Feedback Inhibition of Glutamine Synthetase of *Neurospora crassa* by Nicotinamide–Adenine Dinucleotide*

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ABSTRACT: Glutamine synthetase of *Neurospora crassa* is significantly different from that of *Escherichia coli*. Like the latter, it is subject to feedback inhibition by adenosine monophosphate, glycine, histidine, and cytidine triphosphate, but unlike the *E. coli* enzyme, it is strongly inhibited by guanosine triphosphate and nicotinamide–adenine dinucleotide. The aromatic amino acids, tryptophan, tyrosine, and phenylalanine, do not inhibit the *Neurospora* enzyme, but instead, anthranilic acid, the first compound unique to the tryptophan branch of the aromatic amino acid biosynthetic pathway, is an effective inhibitor. Inhibition by nicotinamide–adenine dinucleotide is apparently competitive toward glutamate, noncompetitive toward adenosine triphosphate and uncompetitive with regard to NH_2OH. Heat inactivation studies and response to pH show that the catalytic site (glutamate binding) and the regulatory site (nicotinamide–adenine dinucleotide binding) are distinct.

Glutamine synthetase (L-glutamate:ammonia ligase, EC 6.3.1.2) catalyzes the amidation of glutamate by the following synthetic reactions: (1) the synthesis of glutamine

$$\text{L-glutamate} + NH_3 + ATP^1 \rightleftharpoons \text{L-glutamine} + ADP + P_i$$

(2) the formation of α-glutamyl hydroxamate when NH_3 is replaced by NH_2OH

$$\text{L-glutamate} + NH_2OH + ATP \rightleftharpoons \gamma\text{-glutamyl hydroxamate} + ADP + P_i$$

the γ-glutamyl transfer from glutamine to hydroxylamine and the cyclization of glutamate to form pyrrolidone carboxylate (Meister, 1965).

Since glutamine serves as the donor of amide group in several reactions leading to the biosynthesis of such diverse compounds as carbamyl phosphate, histidine, tryptophan, glucosamine, purine, and pyrimidine nucleotides, glutamine synthetase is a crucial enzyme occupying the point of divergence of several metabolic pathways. Woolfolk and Stadtman (1964) investigated the effect of several metabolic end products on the activity of glutamine synthetase of *Escherichia coli*. According to their observations eight compounds, namely, tryptophan, AMP, CTP, glucosamine 6-phosphate, histidine, CP, alanine, and glycine, partially inhibited this enzyme when present alone and exerted a cumulative effect in combination. No compounds other than these eight were found to have any effect on this enzyme (Stadtman, 1966).

In the course of an investigation on some allosteric enzymes of *Neurospora crassa* the properties of its glutamine synthetase were studied and compared with those of the *E. coli* enzyme. As a result of this study a number of points emerged indicating a significant difference in the behavior of glutamine synthetase in these two organisms. Most noteworthy among these was the strong inhibition by ADP, one of the reaction products at high concentrations and feedback inhibition by nicotinamide–adenine dinucleotide and anthranilic acid. In this communication the effect of NAD is presented.

Materials and Methods

Growth of Cultures and Preparation of Enzymes. Throughout this study *N. crassa*, pe^m (FGSC No. 37), a predominantly microconidial strain, was employed as a source of glutamine synthetase. All cultures were prepared in Vogel's minimal medium (Vogel, 1956) with 2% sucrose as the carbon source. Conidial suspensions were prepared from inocula grown for 6 days on semisolid minimal medium at 28°. A conidial suspension (5 ml) in sterile, distilled water containing approximately 5×10^7 conidia/ml was transferred to 2800-ml capacity Fernbach flasks containing 1500 ml of liquid medium. The cultures were grown in an environmental rotatory shaker (New Brunswick) at 28° for 24 hr; the mycelia were then harvested, lyophilized, and stored at −20°. Under these conditions the mycelial powder retains activity for almost 2–3 months.

Preparation of the Enzyme. The lyophilized mycelium powder (25 g) of *Neurospora* was extracted with 0.05 M phosphate buffer (5×10^{-4} M in EDTA and 10^{-4} M in β-mercaptoethanol) (pH 7.5) for 30 min. The resulting mixture was strained through four layers of cheesecloth and the supernatant (approximately 400 ml) was centrifuged at 27,000*g* for 15 min in a refrigerated Sor-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CP, carbamyl phosphate; NaMN, nicotinic acid mononucleotide; PEP, phosphoenolpyruvate; PK–LDH, pyruvate kinate–lactate dehydrogenase mixture.

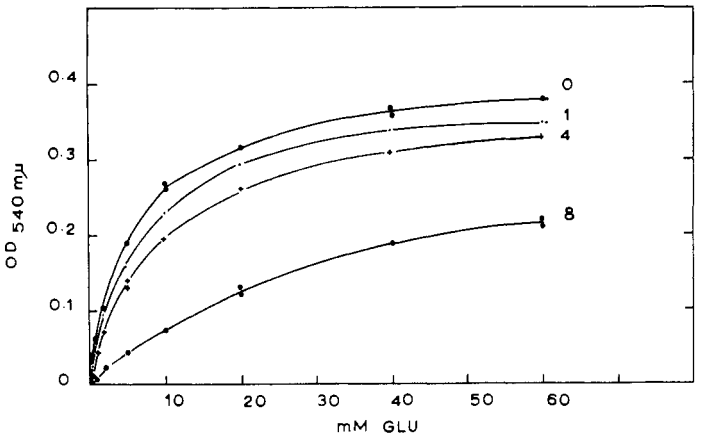

FIGURE 1: Synthesis of γ-glutamyl hydroxamate as a function of glutamate concentration. Reaction velocity is given in optical density units. All points are experimental. The numbers on the right represent millimolar NAD; the top curve contains no additions, the others represent reaction mixtures with 1, 4, or 8 mM NAD.

vall RC-2 centrifuge. Unless otherwise indicated, all operations concerned with the extraction and purification of the enzyme were conducted at 4° in a cold room. To the supernatant obtained from the above-mentioned step an equal volume of a saturated solution of ammonium sulfate (containing 10^{-3} M glutamate) was added; the mixture was stirred in the cold room for 15 min following which it was allowed to stand undisturbed for another 15 min. The precipitate formed was collected by centrifugation at 27,000*g* for 10 min and the protein was dissolved in a small volume of the original buffer. The protein solution was diluted to a concentration of 3–4 mg of protein/ml and, to 480 ml, 14.4 g of alumina Cγ gel (8.5% solid content) was added and the mixture was allowed to stir in the cold room for 30 min. The gel was removed by centrifugation at 8000 rpm for 10 min and to the supernatant another 15 g of the gel was added. After treatment for 40 min the gel was removed from this mixture by centrifugation at 8000 rpm. Most of the enzyme activity was found adsorbed to the second gel. The enzyme was eluted from the gel by treatment with 0.05 M phosphate buffer (5×10^{-4} M EDTA and 10^{-4} M β-mercaptoethanol) (pH 7.5). Three successive elutions were performed using 50 ml of the buffer at each treatment for 20 min. The second eluate contained a large fraction of the activity and showed a 20-fold increase of specific activity over the crude extract. This 20-fold-purified enzyme preparation was used as such in subsequent studies or lyophilized immediately and stored in the powder form. Before use it was dissolved in a small quantity of water and passed through a column of Sephadex G-25 coarse (0.8×35 cm) which had been equilibrated with phosphate buffer (pH 7.5) to reduce the ionic strength to the level of the original buffer. This preparation was diluted before use in enzyme assays.

Enzyme Assays. Glutamine synthetase activity was determined by following the formation of γ-glutamyl hydroxamate from L-glutamate and hydroxylamine in the presence of ATP. The reaction mixture consisting of the following: Tris buffer (pH 7.5), 40 mM; L-glutamate, 20 mM or as indicated; ATP, 3 mM or as indicated; $MgCl_2$, 8 mM; neutralized NH_2OH (freshly prepared), 40 mM; β-mercaptoethanol, 0.1 mM; and 100 μg of enzyme protein in a total volume of 4 ml was incubated at 30° for 20 min. At the end of the incubation period γ-glutamyl hydroxamate formed was determined by adding 1 ml of ferric chloride reagent (Lipmann and Tuttle, 1945) and measuring absorbance of the reaction mixture at 540 mμ in a Spectronic 20 photoelectric colorimeter. For comparison, reference was made to a standard prepared from commercial γ-glutamyl hydroxamate. Under the conditions of assay the reaction was proportional to enzyme concentration of up to 120 μg of protein and the reaction rate was linear up to 20 min.

Protein Determinations. All protein determinations were made by the method of Lowry *et al.* (1951) using serum albumin as a standard.

Reagents. All reagents were commercially obtained and were used without further purification. ATP, ADP, AMP, GTP, and CTP, NAD, and alumina C-γ were obtained from Sigma.

TABLE I: Some Feedback Inhibitors of Glutamine Synthetase of *Neurospora:* Amino Acids and Related Compounds.

Compound	Concn (mM)	Glu (mM)	% Inhibition pH 7.5	% Inhibition pH 8.5
Glucosamine	2	20	6	
	5	20	15	
	2	50		7
	5	50		15
Glycine	20	50	7	
	50	50	13	
Alanine	20	50	0	0
	50	50	0	0
Histidine	20	50	20	7
	50	50	88	
Tryptophan	2	50	0	0
	4	50	0	0
Phenylalanine	2 and 5	50	0	0
Tyrosine	2 and 5	50	0	0
Anthranilic acid	5	20	12	
	10	20	41	
	5	50	3	
	10	50	27	

Results

In an initial experiment the degree of inhibition caused by the eight feedback inhibitors of *E. coli* glutamine synthetase was determined, using the 20-fold-purified *Neurospora* enzyme. Some of these inhibitors

TABLE II: Feedback Inhibition of Glutamine Synthetase by Purine and Pyrimidine Nucleotides and Related Compounds.[a]

Compound	Concn (mM)	Glu (mM)	% Inhibition
None		50 or 20	
ADP	2	50	64
ADP	5	50	96
AMP	2	20	13
AMP	5	20	25
GTP	2	50	9
GTP	5	50	31
CTP	2	50	29
CTP	5	50	64
CP	2	20	5
CP	5	20	17
NAD	2	20	6
NAD	5	20	22
NAD	2	50	6
NAD	5	50	18

[a] The above-mentioned compounds were tested at pH 7.5 in the presence of 16 mM (saturating) $MgCl_2$, the remaining components of the assay system being as specified in Methods.

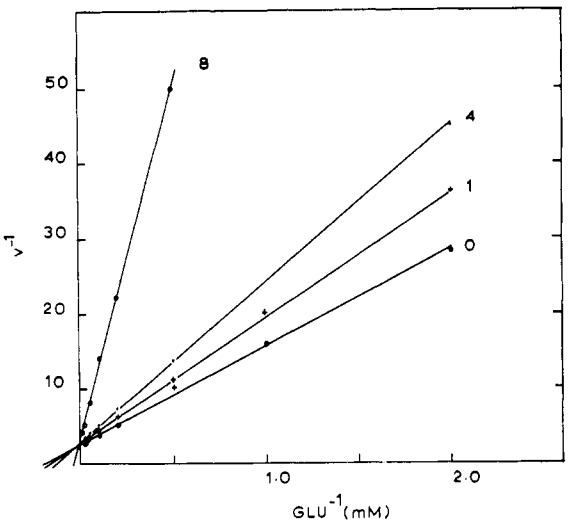

FIGURE 2: A double-reciprocal plot of reaction velocity *vs.* glutamate concentration in the presence of fixed concentrations of NAD.

have been tested with crude extracts of *N. crassa* (Hubbard and Stadtman, 1967). Appropriate quantities of each inhibitor (Tables I and II) were included in a standard assay system containing 20 or 50 mM glutamate and the reaction was performed at conditions specified under Methods, along with substrate and inhibitor controls. Normal enzyme studies were carried out at pH 7.5 but in many cases a preliminary check was also made at pH 8.5. In the case of purine and pyrimidine nucleotides, $MgCl_2$ concentration was increased to 16 mM (saturating).

That the *Neurospora* enzyme is subject to feedback inhibition by glycine, whereas alanine has no effect, is shown in Table I. However, even at concentrations of 50 mM glycine, inhibition of only 13% is achieved. Since the *E. coli* enzyme is inhibited both by glycine and alanine (Stadtman, 1966), it can be concluded that the degree of contribution of glutamine toward the biosynthesis of alanine is not significant in *Neurospora*. The aromatic amino acids, tryptophan, tyrosine, and phenylalanine, are not inhibitory either alone or in various combinations. This observation is again in contradiction to the corresponding situation in *E. coli*. Although tryptophan is completely ineffective, anthranilic acid, the first compound unique to the tryptophan biosynthetic branch of the aromatic pathway, is a significant inhibitor of *Neurospora* glutamine synthetase. Unlike the situation in *E. coli*, glucosamine causes an inhibition of 15% at 5 mM. Glucosamine 6-phosphate was not tested.

A summary of the effect of purine nucleotides, AMP, ADP, and GTP, and the pyrimidine nucleotide, CTP, is given in Table II. GTP, though not an inhibitor of the *E. coli* enzyme proved to be an effective inhibitor of the *Neurospora* enzyme. At 5 mM it causes an inhibition of 31% at pH 7.5. A low level of inhibition by AMP is observed at both the pH levels tested, but ADP is a very strong inhibitor. At pH 7.5 it is inhibitory to an extent of 96%. As ADP is one of the products of the reaction such a high level of inhibition is significant. Inhibition by AMP and CP has been reported for the *E. coli* enzyme (Woolfolk and Stadtman, 1964; Kingdon and Stadtman, 1967; Hubbard and Stadtman, 1967).

We observed significant inhibition by ATP, a substrate, at high concentrations (above 4 mM) but since our enzyme preparation was only partially pure it is possible that contamination with ATPase led to the formation of excessive amounts of ADP which was indirectly responsible for inhibition. This point is being investigated currently. Another significant difference between the *E. coli* and the *Neurospora* glutamine synthetase is the inhibition of the latter by NAD. In view of the considerable inhibition of γ-glutamyl transfer catalyzed by glutamine synthetase of *Neurospora*, a detailed study of the NAD effect was undertaken.

The effect of NAD was first analyzed in relation to the interaction of glutamate with glutamine synthetase. Figure 1 presents the rate of reaction as a function of varying concentrations of glutamate in the presence and absence of NAD. The curves in both the instances are rectangular hyperbolas and even at 8 mM NAD there is no noticeable tendency toward sigmoidicity. Double-reciprocal plots of reaction rate *vs.* glutamate concentration with and without NAD show a set of straight lines intersecting at a common point on the ordinate, indicating an inhibition of the competitive type (Figure 2). NAD increases the apparent K_m of glutamate (app 5.5 mM) thus appearing to affect its binding to the enzyme. However, NAD does not induce any cooperative effects.

Figure 3 shows the effect of increasing concentrations

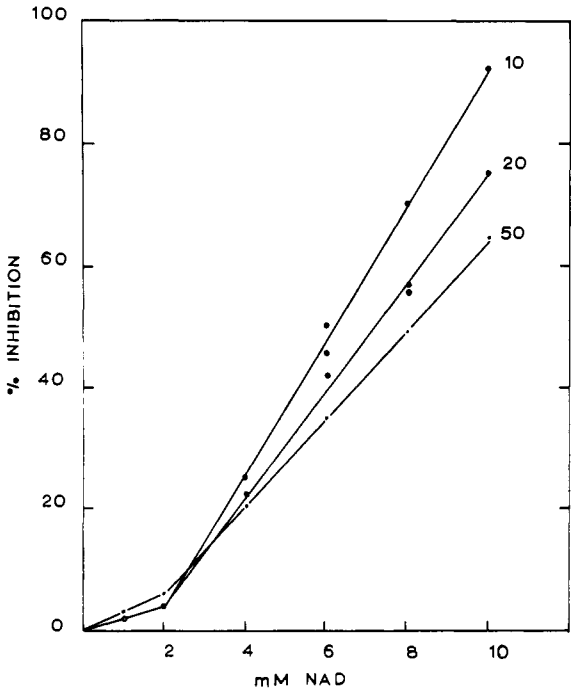

FIGURE 3: The effect of varying concentrations of NAD on the inhibition of γ-glutamyl hydroxamate formation. The numbers on the right next to the curves represent glutamate concentrations of glutamate in millimolar.

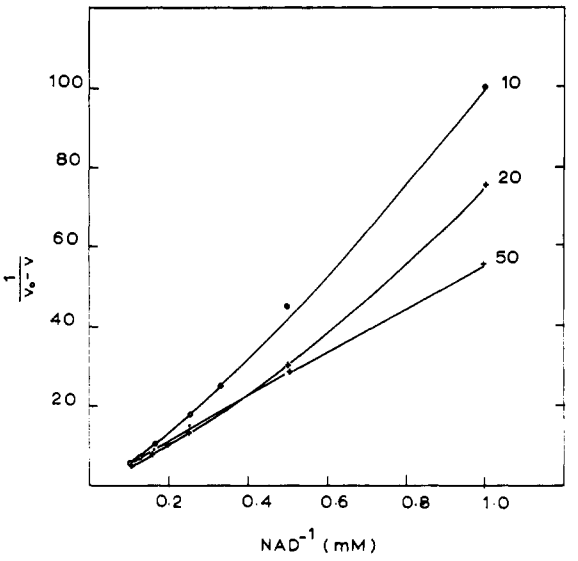

FIGURE 4: Double-reciprocal plots of the rate of reaction *vs.* NAD concentration; v_0 and v represent reaction rates in the absence and presence of NAD, respectively. The numbers on the right next to the curves represent concentrations of glutamate in millimolar.

of NAD on the activity of the enzyme. As is evident, 10 mM NAD causes an inhibition of up to 92% when glutamate is at 10 mM. Raising the concentration of glutamate, even to 50 mM, does not result in a significant diminishing of inhibition. At lower concentrations NAD inhibition is low but at concentrations higher than 2 mM it registers a steeper increase which is linear from 2 to 10 mM. This suggests a cooperative effect in the binding of NAD. A double-reciprocal plot of $v_0 - v$ *vs.* NAD concentration shows a set of curves which concave upwards in the region of high concentrations. The tendency toward upward curvature is evident at all concentrations of glutamate although at saturating levels (50 mM) the curvature is much less pronounced (Figure 4) and the graph appears almost linear. Similar curves obtained at saturating glutamate concentration in the presence of two fixed concentrations (20 and 30 mM) of hydroxylamine also show an upward curvature (Figure 5) showing thereby cooperative interactions with respect to the binding of NAD.

Double-reciprocal plots of reaction velocity *vs.* ATP and NH_2OH (not shown) in the presence and absence of NAD suggest that NAD inhibition is noncompetitive with regard to ATP and uncompetitive with relation to NH_2OH.

As NAD is a feedback inhibitor and bears no obvious structural resemblance to glutamate, the apparent competitive inhibition is less clear. However, studies with certain other allosteric enzymes have also shown that on kinetic analysis such inhibitions may appear to be competitive (Caskey *et al.*, 1964; Rothman and Cabib, 1967). Kinetic studies, therefore, may not yield adequate information regarding the true nature of the interaction.

Question arose as to whether the inhibition exerted by NAD was, in fact, indirectly caused by ADP which might be produced from the breakdown of NAD. NADase (DPNase), an enzyme catalyzing the cleavage of NAD into nicotinamide and ADP–ribose moiety, has been demonstrated in *Neurospora* (Nason *et al.*, 1951; Kaplan, 1955) cultures grown on zinc-deficient media. It is conceivable that ADP produced by the action of enzymes on ADP–ribose may indirectly cause the inhibition of glutamine synthetase. To test this possibility, we incubated enzyme preparations normally used in glutamine synthetase assays with 1–5 mM of NAD and followed the NAD cleavage by means of the cyanide reaction (Colowick *et al.*, 1951). Since the cyanide reaction is specific for the presence of intact *N*-ribosyl bond and is not given by free nicotinamide or by reduced NAD or reduced NADP (Kaplan, 1955), this was considered a suitable method for estimating the extent of NAD cleavage in our preparations. Under these conditions of assay and with the 20-fold-purified enzyme preparation that was used in this study, no NAD cleavage was evident by the cyanide reaction.

To ascertain if there was any ADP formed in the system in the presence of NAD, the enzyme preparations were incubated with NAD under the conditions employed for glutamine synthetase assay and the system was coupled with a pyruvate kinase–lactate dehydrogenase reaction. For ADP determinations the following reaction mixture in a glass cuvet of 1-cm light path (final volume 3 ml) was prepared: Tris-HCl buffer (pH 8.0), 100 μmoles; $MgCl_2$, 20 μmoles; PK–LDH (Sigma), 0.1 mg; PEP, 1.0 μmole; and reduced NAD, 0.15 μmole. An aliquot of glutamine synthetase preparation which had been incubated with NAD (5 mM) was used instead of ADP in the system. The rate of reaction was followed by the decrease in absorbance at 340 mμ accompanying the disappearance of reduced NAD by means of a Beckman

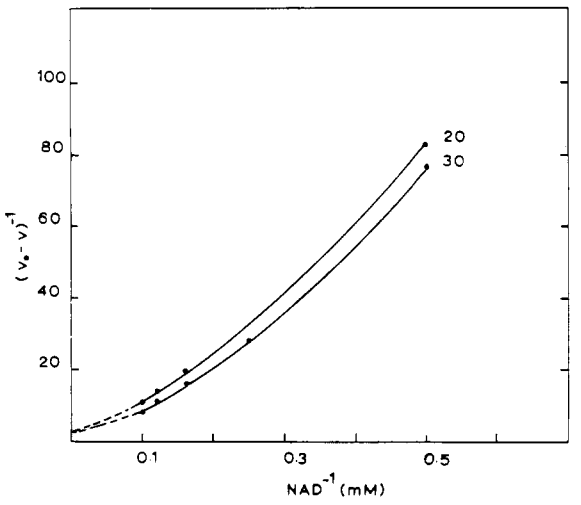

FIGURE 5: A double-reciprocal plot of reaction rate *vs.* NAD concentration in the presence of 20 and 30 mM NH_2OH; v_0 and v are reaction rates in the absence and presence of NAD.

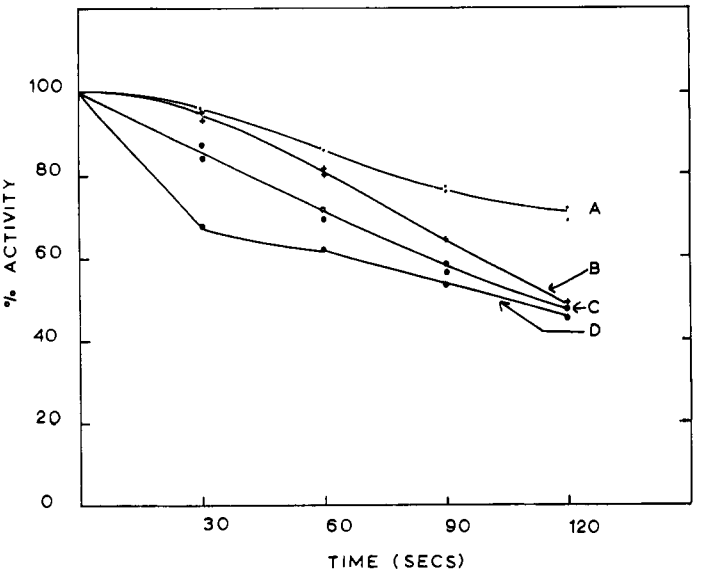

FIGURE 6: Heat inactivation of glutamine synthetase. An inactivation mixture (2 ml) containing a constant final amount of enzyme in phosphate buffer at pH 7.5 was incubated in a water bath heated to 50°. Aliquots were withdrawn at appropriate intervals, chilled rapidly, centrifuged to remove all precipitated protein and the supernatant was assayed for glutamyl transfer activity with a standard assay mixture. Per cent activity remaining after heat inactivation is plotted against treatment time at 50° in seconds. Curve B: no additions; enzyme in buffer (50 mM) (inactivation mixture). Curve A: inactivation mixture with 120 mM glutamate and 20 mM Mg^{2+}. Curve C: inactivation mixture with 120 mM glutamate, 20 mM Mg^{2+}, and 7.5 mM NAD. Curve D: Inactivation mixture and 7.5 mM NAD.

DU monochromator connected to a Gilford Model 2000 recorder. No ADP could be detected in the alumina C-γ gel eluate, the lyophilized, and Sephadex-treated gel eluate. Since the enzyme preparations used in the course of this study did not show any NAD cleavage or the presence of ADP resulting from a breakdown of NAD, the inhibition of the γ-glutamyl transferase activity is attributed to NAD.

Heat inactivation of glutamine synthetase was studied by determining the amount of activity remaining after exposure to 50° for intervals of up to 120 sec. The conditions of heat treatment are given in the legend to Figure 6. Glutamate and Mg^{2+} when present together diminish inactivation (curve A) but NAD enhances inactivation (curve D). These observations are consistent with the interpretation that NAD and glutamate could not be binding to the same site on the enzyme and in the same manner.

A comparison of the pH optimum of the catalytic reaction and that of NAD inhibition further supports the conclusion that the substrate binding site may be different from the regulatory or the NAD binding site. With phosphate buffer the catalytic activity shows a broad range of pH from 6.5 to 8.5 within which catalytic activity does not differ remarkably from one point to another. It does appear to show an inconspicuous peak around 7.5 and sometimes another one in the vicinity of pH 8.5. As compared with this, if NAD inhibition is measured in the same pH range, a well-defined maximum is observed at 6.5 beyond which percentage inhibition registers a steady decline and comes to a minimum at 8.5. Using *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, the γ-glutamyl transferase activity exhibits a clear pH optimum at 7.75. In the same pH range, the NAD inhibition is seen to diminish steadily with increasing pH being less than 10% at the pH optimum for the catalytic activity (Figure 7). With 2-amino-2-methyl-1,3-propanediol buffer the pH optimum of the catalytic reaction is shifted close to 9.0, whereas the maximum value for NAD inhibition is realized at 8.5. At pH higher than 8.5, susceptibility to NAD inhibition declines sharply till there is no inhibition at pH 9.2 (Figure 8). At 9.5 the catalytic activity is approximately 75% that at the optimum pH but instead of being inhibitory, NAD leads to an increase of 9.0% in activity over that shown by the controls. This pH-induced variation in the effect of NAD, evident by inhibition and activation below and above 9.2, is indicative of a conformational change in the protein reflecting an alteration in the capacity to bind NAD. At the point of transition (pH 9.2), the catalytic activity is still as much as 87% that at the optimum pH.

Ageing of the enzyme preparations results in a gradual loss of catalytic activity over a given period. However, the loss in NAD inhibitability occurs more rapidly. Furthermore, susceptibility to inhibition by NAD seems to vary somewhat in different batches of enzyme prepared at different times from the same stock of mycelium powder and frequently with enzyme extracted from different lots of mycelia. Similar results could also be obtained if the enzyme preparation contained two enzyme forms with different pH optima. However, this explanation seems to be very unlikely in view of the fact that column chromatography on DEAE-cellulose and DEAE-Sephadex and sedimentation on sucrose density gradients show the enzyme emerging as a single peak. Although these observations suggest that probably there are distinct catalytic and regulatory sites for glutamate and

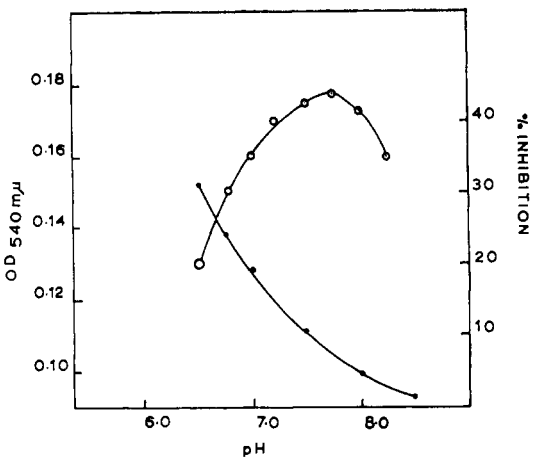

FIGURE 7: Effect of pH on the γ-glutamyl transferase activity (○———○) and NAD inhibition (●———●) with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer. The reaction mixtures contained 50 mM glutamate and 3 mM NAD; the buffer being 40 mM.

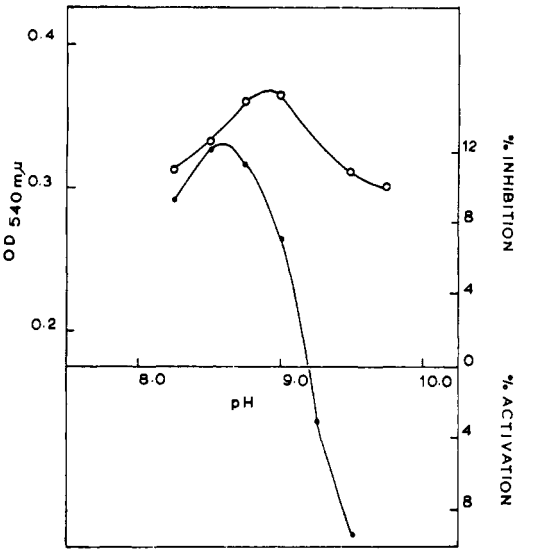

FIGURE 8: Effect of pH on the γ-glutamyl reaction (○———○) and the influence of NAD (●———●). Buffer 2-amino-2-methyl-1,3-propanediol, 40 mM; NAD, 3 mM; and glutamate, 50 mM.

NAD, convincing evidence for the validity of such a conclusion can only be obtained by direct binding studies. Investigations along these lines are in progress.

Discussion

The differences between the glutamine synthetase of *E. coli* and *Neurospora* are evident upon consideration of the feedback inhibition elicited by the compounds known to inhibit the *E. coli* enzyme. The regulation of the activity of glutamine synthetase of *Neurospora* appears to be more complex and is effected by some end products of the purine nucleotide biosynthesis pathway, namely, AMP and GTP. Since glutamine is the amide donor of the nitrogen atoms 3 and 9 of the purine ring (Buchanan, 1960) common to both AMP and GMP and in addition of the 6-amino group of GMP (Magasanik, 1962), feedback inhibition in *Neurospora* by GTP offers another fine control point. The extent of inhibition by GTP is much greater than that by AMP. In *E. coli*, too, AMP by itself is only slightly inhibitory but cumulative inhibition exerted by more than one inhibitor in combination results in a pronounced over-all effect. It is possible that the *Neurospora* enzyme, too, is inhibited in a cumulative manner by compounds (like AMP and glycine) which alone do not produce a marked effect.

The lack of feedback inhibition by the aromatic amino acid tryptophan is compensated for by the effectiveness of anthranilic acid. Anthranilic acid is the first compound unique to the tryptophan biosynthetic branch of the aromatic amino acid pathway. The amino group of anthranilic acid is derived from glutamine (Baker and Crawford, 1966), therefore, anthranilate can logically be regarded as the end product of a metabolic branch the first enzyme of which is glutamine synthetase.

Stadtman (1966) has predicted that compounds derived from glutamine or their immediate products would be expected to act as feedback inhibitors of glutamine synthetase. In microorganisms the biosynthesis of nicotinamide–adenine dinucleotide proceeds through the nicotinate pathway or the nicotinamide pathway (Dahmen *et al.*, 1967). Glutamine participates in the nicotinate pathway where deamino-NAD is converted in the terminal step into NAD, subsequent to amidation. NAD should, therefore, not be involved with the regulation of glutamine synthetase of those organisms which use the nicotinamide pathway for the synthesis of NAD. This point should be elucidated with *Lactobacillus fructosus* which is known to utilize the nicotinamide pathway (Ohtsu *et al.*, 1967).

Quinolinate is another precursor of NAD found in animal systems. According to Nishizuka (1964) the enzyme catalyzing phosphoribosyl pyrophosphate dependent formation of NaMN from quinolinate is encountered commonly in nicotinate auxotrophs of *E. coli*. Starting with quinolinate, too, a terminal amidation of deamino-NAD is necessary to produce NAD. The NAD synthetase of *E. coli*, however, uses NH_3 predominantly, as a source of the amino group in the amidation reaction (Spencer and Preiss, 1967).

Evidence obtained from studies of heat inactivation, pH optima, and susceptibility of the aged enzyme toward NAD inhibition suggest that the catalytic site is distinct from the regulatory (NAD binding) site. In response to pH greater than the reaction optimum, there appears to be a conformational modification of the protein so that the catalytic site remains apparently unchanged while the binding of NAD is affected drastically, consequently NAD inhibitability is reduced markedly. This modification culminates in a state of the enzyme in which the activity of the catalytic site is enhanced by NAD at pH 9.5. The interpretation of this conformatory change must remain tentative in the absence of direct binding studies, the results of which are expected to provide more conclusive evidence of this. On the basis of kinetic and inactivation studies, Shapiro and Stadtman (1967) have postulated the existence of at least three, and possibly more, regulatory sites on the

glutamine synthetase of *E. coli*. It would be interesting to see if the *Neurospora* enzyme also possesses separate sites for each of the inhibitory compounds.

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