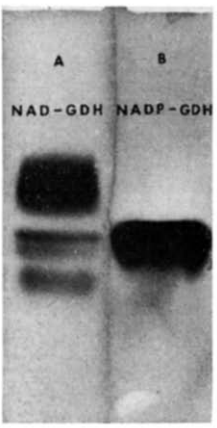


Fig. 2. Polyacrylamide electrophoresis of purified NAD-specific glutamate dehydrogenase (NAD-GDH) from Neurospora.

Fig. 3. Starch-gel electrophoresis of a crude extract of *N. crassa* (W.T.) grown under induced conditions (see text). Gel strip: (A) stained specifically for NAD-glutamate dehydrogenase (NAD-GDH); (B) was stained specifically for NADP-glutamate dehydrogenase (NADP-GDH) (according to MARKERT AND MOLLER[20]).

Zone centrifugation of the enzyme in sucrose density gradients

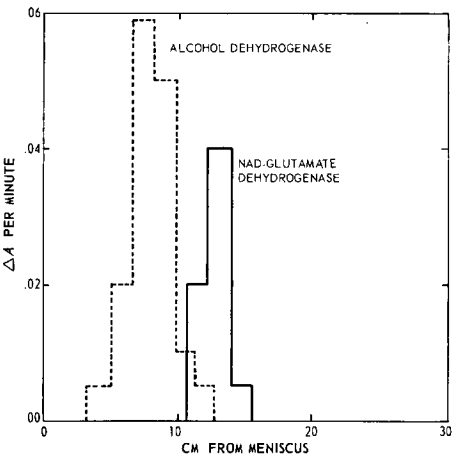
The sedimentation behaviour of the enzyme in density gradients, using yeast alcohol dehydrogenase as a marker, is shown in Fig. 4. Assuming a mol. wt. of 150 000 for alcohol dehydrogenase[23], NAD-specific glutamate dehydrogenase can be assigned an average mol. wt. of 330 000.

Optimum pH

The pH optimum for the oxidative deamination of L-glutamate was found to be 9.5, whereas, the optimum for the reductive amination of α-ketoglutarate was 8.0.

Coenzyme specificity

Reduced NAD in the reductive amination, and oxidized NAD in the oxidative deamination assay, could not be replaced by NADPH or NADP, respectively, even at concentrations 15 times that of NAD used normally in assays. Of the NAD analogues tested, (deamino-NAD, 3-pyridinealdehyde-NAD, 3-pyridine aldehyde-deamino-NAD and 3-acetylpyridine-NAD) only deamino-NAD functioned as a coenzyme, and with only $^1/_{20}$ the activity of that obtained with NAD (Fig. 5). 3-Pyridinealdehyde-NAD at a concn. of 10^{-4} M caused a 65% inhibition of activity in both the oxidative (NAD concn., $5 \cdot 10^{-3}$ M) and reductive (NADH concn., 10^{-3} M) deamination assay.



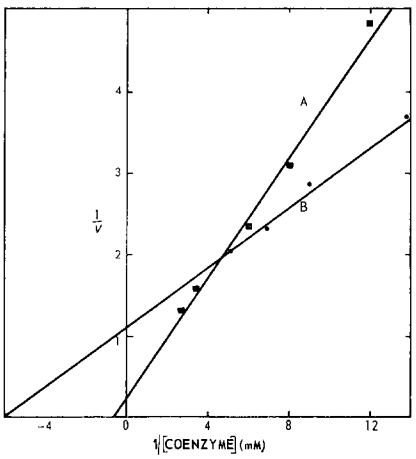


Fig. 4. Molecular weight determination of NAD-specific glutamate dehydrogenase by ultracentrifugation in sucrose density gradient. Crystalline alcohol dehydrogenase as reference protein.

Fig. 5. Double reciprocal plot of the rate of oxidative deamination of L-glutamate as a function of (A) deamino-NAD and (B) NAD by NAD-glutamate dehydrogenase.

Ammonia analogues

None of the following compounds could be substituted for ammonia in the reductive amination assay: Methylamine (0.1 M), dimethylamine (0.1 M), ethylamine (0.1 M) and urea ($2 \cdot 10^{-3}$ M).

Substrate specificity

In view of the report that beef-liver glutamate dehydrogenase exhibits multiple substrate specificity[24], various amino and β-keto acids were tested as substrates for the NAD-specific glutamate dehydrogenase. Assays were conducted at pH's 7, 8, 9 and 10 at a concn. of $6.6 \cdot 10^{-3}$ M using an enzyme concentration twice that used in normal assays. The amino acids, L-alanine, L-leucine, L-valine, L-isoleucine, L-aspartate or their keto acid analogues were found to be inactive as substrates.

Effect of inhibitors

Of a number of metal chelating agents, substrate analogues and other compounds tested as inhibitors, only *p*-chloromercuribenzoate (PCMB) (10^{-3} M), EDTA (10^{-3} M) and glutaric acid ($3 \cdot 10^{-2}$ M) inhibited the enzyme activity significantly. The nucleotides, GTP, GMP and IMP at a concn. of 10^{-3} M inhibited[25] the activity 100%.

Equilibrium constant

The $K_{eq.}$ of the reaction

$$K_{eq.} = \frac{[NADH]\,[NH_4^+]\,[\alpha\text{-ketoglutarate}]\,[H^+]}{[\text{glutamate}]\,[NAD^+]}$$

uncorrected for the ionization of the reaction at pH 9.5 was found to be $5.2 \cdot 10^{-12}$.

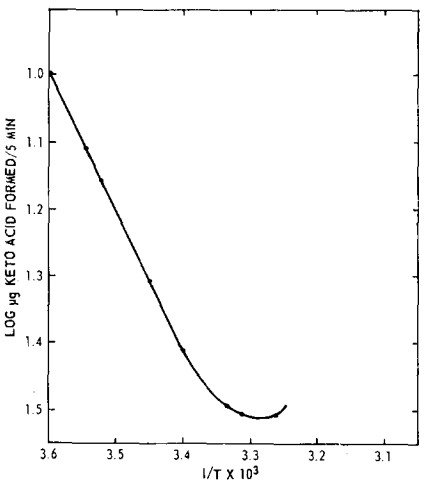
Energy of activation

The effect of temperature on the velocity of the oxidative deamination of L-

glutamate is shown in Fig. 6 in the form of an Arrhenius plot. The energy of activation for NAD-specific glutamate dehydrogenase was calculated to be 11 835 calories/mole.

Binding of coenzymes to the enzyme

The enzyme, when excited at a wavelength of 290 mμ, yields a fluorescence emission band at 350 mμ and this emission is quenched by NAD and NADH, but not by glutamate. When a fixed amount of NADH was fluorimetrically titrated by increasing amounts of NAD-specific glutamate dehydrogenase, a characteristic shift



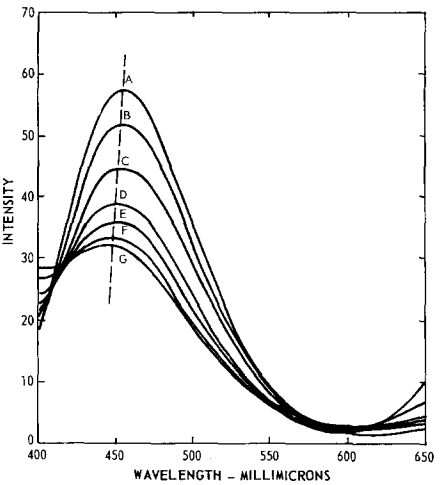


Fig. 6. Arrhenius' plot of NAD-specific glutamate dehydrogenase activity of *N. crassa*.

Fig. 7. Fluorescence tritration of NADH in the presence of increasing amounts of NAD-specific glutamate dehydrogenase. Reduced nucleotide was excited at 350 mμ, (emission at 460 mμ). Purified NAD-specific glutamate dehydrogenase concentrations were: (A) none; (B) $0.61 \cdot 10^{-6}$ M; (C) $1.18 \cdot 10^{-6}$ M; (D) $1.72 \cdot 10^{-6}$ M; (E) $2.22 \cdot 10^{-6}$ M; (F) $2.66 \cdot 10^{-6}$ M; (G) $3.13 \cdot 10^{-6}$ M. Vertical dashed line illustrates characteristic shift in emission peak.

in the emission peak of NADH occurred at 460 mμ (excitation at 350 mμ) which is shown in Fig. 7. The dissociation constant for NAD was calculated from this experiment to be 0.167 mM.

Comparison of purified constitutive and induced NAD-specific glutamate dehydrogenase

Before a mechanism for the concurrent regulation of NADP- and NAD-specific glutamate dehydrogenase (*cf.* INTRODUCTION) can be postulated, it must be investigated whether the NAD-specific enzyme induced in the cells is the same as that present in basal (or 'constitutive') levels[7,8], under uninduced conditions. In view of the findings of DUERKSEN AND FLEMING[26] in the case of the β-glucosidases in yeast, and the presence of isozymes demonstrated earlier it may be that there are two types of NAD-specific glutamate dehydrogenases.

Immunological comparison

Precipitation reaction was carried out at 6° for 18 h in 0.15 M NaCl containing

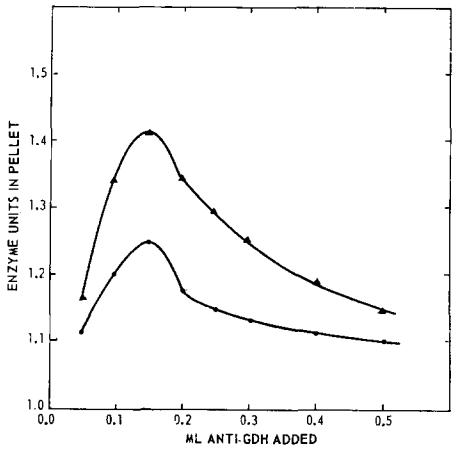


Fig. 8. Immunological comparison of precipitation pattern of induced and constitutive NAD-specific glutamate dehydrogenase (see text for procedure). ▲—▲, induced NAD-glutamate dehydrogenase; ●—●, constitutive NAD-glutamate dehydrogenase.

$5 \cdot 10^{-3}$ M GSH in a final vol. of 1.5 ml. Non-immune serum was used as a control. The enzyme–antibody complexes were found to be completely active. The overall precipitation patterns of both 'constitutive' (purified as described before) and induced glutamate dehydrogenases (Fig. 8) were similar. Such results are expected in cases where two protein molecules are antigenically related. The antibody did not show any cross reaction with the NADP-specific glutamate dehydrogenase.

Kinetic comparison

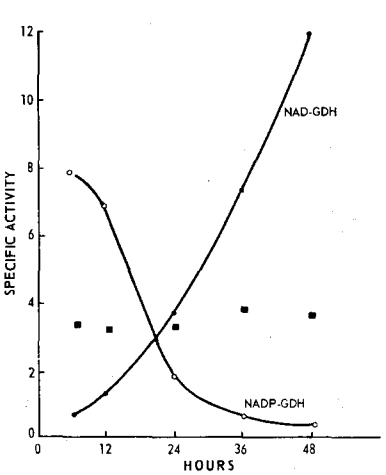
The Michaelis constants of the purified 'constitutive' and induced NAD-specific glutamate dehydrogenases were calculated graphically from double reciprocal plots by varying one substrate in the presence of constant amounts of the other substrates. The non-varied substrates were not completely saturating but the constants were calculated for both enzyme preparations under identical conditions. The results are presented in Table II. No significant differences in the kinetic parameters could be found for the 'constitutive' and induced enzymes.

TABLE II

COMPARISON OF SOME KINETIC CONSTANTS OF INDUCED AND CONSTITUTIVE NAD-GLUTAMATE DEHYDROGENASE OF *Neurospora crassa*

Substrate	K_m *values (moles/l)**		
	Wild type (non-induced)	*am⁻(525) (induced)*	*am⁻i⁻(1499-12) (induced)*
L-Glutamate	$5.5 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$	$3.8 \cdot 10^{-3}$
NAD	$3.3 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$	$2.3 \cdot 10^{-4}$
α-Ketoglutarate	$4.6 \cdot 10^{-3}$	$3.0 \cdot 10^{-3}$	$4.8 \cdot 10^{-3}$
$(NH_4)_2SO_4$	$17.0 \cdot 10^{-3}$	$8.3 \cdot 10^{-3}$	$5.2 \cdot 10^{-3}$
NADH	$5.5 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	$6.6 \cdot 10^{-4}$

* The values presented here are an average of three separate determinations.



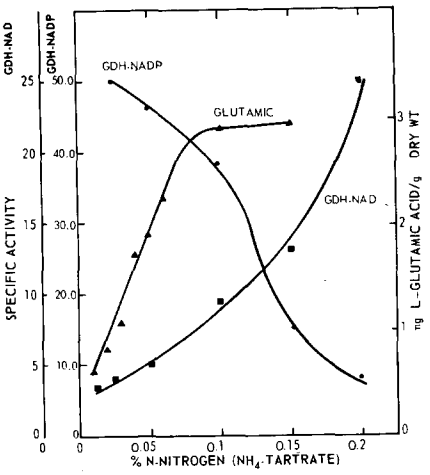


Fig. 9. Effect of urea on the induction and repression of both glutamate dehydrogenases (GDH) of *N. crassa*. Wild-type strain (262) was grown in Vogels' minimal medium N-supplemented with 0.062 M urea (sterilized by Millipore filtration). Solid squares represent urease specific activity.

Fig. 10. Effect of increasing ammonia concentrations on the induction and repression of NAD-glutamate dehydrogenase (NAD-GDH) and NADP-glutamate dehydrogenase (NADP-GDH), respectively from *N. crassa* (STA4). Endogenous L-glutamate concentrations were determined manometrically with commercial glutamate decarboxylase (Sigma Chemical Co., U.S.A.). L-Glutamate was extracted from mycelia in boiling water and concentrated *in vacuo* prior to determination.

The regulation of NAD- and NADP-specific glutamate dehydrogenases by nitrogenous precursors of glutamate

In accord with our earlier findings[8], addition of urea to the growth medium of the wild-type strain (STA 4) brought about a concurrent regulation of the two dehydrogenases (Fig. 9). Under the same conditions, the specific activity of urease remained unchanged. This regulation could be due to a conversion of urea into other metabolites rather than due to urea itself. To investigate this possibility the effect of urea on the induction of the NAD-specific glutamate dehydrogenase in Am[3] and U[25] (a strain-lacking urease) was tested.

TABLE III

COMPARISON OF NAD-GLUTAMATE DEHYDROGENASE INDUCTION BY UREA (0.062 M) IN *am*$^-$ AND *urease*$^-$ STRAINS OF *Neurospora crassa*

Supplement	*NAD-glutamate dehydrogenase specific activity strain*	
	Urease$^-$	*Am*$^-$
None	6.0	7.3
Urea (0.062 M)	6.8	72.0
Methyl urea (0.05 M)	5.3	6.3
L-Alanine (0.05 M)	41.0	52.0
Ammonium succinate (0.05 M)	35.0	43.4

As shown in Table III, urea was indeed incapable of inducing the enzyme in U[25] which means that urea *per se* is not an inducer *in vivo*. Since urease splits urea into ammonia, it is clear that regulation of the enzymes could be either brought about by ammonia or a compound (such as glutamate) derived from it. Accordingly, the effect of increasing ammonia concentrations was tested on the regulation of NAD- and NADP-specific dehydrogenases in the wild-type strain. Fig. 10 presents the results of this investigation. The results clearly indicate an induction of NAD-specific glutamate dehydrogenase and a concurrent repression of the NADP-specific enzyme in the presence of increasing concentrations of ammonia. It is also clear from Fig. 10 that with increasing ammonia in the medium concentration *in vivo* of L-glutamate also increases, till it reaches a plateau at an external ammonia concentration equivalent to 0.1 g/100 ml. However, the most significant finding is that increments of ammonia beyond the level where glutamate concentration does not increase any further *in vivo*, lead to a further induction of NAD-specific enzyme. This may mean that ammonia rather than glutamate serves as a regulator of the two enzymes[27].

Effect of actinomycin D on the induction of NAD-specific glutamate dehydrogenase

Since ammonia possibly serves as an inducer, as well as a substrate for the enzyme, the possibility exists that it may complex within the 'nascent' enzyme and

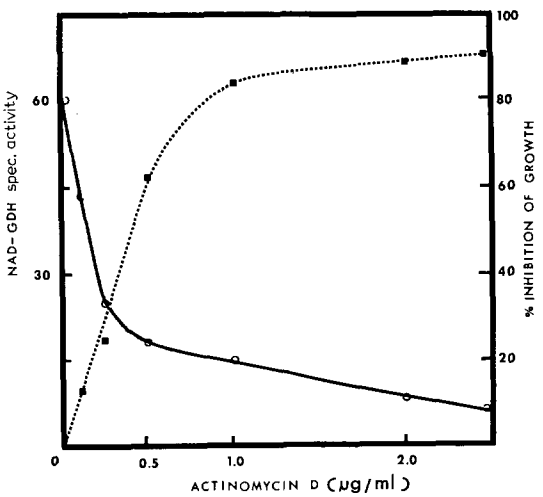


Fig. 11. Effect of various actinomycin-D concentrations on the induction of NAD-glutamate dehydrogenase (NAD-GDH) of *N. crassa* (STA4). Solid line denotes specific activity of NAD-glutamate dehydrogenase and the dotted line denotes percent inhibition of growth as measured by dry weight.

detach it from its site of synthesis in the cytoplasm. The possibility has, indeed, been suggested for the induction of alanine dehydrogenase of *Bacillus subtilis* by FREESE AND OSTERWYK[28]. Thus the decrease or increase in enzyme levels (such as shown in Fig. 10) could be due to a simple interaction of enzymes and inducers in the cytoplasm and not due to interactions at the genetic level.

It has been shown that actinomycin D selectively inhibits synthesis of messenger RNA from the DNA template[29]. If induction process occurs at the genetic level, the antibiotic should inhibit this induction. To test this possibility, conidia from a 5-day-old culture of wild-type strain were inoculated into 25 ml of minimal medium supplemented with 0.05 M urea and various concentrations of actinomycin D. The flasks were aerated at 28° for 36 h. It can be seen from Fig. 11 that 0.25 μg/ml of actinomycin D retarded enzyme induction by 58% whereas growth only decreased by 25%.

Possible absence of interconversion of NADP-specific and NAD-specific glutamate dehydrogenases

It will be noted from Figs. 9 and 10 that exogenously supplied metabolites cause a derepression of NAD-specific enzyme and a corresponding repression of the NADP-specific dehydrogenase. The possibility arises that, in the presence of inducer, the NADP-specific glutamate dehydrogenase is converted or modified to NAD-specific dehydrogenase during growth. To test this hypothesis an experiment was designed in which the NADP-specific enzyme was labelled by exposing the culture to [1-^{14}C]leucine and then transferred to a label-free medium containing inducer (for detail see METHODS). If conversion of one enzyme to another occurred, the label incorporated in the NAD-specific enzyme should be equal to that in the NADP-specific

TABLE IV

CONVERSION OF NADP-SPECIFIC GLUTAMATE DEHYDROGENASE DURING INDUCTION

Samples were counted for 100 min with a Nuclear Chicago scintillation counter. Counts reported are corrected for background. For details of procedure see text.

Enzyme	*Incorporation of (1-^{14}C)leucine into enzyme (disint./mg protein)*
NADP-glutamate dehydrogenase	595
NAD-glutamate dehydrogenase	293

enzyme. The incorporation of radioactivity into NAD-specific glutamate dehydrogenase was less than 50% of that of the NADP-specific enzyme (Table IV). This indicates that interconversion is unlikely, although the experiment does not completely exclude the possibility.

DISCUSSION

The results presented here indicate it is likely that the actual effector *in vivo* for the concurrent regulation of the NAD- and NADP-specific glutamate dehydrogenases is ammonia. These observations are in accord with the results obtained by BARRATT[27] who demonstrated that ammonia represses the NADP-specific enzyme in nitrogen-starved mycelia.

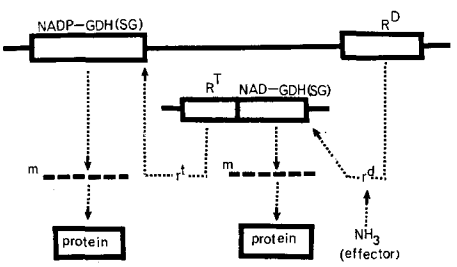
The effect of actinomycin D on the derepression of NAD-specific glutamate dehydrogenase coupled with the earlier evidence that serological cross-reacting

material related to the NADP-dependent dehydrogenase in mutants (Am) lacking this enzyme[9] is regulated in the same manner as the active enzyme indicates that regulation of these enzymes is independent of cytoplasmic events, and must occur at the primary template level. That there is very little structural similarity between the two dehydrogenases, is demonstrated by the fact that antibody against the NAD-specific dehydrogenase does not cross react with the NADP-specific enzyme, and *vice versa*[9]. Further, the structure of the two enzymes is determined by two completely unlinked structural genes (AHMED AND B. D. SANWAL, unpublished observations).

It has already been mentioned elsewhere[7-9] that the two glutamate dehydrogenases show a pattern of regulation which is unusual. Under all environmental conditions tested[7-9], derepression of the NAD-specific glutamate dehydrogenase leads invariably to a corresponding repression of the NADP-specific enzyme. A better known case of this kind is the regulation of hemoglobin chains in humans during foetal development. It is well established that HbF is composed of equal numbers of α and γ polypeptide chains. During development this is gradually replaced by HbA which is constituted of α and β chains. As in Neurospora system, the related peptides, β, α, γ, show a 'compensatory' increase in one type of hemoglobin (HbA) when the rate of synthesis of the other type falls (HbF). One explanation that can be given for this inverse concurrent regulation is that perhaps one chain is being converted into another, or in the case of Neurospora, one enzyme is being converted into another. This possibility can be ruled out for both hemoglobin and glutamate dehydrogenases. In hemoglobin the amino acid composition is sufficiently different to rule out this presumed interconversion. Also in *N. crassa* results of tracer experiments although admittedly not conclusive, lead to the inference that there is no interconversion of the two glutamate dehydrogenases.

Since the results indicate that under derepressed conditions Neurospora contains isozymes of NAD-specific glutamate dehydrogenase, a comparison of the uninduced and induced enzymes was undertaken to see whether both enzymes were similar. Comparison of kinetic parameters, heat sensitivity and antigenic relationships of the NAD-dependent glutamate dehydrogenases show that uninduced and induced enzymes are identical.

In the present state of our knowledge regarding the concurrent regulation of the two glutamate dehydrogenases of Neurospora, it is difficult to postulate a genetic



SCHEME I

GDH, glutamate dehydrogenase; SG, structural gene; R, regulatory gene; r, repressor; m, messenger RNA; superscripts D,d, NAD-glutamate dehydrogenase; T, t, NADP-glutamate dehydrogenase.

mechanism of regulation. As a pure speculation it may, however, be proposed in analogy with the hemoglobin system that two or more regulatory genes are involved in this regulation. Thus, if it is postulated that there is a regulatory gene (R^T) for the NADP-specific enzyme and another (R^D) for the NAD-specific dehydrogenase, and further that the R^T gene and the structural gene for the NAD-specific enzyme form an operon[30] the mechanism of concurrent regulation can be easily explained (Scheme I).

In accordance with this model when ammonia levels are too low maximum amount of NADP-specific enzyme and very low amounts of NAD-specific enzyme would be produced. High concentrations of ammonia would inactivate repressor r^d so that NAD-specific enzyme and repressor r^t would consequently be produced. The latter would repress the NADP-specific dehydrogenase.

Alternatively, concurrent regulation could also be brought about by the interaction of three regulatory genes acting in a 'cascade' manner as suggested by PONTECORVO[31]. The only virtue of these models is that they predict certain types of mutations and their phenotypes so that a diligent search for such mutants is possible.

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