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INVESTIGATIONS OF THE PROPERTIES OF SOME ENZYMES IN *NOCARDIA CORALLINA* AND TOBACCO TISSUE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

The properties and metabolic regulations of some enzymes from Nocardia corallina (a bacterium) and tobacco tissue (a plant) were investigated.

Feedback inhibitions of glutamine synthetase from Nocardia corallina were studied with respect to the end products or compounds related to glutamine metabolism.

Among the eight compounds that are known to inhibit glutamine synthetase from E. coli and other sources, all but histidine showed definite inhibition of the enzyme from N. corallina.

GTP, NAD^+, $NADP^+$ and ADP were also found to demonstrate strong inhibitions. The results suggest that glutamine synthetase from Nocardia corallina differs significantly from that of other bacteria.

The properties of D-3-phosphoglycerate dehydrogenase from N. corallina were studied. The D-3-phosphoglycerate saturation function for the enzyme was non-sigmoidal and hyperbolic. The enzyme was not subject to feedback inhibition by the end product serine. Activity of D-glycerate dehydrogenase from the same organism was detected as very low or absent, whereas under the same condition great activity of D-3-phosphoglycerate dehydrogenase was observed, indicating that the phosphorylated pathway rather than the non-phosphorylated pathway is the major route of serine biosynthesis in this organism.

Glutamine synthetase from tobacco tissue was investigated for its regulatory properties. Thirteen metabolic compounds that may be related to glutamine metabolism were tested for their effect on the enzyme. AMP and L-tryptophan did not display any effect on the enzyme. The strongest inhibitors were found to be the purine and pyrimidine nucleoside triphosphates.

In order to elucidate the possible mode of action of wildfire toxin, the influence of L-methionine sulphoximine (MSO) on glutamine synthetase from tobacco tissue was studied. It was found that MSO inhibited the enzyme and that MSO bound to the enzyme irreversibly.

Glucose-6-phosphate dehydrogenase from tobacco tissue was studied for its regulations. It was found that scopoletin, scopolin, esculin and ferulic acid inhibited the enzyme. Similar inhibitions by scopoletin and scopolin were observed for three other enzymes from the same plant; namely, 6-phosphogluconate dehydrogenase, $NADP^+$ specific isocitrate dehydrogenase and $NADP^+$ specific malate dehydrogenase. A possible physiological significance of this inhibition is suggested.

INVESTIGATIONS OF THE PROPERTIES OF SOME ENZYMES IN NOCARDIA CORALLINA AND TOBACCO TISSUE

CHAPTER I

GLUTAMINE SYNTHETASE FROM NOCARDIA CORALLINA

Introduction

It has been demonstrated that glutamine synthetase (L-glutamate-ammonia ligase, E C 6.3.1.2) is an enzyme involved in the starting point of several important metabolic pathways. The amide group of glutamine, a product of glutamine synthetase reaction, is used in the biosynthesis of such compounds as histidine, carbamyl phosphate, tryptophan, glucosamine-6-phosphate, and purine and pyrimidine nucleotides. Furthermore, Woolfolk and Stadtman (1964) have shown that glutamine synthetase from E. coli is subject to feedback inhibition by eight of these compounds: namely, histidine, carbamyl phosphate, tryptophan, glucosamine-6-phosphate, AMP, CTP, alanine and glycine. None of these compounds would completely inhibit the enzyme, but two or more of the compounds together exerted a "cumulative" inhibition of the enzyme.

Hubbard and Stadtman (1967) observed similar patterns of feedback inhibition on glutamine synthetase from numerous other microorganisms such as Neurospora crassa, Chlorella pyrenoidosa, Candida utilis, Rhodospirillum rubrum, Clostridium pasteurianum, Pseudomanas fluorescens,

Bacillus cereus, Bacillus licheniformis, Micrococcus sodonensis and Salmonella typhimurium. Glutamine was also found to inhibit the enzyme from some of these microorganisms. They also reported that p-aminobenzoic acid, anthranilic acid, methionine, isoleucine, GMP and NAD^+ were either non-inhibitory or only slightly inhibitory, causing less than 5% diminition in activity. Glutamine synthetase from Neurospora crassa, studied by Kapoor and Bray (1968), was found to be slightly different from the E. coli enzyme in that the Neurospora enzyme was strongly inhibited by ADP, one of the reaction products, and by NAD^+, but was not inhibited by L-alanine or L-tryptophan.

It has also been reported (Kingdon and Stadtman, 1967) that the regulatory properties of E. coli glutamine synthetase are influenced by the manner in which the bacterium is grown. The enzyme from the cells grown on glycerol and glutamate is strongly inhibited by each of the eight compounds known to affect the enzyme, but the enzyme from cells grown on glucose and growth-limiting concentrations of NH_4Cl is stimulated by AMP, histidine and tryptophan. It was shown that these two enzymes are different enzymes: adenylated and non-adenylated enzymes (Shapiro, Kingdon and Stadtman, 1967; Kingdon, Shapiro and Stadtman, 1967).

In the course of his investigation on the metabolism of Nocardia corallina, the author began a study of glutamine synthetase. It was found that the feedback inhibition pattern of glutamine synthetase from Nocardia corallina differs significantly from that of most of the other microorganisms reported thus far. For the experiments reported here, glutamine synthetase from Nocardia corallina was partially purified and the patterns of feedback inhibition by various important metabolic compounds

related to glutamine metabolism were studied.

Materials and Methods

Growth of Cultures

All cultures of N. corallina were grown on nutrient broth agar supplemented with 1% fructose. Generally the microorganism was grown in Roux bottles for 60 hr at 30 - 31°C. Similar growth was observed when Petri dishes were used. After growth, the cells were washed from the agar into centrifuge bottles with 0.1 M imidazole-HCL buffer (pH 7.5) and centrifuged at 16,300 X g (average) for 10 min. The supernatant solution was decanted and the cells were used directly or frozen for future use.

Enzyme Preparation

The frozen cells (about 20 g) were thawed and mixed with equal weights of 0.1 M imidazole-HCL buffer (pH 7.5) and glass beads (Aloe # 12). The slurry was placed in the pre-cooled metallic cup of a blender (Sorvall Omnimixer) and blended at 5000 rpm for 30 min with the metallic cup submerged in an ice water bath. The crude enzyme solution was then centrifuged at 114,700 X g (average) for 2 hr. All operations concerned with the preparation and purification of the enzyme were performed at approximately 4°C unless otherwise indicated.

To the supernatant obtained by ultracentrifugation, solid $(NH_4)_2SO_4$ (24.2 g per 100 ml of preparation) was added slowly with constant stirring. Stirring was continued for 5 min and the precipitated protein was collected by refrigerated centrifugation and discarded. The remaining supernatant solution was brought to about 50% saturation in $(NH_4)_2SO_4$

by slowly adding additional $(NH_4)_2SO_4$ to the level of 31.5 g per 100 ml of preparation. The precipitated protein was collected by refrigerated centrifugation and resuspended in 5 ml of 0.1 M imidazole-HCL buffer (pH 7.5) and dialyzed overnight against 500 ml of the same buffer. After dialysis the solution was diluted with the appropriate volume of buffer to obtain a final concentration of 3 mg of protein per ml of preparation. The resulting solution, which had a specific activity of ten times that of the initial broken cell preparation, was used for all the assays presented in this dissertation.

Enzyme Assay

Glutamine synthetase activity was determined by following the formation of orthophosphate from ATP in the presence of the other substrates, L-glutamate, Mg^{++} and NH_4^+. The pH optimum of glutamine synthetase was determined and found to have a broad peak between pH 7 and 8. The reaction mixture for a typical assay consists of the following (Stadtman, 1966): 0.25 M monosodium L-glutamate (Sigma); 38.0 mM ATP (Sigma); 0.25 M NH_4Cl; 0.25 M imidazole buffer (pH 7.5)(Sigma); 0.25 M $MgCl_2$; and 0.1 ml of the enzyme solution. The total volume of the solution was 0.4 ml. The reaction was initiated by addition of the enzyme.

The reaction mixture was incubated for 10 min at room temperature. The reaction was stopped by the addition of 3.6 ml of a freshly prepared solution containing 0.8% $FeSO_4 \cdot 7H_2O$ in 0.015 N H_2SO_4. The color was developed by adding 0.3 ml of a solution containing 6.3% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 N H_2SO_4. After 7 min of color development, the solution was centrifuged to remove some precipitated protein. The optical density of the

supernatant solution was then measured at 660 mμ using the Hitachi Perkin-Elmer UV-visible spectrophotometer (Model 139-PM).

Protein Determination

Protein concentrations were estimated by the micro-biuret method (Itzhaki and Gill, 1964) using bovine serum albumin as a standard.

Results

The saturation curve shown in Fig. 1-1 indicates that glutamine synthetase isolated from N. corallina has a very high K_m of 90 X 10^{-3} M. Similar non-sigmoid saturation curves were obtained for the other two substrates, ATP and NH_4^+, as shown in Fig. 1-2 to Fig. 1-5. Again both of the K_m values are unusually high, being 17 X 10^{-3} M and 3 X 10^{-3} M, respectively.

Also shown in Fig. 1-1 is the inhibition caused by glutamate at concentrations higher than 0.25 M. To demonstrate that this inhibition was not caused by the higher ionic strength of the reaction mixture but by the elevated substrate concentration, NaCl (up to 0.5 M) was added to reaction mixtures containing glutamate at a concentration of 0.25 M. Even at the highest NaCl concentration, the reaction rate was not diminished, indicating that inhibition was due to the very high substrate concentration.

In an attempt to determine the significance of glutamine synthesis in N. corallina metabolism, 14 metabolic compounds that are known to be or are believed to be related to glutamine metabolism were tested for feedback inhibition of the enzyme. All the compounds investigated and the concentrations used are listed in Table 1-1 along with the percentage of inhibition at various concentrations of the substrate, L-glutamate (at V_M, 0.25 M; 80% V_M, 0.20 M; 40% V_M, 0.10 M; 10% V_M, 0.025 M).

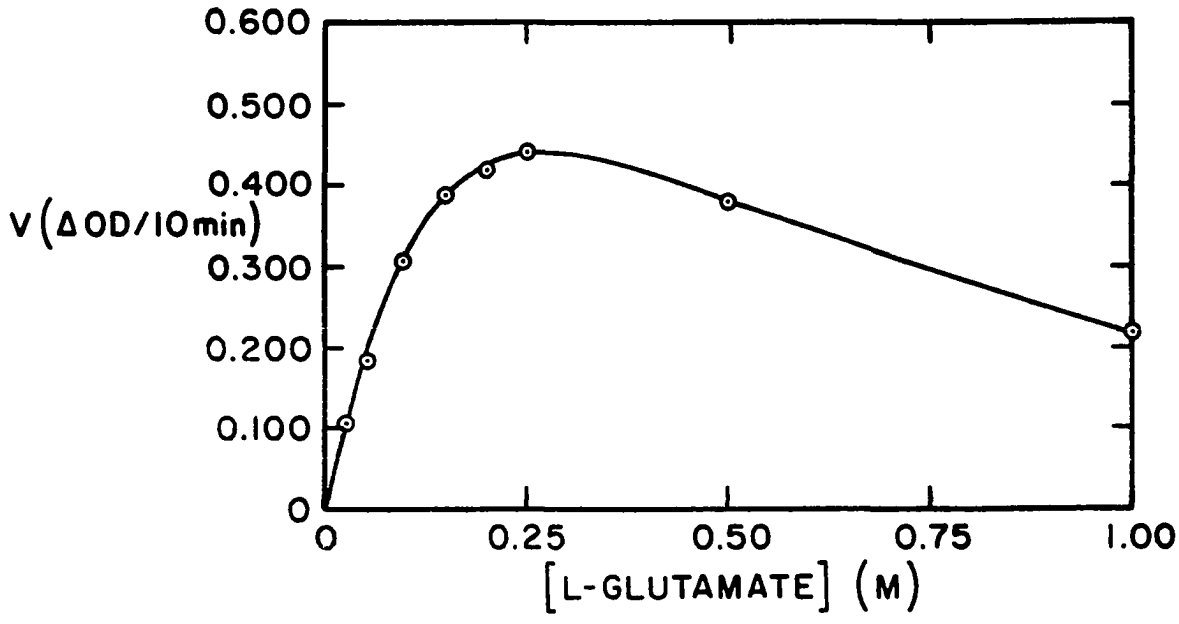
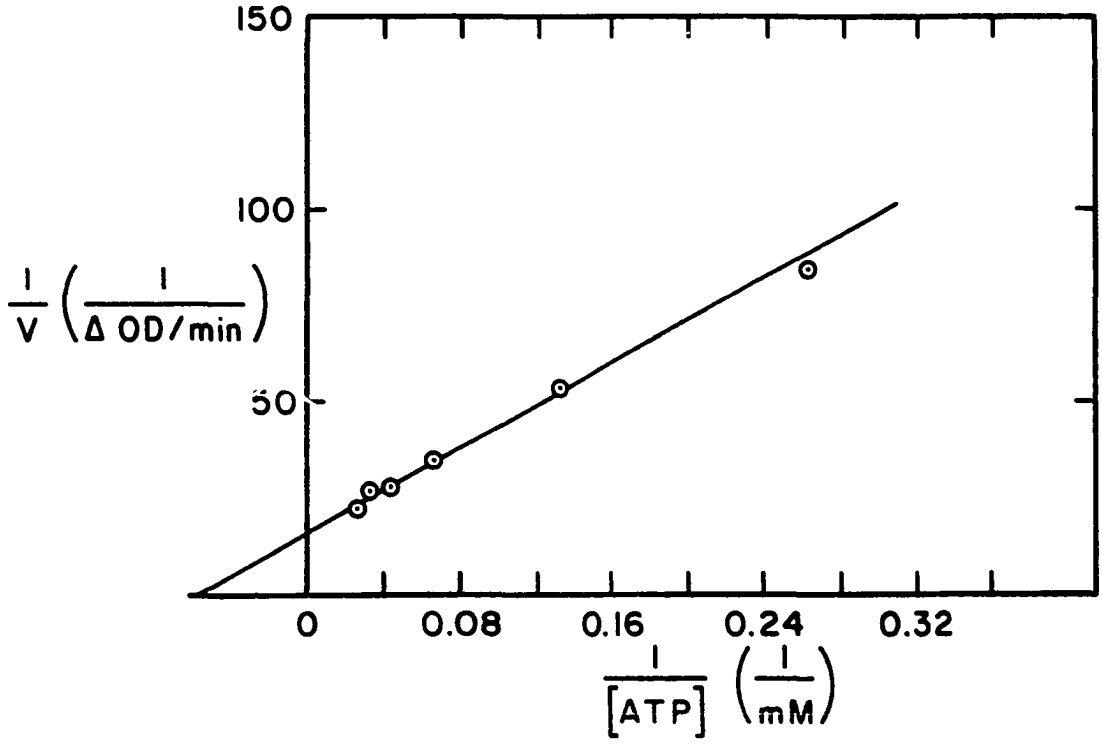


Fig. 1-1. The saturation function of L-glutamate for glutamine synthetase from N. corallina. The reaction velocity is given in optical density units. All points are experimental.



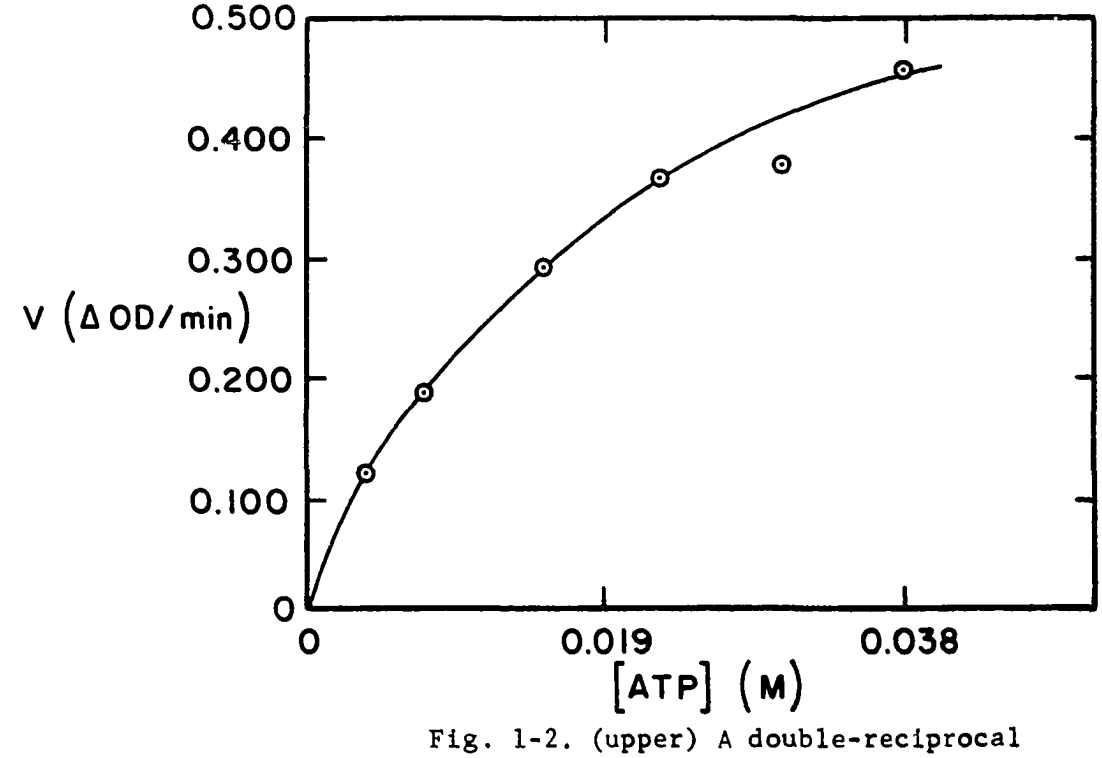
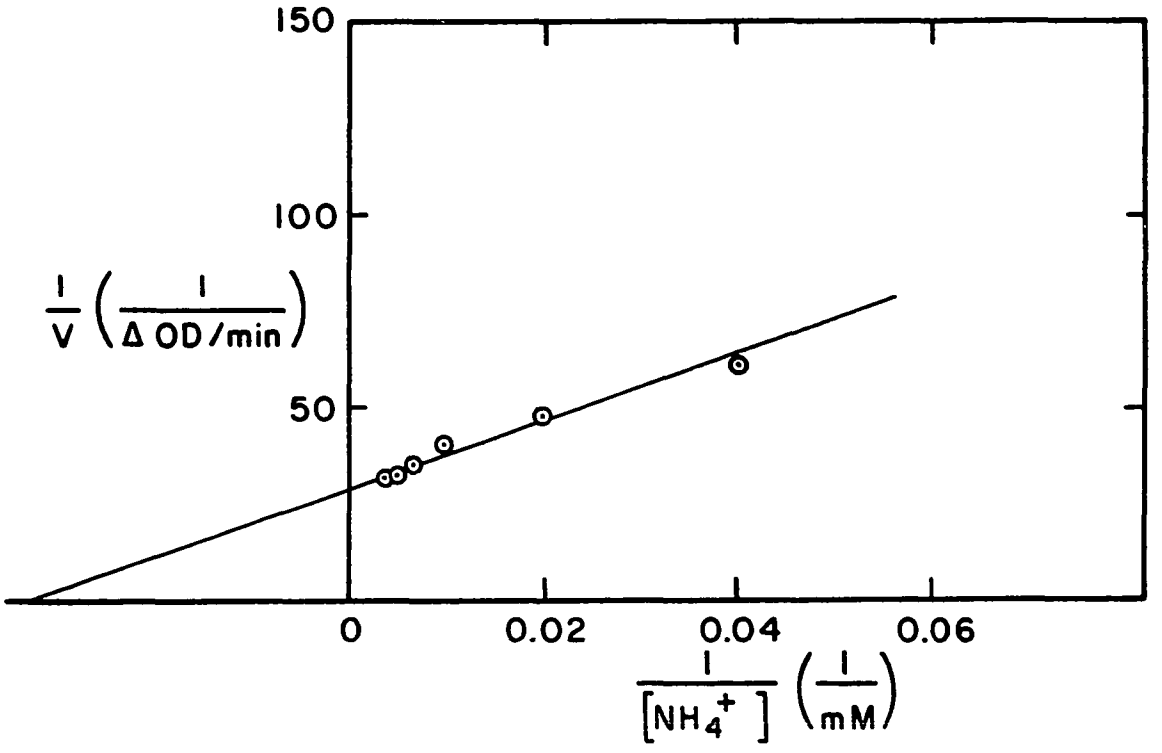


Fig. 1-2. (upper) A double-reciprocal plot of reaction velocity vs. ATP concentration for glutamine synthetase from N. corallina.

Fig. 1-3. (lower) The saturation function of ATP for glutamine synthetase from N. corallina.



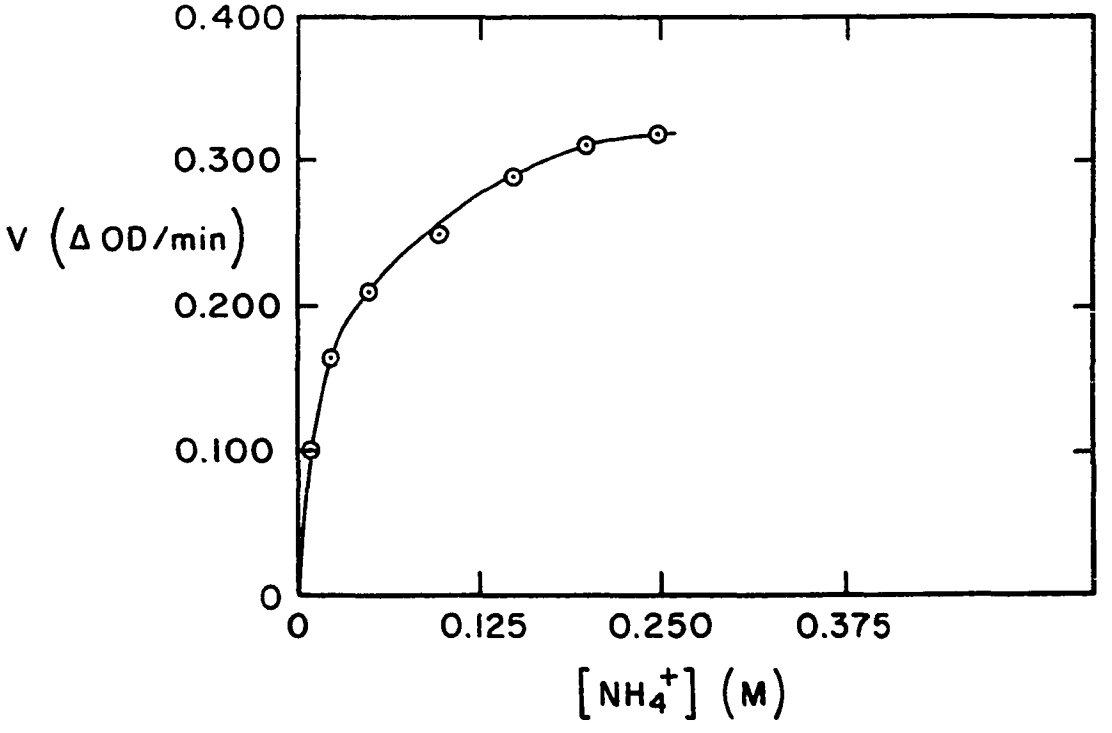


Fig. 1-4. (upper) A double reciprocal plot of reaction velocity vs. NH_4^+ concentration for glutamine synthetase from N. corallina.

Fig. 1-5. (lower) The saturation function of NH_4^+ for glutamine synthetase from N. corallina.

TABLE 1-1

PERCENTAGE OF INHIBITION OF GLUTAMINE SYNTHETASE IN N. CORALLINA

Compounds	Concentration of L-Glutamate 0.025 M	0.10 M	0.20 M	0.25 M	Mode of Inhibition
15.5 mM AMP	56%	49%	44%	43%	N
15.5 mM CTP	8	5	3	3	C
25 mM L-histidine	0	0	0	0	-
25 mM L-tryptophan	41	45	37	33	C
25 mM Glycine	23	27	20	17	C
12.5 mM L-alanine	15	15	14	14	N
6.25 mM Carbamyl-P	16	14	12	12	N
25 mM L-glutamine	41	29	20	17	C
25 mM Glucosamine-6-P	31	28	26	26	N
12.5 mM ADP	64	53	44	39	C
12.5 mM GTP	63	51	41	36	C
12.5 mM NAD^+	86	80	74	71	C
12.5 mM $NADP^+$	18	12	9	8	C
12.5 mM ITP	0	0	0	0	-

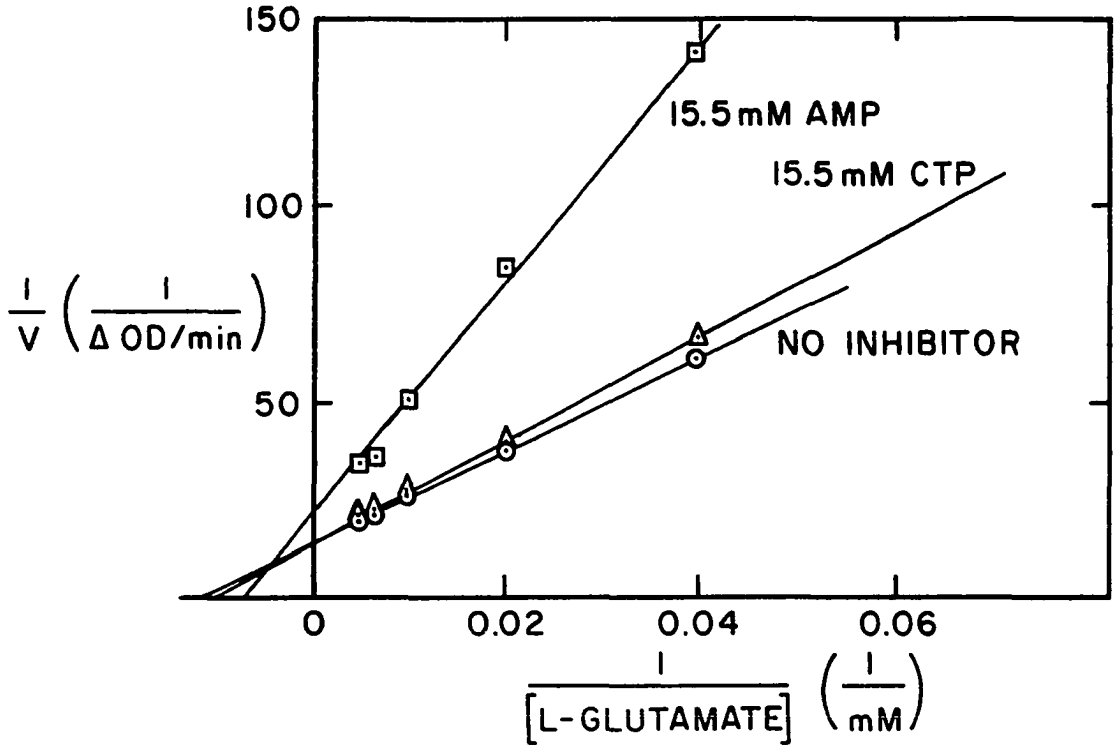
Note. Mode of inhibition was determined by Lineweaver-Burk plots.
N stands for noncompetitive inhibition and C for competitive inhibition.

Lineweaver-Burk plots (1934) for the various inhibitors were drawn to determine the modes of inhibition with respect to the substrate. These results are also listed in Table 1-1 and the plots are shown in Fig. 1-6 to Fig. 1-13. Among the 14 compounds examined, only histidine and ITP were non-inhibitory, while CTP and carbamyl phosphate had little effect on this enzyme.

The results shown in Table 1-2 indicate the inhibition percentage for each inhibitor at different levels of inhibitor concentration. As expected, the inhibition decreases as the inhibitor concentration is lowered. In fact, at concentrations of 2.5 mM L-alanine, carbamyl phosphate, L-glutamine and glucosamine-6-phosphate are not inhibitory.

From Table 1-1 it can be seen that among those eight metabolic compounds that were known to inhibit E. coli glutamine synthetase (Woolfolk and Stadtman, 1964), only histidine was demonstrated not to inhibit N. corallina glutamine synthetase. L-tryptophan and L-alanine, which were known to be non-inhibitory for glutamine synthetase from Neurospora crassa, showed definite inhibitions. Glycine and L-tryptophan displayed bimodal reciprocal plots (Fig. 1-7 to Fig. 1-9) of reaction velocity vs. glutamate concentration similar to the E. coli enzyme (Woolfolk and Stadtman, 1967) as evidenced in their highest percentage of inhibitions at 0.10 M of L-glutamate concentration (see Table 1-1).

In addition to these eight compounds, five other metabolic compounds; NAD^+, $NADP^+$, GTP, ADP and L-glutamine, displayed definite inhibitory patterns. These observations clearly reveal that the Nocardia enzyme is remarkably unique among the other microorganisms already investigated.



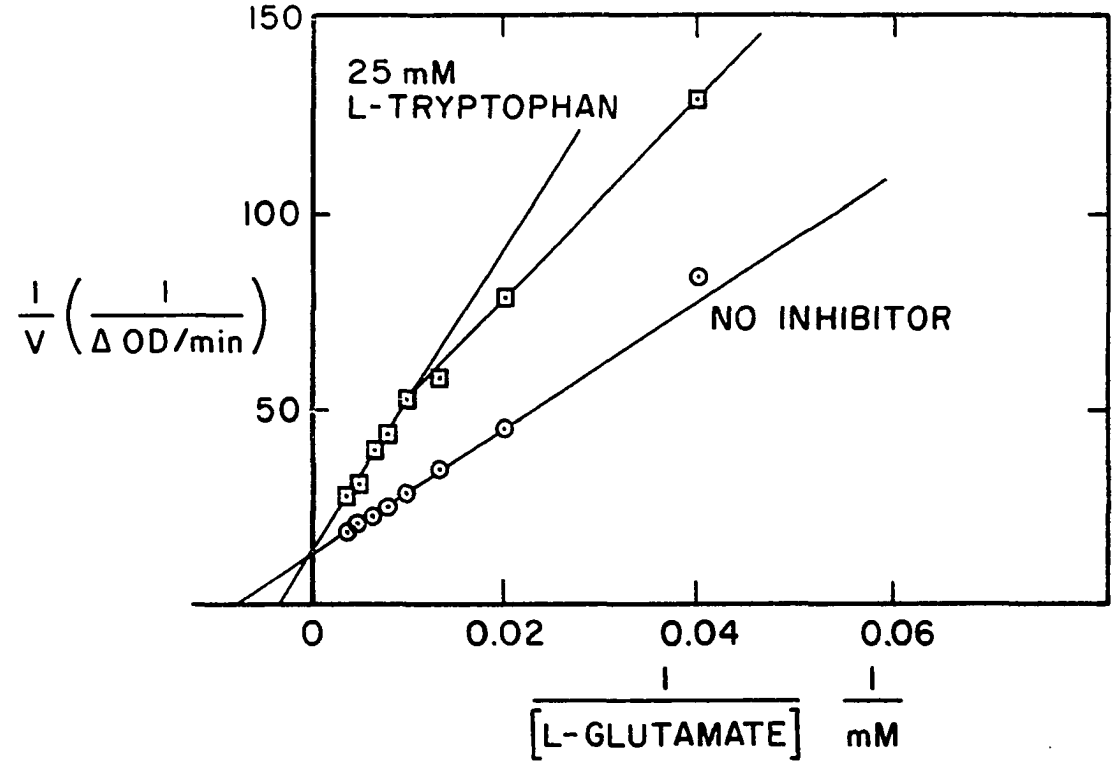
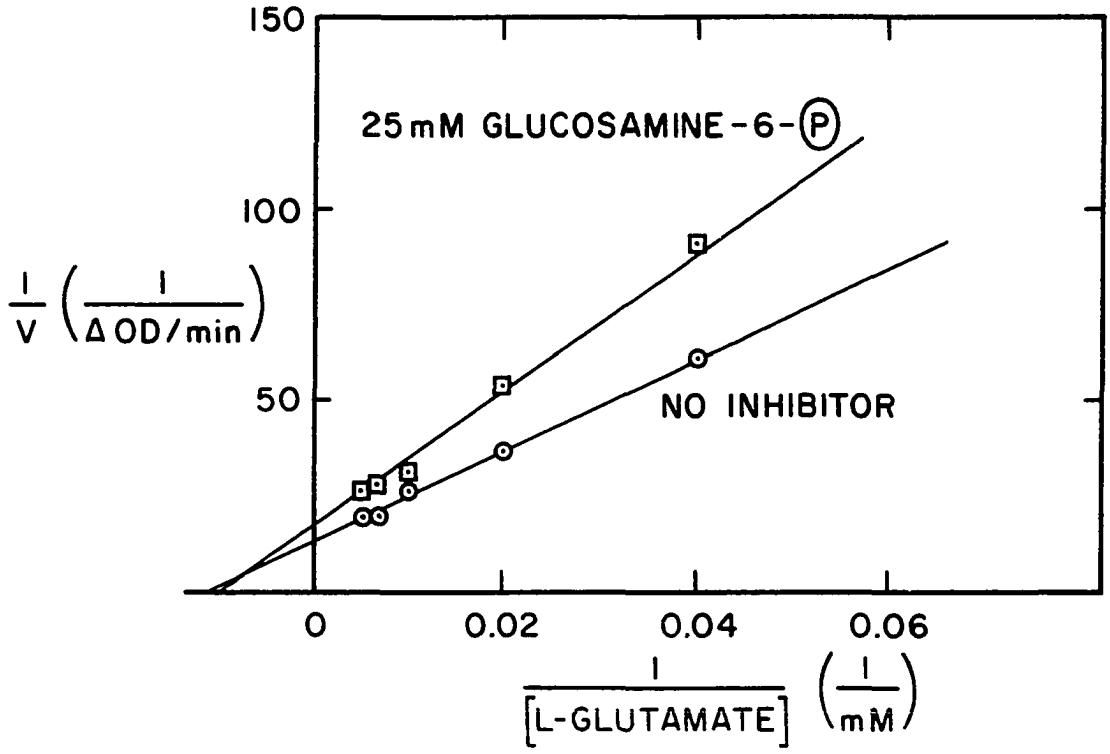


Fig. 1-6. (upper) Double reciprocal plots of reaction velocity vs. glutamate concentration in the presence of fixed concentrations of AMP and CTP for glutamine synthetase from N. corallina.

Fig. 1-7. (lower) A double reciprocal plot of reaction velocity vs. glutamate concentration in the presence of fixed concentrations of L-tryptophan for glutamine synthetase from N. corallina.



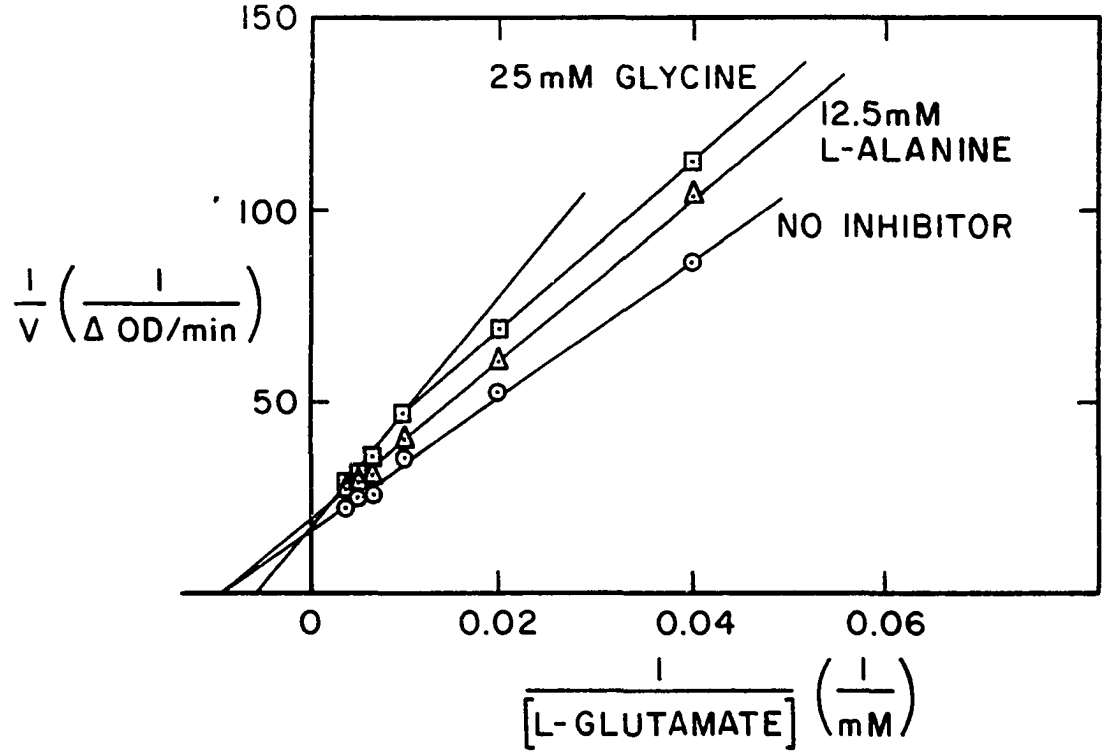
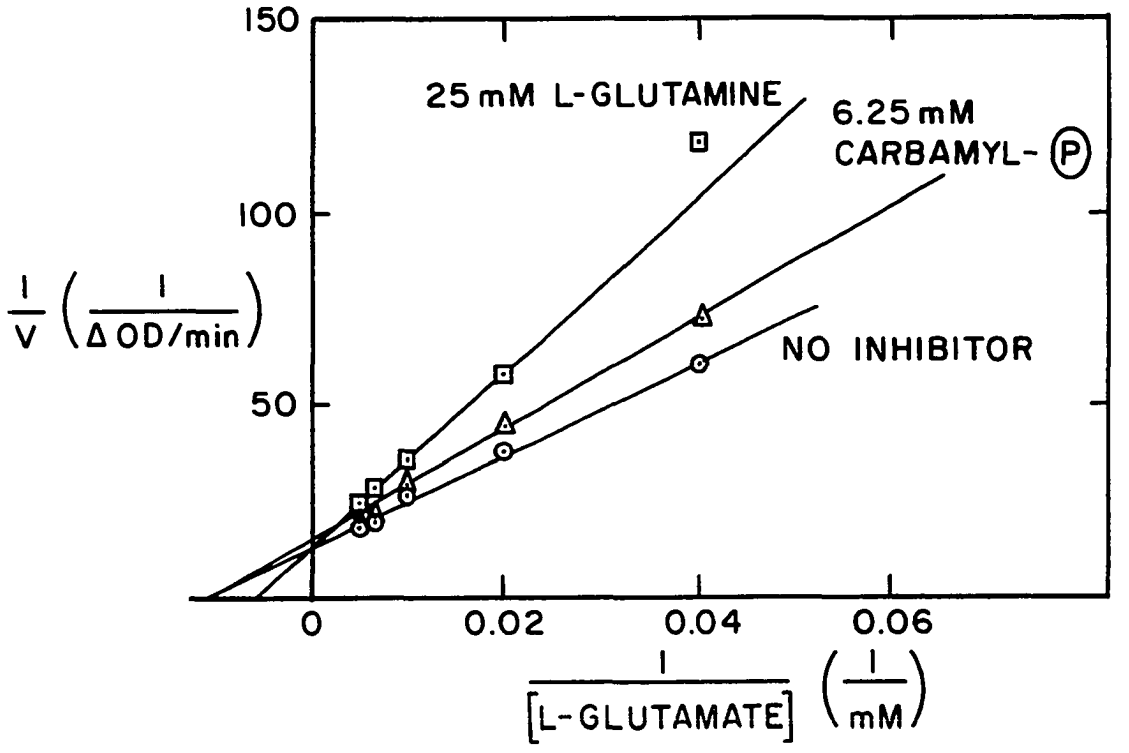


Fig. 1-8. (upper) A double reciprocal plot of reaction velocity vs. glutamate concentration in the presence of fixed concentration of glucosamine-6-phosphate for glutamine synthetase from N. corallina.

Fig. 1-9. (lower) Double reciprocal plots of reaction velocity vs. glutamate concentration in the presence of fixed concentrations of glycine and L-alanine for glutamine synthetase from N. corallina.



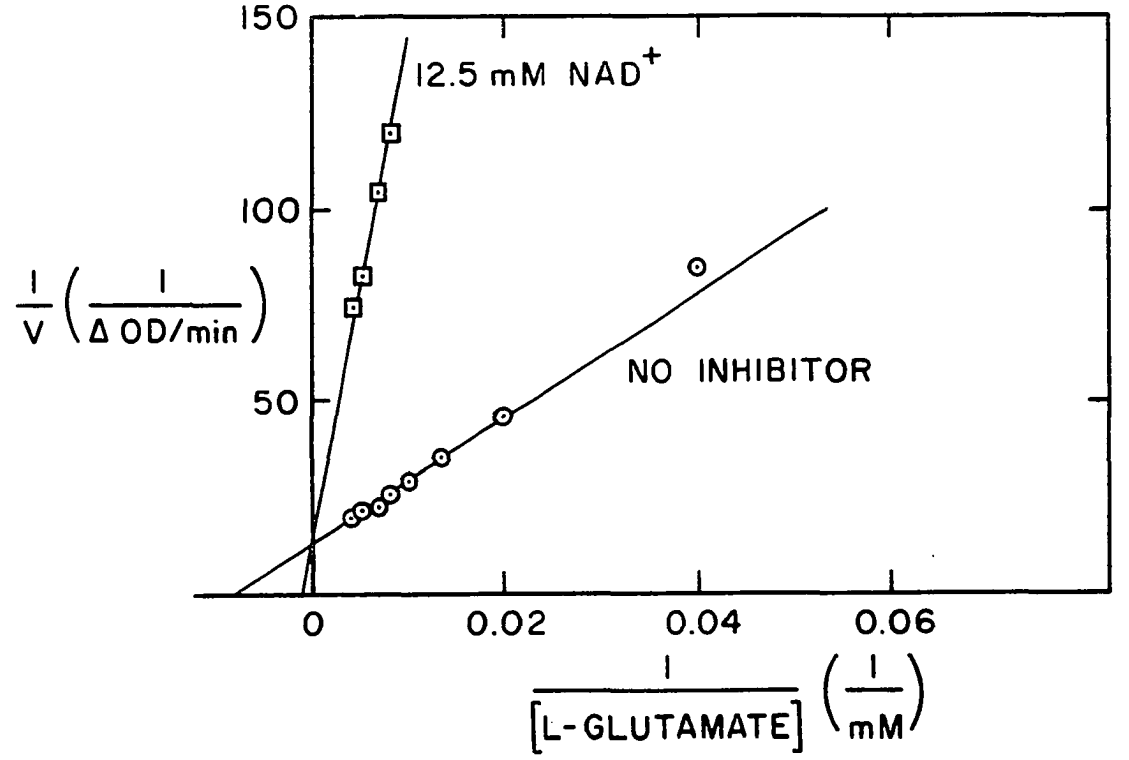
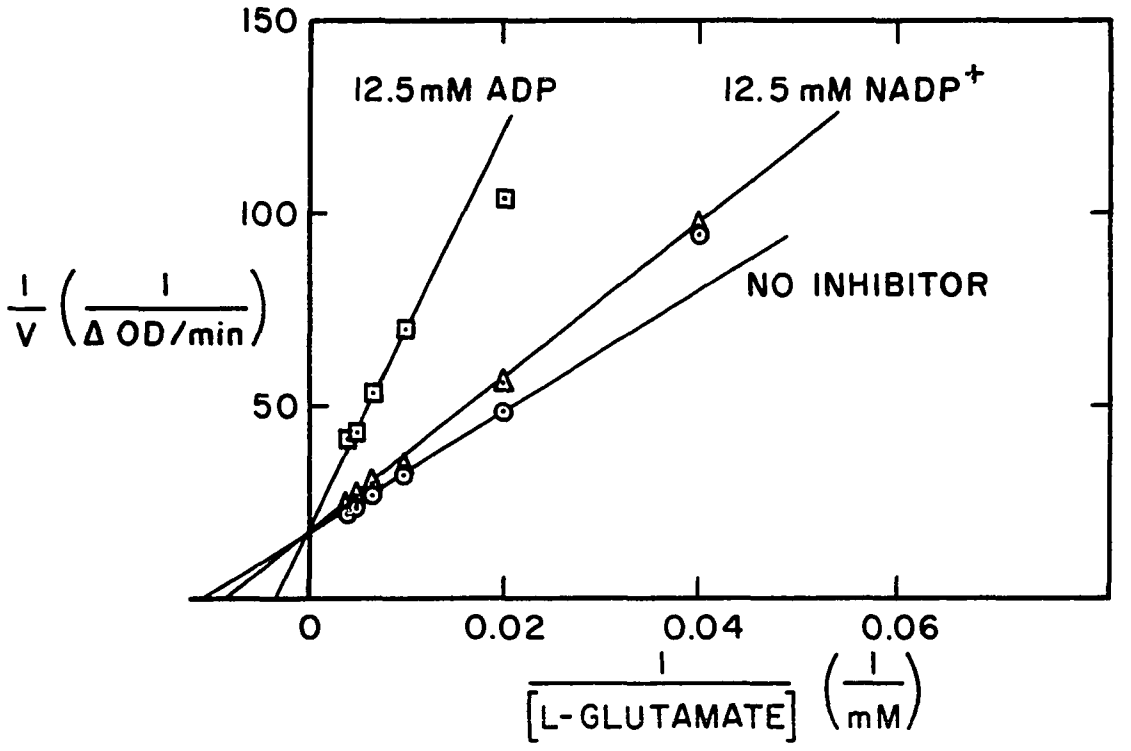


Fig. 1-10. (upper) Double reciprocal plots of reaction velocity vs. glutamate concentration in the presence of fixed concentrations of L-glutamine and carbamyl phosphate for glutamine synthetase from N. corallina.

Fig. 1-11. (lower) A double reciprocal plot of reaction velocity vs. glutamate concentration in the presence of a fixed concentration of NAD^+ for glutamine synthetase from N. corallina.



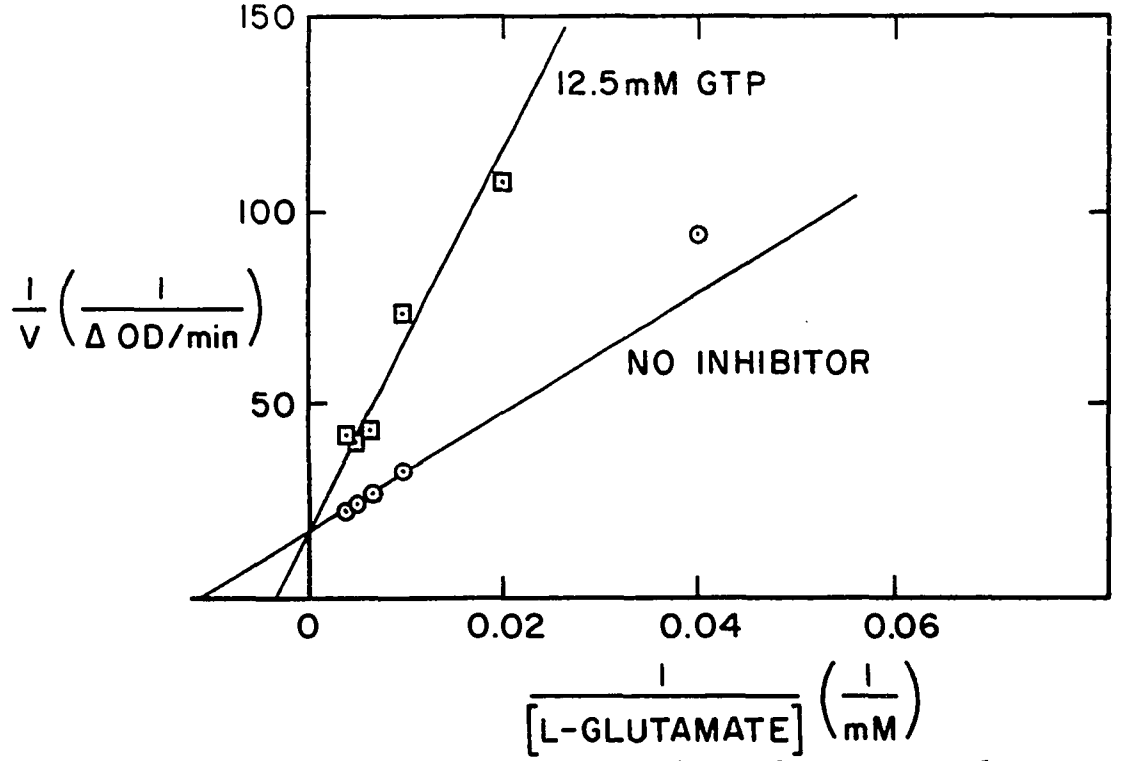


Fig. 1-12. (upper) Double reciprocal plots of reaction velocity vs. glutamate concentration in the presence of fixed concentrations of ADP and $NADP^+$ for glutamine synthetase from N. corallina.

Fig. 1-13. (lower) A double reciprocal plot of reaction velocity vs. glutamate concentration in the presence of a fixed concentration of GTP for glutamine synthetase from N. corallina.

TABLE 1-2

PERCENTAGE OF INHIBITION OF GLUTAMINE SYNTHETASE IN N. CORALLINA

Compounds	% Inhibition		
	15.5 mM	6.2 mM	3.1 mM
AMP	43%	30%	29%
	25 mM	10 mM	5 mM
L-tryptophan	33	25	14
	25 mM	10 mM	5 mM
Glycine	17	16	13
	12.5 mM	5 mM	2.5 mM
L-alanine	14	2	0
	6.25 mM	2.5 mM	1.25 mM
Carbamyl-P	12	0	0
	25 mM	10 mM	5 mM
L-glutamine	17	0	0
	25 mM	10 mM	5 mM
Glucosamine-6-P	26	14	0
	12.5 mM	5 mM	2.5 mM
ADP	39	31	22
	12.5 mM	5 mM	2.5 mM
GTP	36	21	18
	12.5 mM	5 mM	2.5 mM
NAD^+	71	38	28
	12.5 mM	5 mM	2.5 mM
$NADP^+$	8	8	6

Note. Three different concentrations for each inhibitor used are underlined. Standard conditions of the forward assay were used. CTP had very little effect. ITP and L-histidine had no effect on the enzyme.

The degree of inhibition caused by NAD^+ was the greatest of all compounds tested. It was inhibitory to the extent of 71% at a concentration of 12.5 mM and 38% at 5 mM. The Neurospora enzyme was reported (Kapoor and Bray, 1968) to be subject to 18 - 22% inhibition at 5 mM of NAD^+. A compound similar in structure to NAD^+, $NADP^+$, also inhibited the Nocardia enzyme significantly, but less than NAD^+. Inhibition by $NADP^+$ has not been reported for glutamine synthetase from other sources. Reciprocal plots of reaction velocity vs. glutamate concentration for these two compounds are given in Fig. 1-11 and Fig. 1-12. As can be easily seen from the plots, both compounds act as effective competitive inhibitors.

The purine nucleotides ADP and GTP dictated a high degree of inhibition compared to a low degree of inhibition by a pyrimidine nucleotide CTP (Table 1-1). ADP and GTP showed 39% and 36% inhibition of the enzyme at concentrations of 12.5 mM and 31% and 21%, respectively, at 5 mM. For glutamine synthetase in Neurospora crassa, an extremely high level of inhibition of 96% by 5 mM ADP and a moderately high level of inhibition of 31% by 5 mM GTP are reported (Kapoor and Bray, 1968).

Although the degree of inhibition by ADP and GTP for the Nocardia enzyme is similar to that by AMP, inhibitions by these two compounds were competitive, as shown in Fig. 1-12 and Fig. 1-13, in contrast to the non-competitive pattern of AMP. ADP is an immediate reaction product from ATP and may play a direct role of metabolic control of the enzyme activity.

Another immediate reaction product, L-glutamine, was found to inhibit the enzyme moderately at the relatively high concentration of

25 mM, and not to inhibit at the level of 5 mM or 10 mM. The reciprocal plot is shown in Fig. 1-10. L-glutamine is a competitive inhibitor like the other reaction product, ADP.

Since L-histidine and glucosamine-6-phosphate display competitive inhibition with respect to NH_4^+ for glutamine synthetase in E. coli, the inhibition of the N. corallina enzyme by these two compounds was investigated at several saturation levels (½ saturation and ¼ saturation of NH_4^+). Glucosamine-6-phosphate showed slightly more inhibition at the low level of NH_4^+ concentration, but histidine still had no inhibitory effects.

Discussion

From the results obtained, it is apparent that the inhibition pattern of glutamine synthetase from N. corallina is quite unique among the microorganisms studied so far.

The observation of a quite high K_m of the enzyme with respect to L-glutamate suggests that in this microorganism the physiological concentration of glutamate may be comparatively high.

Recently Deuel et al. (1969) studying Bacillus subtilis glutamine synthetase observed that with Mn^{++} in place of Mg^{++}, the optimal activity was obtained at a low concentration of glutamate and NH_4^+ (5 - 10 mM), but when equal concentrations of ATP and Mn^{++} were used, high substrate inhibition occurred. Furthermore, they report that with Mg^{++} as the cofactor, glutamate and NH_4^+ at 100 mM do not saturate the enzyme. Similar phenomena were observed with N. corallina glutamine synthetase and a high substrate inhibition by glutamate also was detected.

Such a high K_m and high substrate inhibition have not been reported for the E. coli and N. crassa enzymes.

The activity of N. corallina glutamine synthetase seems to be controlled by the five major end products of glutamine metabolism: L-tryptophan, carbamyl phosphate, glucosamine-6-phosphate, AMP and CTP. The inhibition patterns by these five compounds for the N. corallina enzyme are very similar to those of the E. coli and N. crassa enzymes, except that a very low inhibition by CTP for N. corallina was observed and that L-tryptophan did not inhibit the N. crassa enzyme.

It is interesting to note that L-histidine did not inhibit N. corallina glutamine synthetase. It is possible that histidine biosynthesis in this microorganism is accomplished without involvement of glutamine and that nitrogens of histidine are supplied by some other sources, such as NH_4^+, asparagine, etc. Research on histidine biosynthesis should reveal this answer.

It is thought that glycine is formed from serine, and that alanine is formed from pyruvate in the presence of glutamate and transaminase. However, the fact that glycine and alanine inhibit glutamine synthetase indicates that they may somehow be related to glutamine metabolism in N. corallina. It appears that, as in mammalian tissue (Meister and Tice, 1950), there may be glutamine-dependent transaminase which catalyzes the reactions in which glycine and alanine are synthesized from glyoxalate and pyruvate. Inhibitions by glycine and alanine are also observed for E. coli glutamine synthetase, whereas alanine was found to be noninhibitory for the N. crassa enzyme.

The strong inhibition shown by NAD^+ and the definite inhibition

by $NADP^+$ is evidence that in this organism the biosyntheses of these two compounds are probably accomplished through the nicotinate pathway where deamido-NAD^+ is converted to NAD^+ at the final step, receiving the amide-amino group of glutamine. A strong inhibition by NAD^+ was also observed for the N. crassa enzyme, but not for the E. coli enzyme.

The immediate reaction products, L-glutamine and ADP, inhibit N. corallina glutamine synthetase competitively, indicating that they may act as product inhibitors. Inhibition by glutamine is also observed in other organisms such as Lactobacillus arabinosus (Ravel, Humphreys and Shive, 1965) and some Bacillus and Clostridium species (Hubbard and Stadtman, 1967). Whether or not the inhibitions by these two reaction products have any physiological significance remains to be established.

The high inhibitory capacity of GTP indicates that it may play a regulatory role, whereas the other nucleotide, ITP, did not display any effect. Since GTP inhibited competitively to the same extent as ADP, GTP probably binds at the ATP site to influence the enzyme reaction, whereas the non-competitive inhibitor AMP may bind at the allosteric site to affect the catalytic site.

Glutamine synthetase of N. corallina, therefore, seems to be controlled altogether by several compounds, suggesting that the nature of enzyme regulation is quite complex. Future experiments will demonstrate whether these compounds show cumulative inhibitions similar to those shown in E. coli. It has not been revealed yet whether there are two kinds of glutamine synthetase, adenylated and non-adenylated, for N. corallina as there are for E. coli. If two different forms of N. corallina glutamine synthetase exist, the regulatory properties of the enzyme will

be more complex. The enzyme studied here was from N. corallina grown on nutrient broth agar supplemented with 1% fructose and it was not activated by AMP, tryptophan and histidine, unlike the non-adenylated enzyme from E. coli, and in fact it was inhibited strongly by AMP and tryptophan like the adenylated enzyme, although histidine had no effect on the enzyme. It is probably not a mixture of the two forms because it is very unlikely to have two forms mixed in such a way as to show neither inhibition nor activation by histidine. It is quite possible, therefore, that the enzyme studied here may be the equivalent of an adenylated enzyme in E. coli.

Another possible aspect of regulation of glutamine synthetase may involve the control of enzyme activity by the charge of the cell of the adenylate system, as in the theory proposed by Atkinson et al. (1967a, 1967b) for other ATP-utilizing enzymes such as the citrate cleavage enzyme, phosphoribosylpyrophosphate synthetase, etc. Future experiments are needed to prove whether this type of regulation of glutamine synthetase exists.

Finally, as inhibition patterns shown in Table 1-3 reveal, this mold-like bacterium N. corallina resembles both the bacterium E.coli and the mold N. crassa in some respects and differs from both in other respects. This implies that N. corallina may possibly lie between E. coli and N. crassa in the evolutionary process.

Summary

Feedback inhibitions of glutamine synthetase from Nocardia corallina were studied with respect to the end products or compounds related to glutamine metabolism: glucosamine-6-phosphate, histidine, AMP, CTP, carbamyl phosphate, tryptophan, alanine, glycine, ITP, GTP, NAD^+, $NADP^+$, ADP and glutamine.

TABLE 1-3

INHIBITION PATTERNS OF N. CORALLINA COMPARED TO THOSE OF E. COLI AND N. CRASSA

Compounds	E. coli[a] (bacterium)	N. corallina (mold-like bacterium)	N. crassa[b] (mold)
Similarities			
Carbamyl- P	+	+	+
Glucosamine-6- P	+	+	+
AMP	+	+	+
Glycine	+	+	+
CTP	+	+	+
Dissimilarities			
L-tryptophan	+	+	-
L-histidine	+	-	+
L-alanine	+	+	-
NAD^+		+	+
$NADP^+$		+	
ADP		+	+
L-glutamine		+	
GTP		+	+
ITP		-	

[a]The results were taken from Woolfolk and Stadtman, Biochem. Biophys. Res. Commun. 17, 313 (1964).

[b]The results came from Kapoor and Bray, Biochem, 7, 3583 (1968).

Note. + indicates inhibition. - indicates non-inhibition. No sign indicates that results have not yet been reported.

Among the first eight compounds that are known to inhibit glutamine synthetase from E. coli and other sources, all but histidine showed definite inhibition of the enzyme. Histidine did not inhibit the enzyme even when the concentration of NH_4^+ was lowered to one-fourth of saturating (standard) NH_4^+ concentration.

GTP, NAD^+, $NADP^+$ and ADP were also found to demonstrate strong inhibitions. These results suggest that glutamine synthetase from Nocardia corallina differs significantly from that of other bacteria.

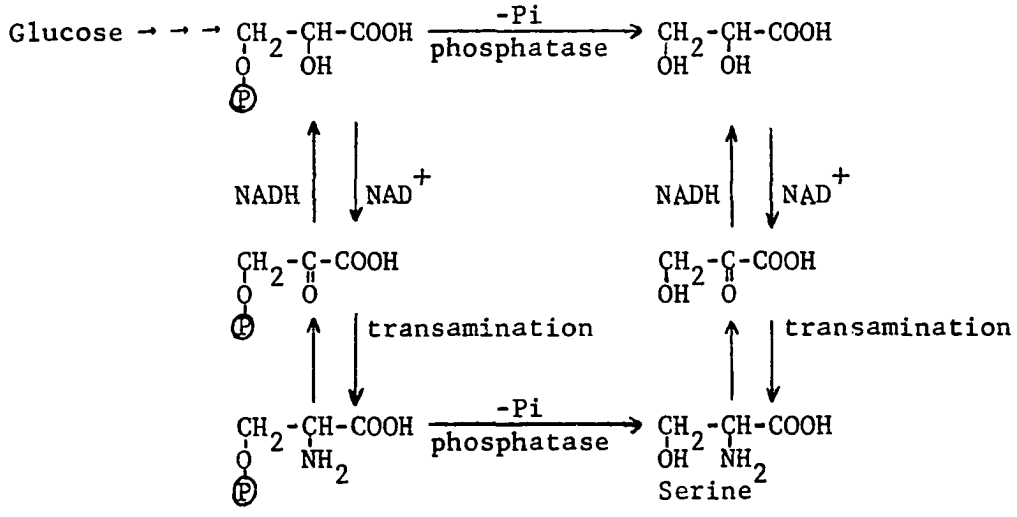
CHAPTER II

D-3-PHOSPHOGLYCERATE DEHYDROGENASE FROM NOCARDIA CORALLINA

Introduction

D-3-phosphoglycerate dehydrogenase is the enzyme which catalyzes the first step of the serine biosynthetic pathway, starting from 3-phosphoglycerate, one of the intermediary metabolites of glycolysis. Ichihara and Greenberg (1957) first reported the occurrence of this enzyme in mammalian systems.

It has been known that serine synthesis occurs through the phosphorylated pathway as well as through the non-phosphorylated one, as shown in the following diagram. Ⓟ stands for a phosphate.



In the phosphorylated pathway 3-phosphoglycerate is oxidized through 3-phosphoglycerate dehydrogenase and the cofactor NAD^+ to 3-phosphohydroxypyruvate, which in turn is aminated through transamination to phosphoserine and finally dephosphorylated to the end product, serine. In the non-phosphorylated pathway 3-phosphoglycerate undergoes a similar transformation, except that it is dephosphorylated first.

It has been reported from studies of enzymes in the liver of vertebrates that both the phosphorylated and non-phosphorylated pathways occur in rats, guinea pigs and chickens, whereas in pigeon liver only the phosphorylated pathway is operative. In the bacteria E. coli and S. typhimurium, the phosphorylated pathway is considered the major route of serine biosynthesis.

Pizer (1963) observed that 3-phosphoglycerate dehydrogenase from E. coli is inhibited by serine: 4×10^{-5} M serine causes 50% inhibition of the enzyme. The same enzyme from Salmonella typhimurium was also found to be sensitive to the feedback inhibition by the end product, serine (Umbarger and Umbarger, 1962). The enzyme from chicken liver (Walsh and Sallach, 1965) and from mouse brain extracts (Bridges, 1965) was found not to be affected by serine.

For this study, D-3-phosphoglycerate dehydrogenase from Nocardia corallina was partially purified and the attempt was made to elucidate some properties of the enzyme and its sensitivity to feedback inhibition by the end product, L-serine, and by other compounds. The occurence of D-glycerate dehydrogenase was also studied to determine the degree of importance of the non-phosphorylated pathway in this organism.

Materials and Methods

The cultures of Nocardia corallina were grown in a manner similar to that described in Chapter I. The enzyme preparation was also the same, except that 0.1 M Tris·HCl-0.03 M β-mercaptoethanol (pH 7.4) was used as a buffer solution. Protein concentrations were again estimated by the micro-biuret method.

Enzyme Assay

The 3 ml standard reaction mixture contained the following (Willis and Sallach, 1964): 167 m M Tris·HCl (pH 9.0); 133 m M hydrazine acetate (pH 9.0); 1.67mM GSH; 0.5 m M NAD^+; 8.3 mM D-3-phosphoglycerate; and the enzyme. The reaction was started by the addition of NAD^+ and followed by the measurement of the absorbency at 340 mμ. D-glycerate dehydrogenase was assayed using D-glycerate in place of D-3-phosphoglycerate for the standard reaction mixture.

Results

To determine the pH optimum of the enzymic oxidation of D-3-phosphoglycerate, Tris·HCl and hydrazine-acetate at different pH values were used. The pH optimum was found to be around 9.5, as is shown in Fig. 2-1.

The initial velocity of this enzymic reaction changes in hyperbolic fashion as the concentration of the substrate D-3-phosphoglycerate increases (Fig. 2-3). This indicates non-sigmoidal, simple Michaelis-Menten kinetics. The Michaelis constant K_m was determined to be 0.8 mM from the reciprocal plot of the reaction rate vs. substrate concentration (Fig. 2-2). The K_m for E. coli is 25 mM (Pizer, 1963).

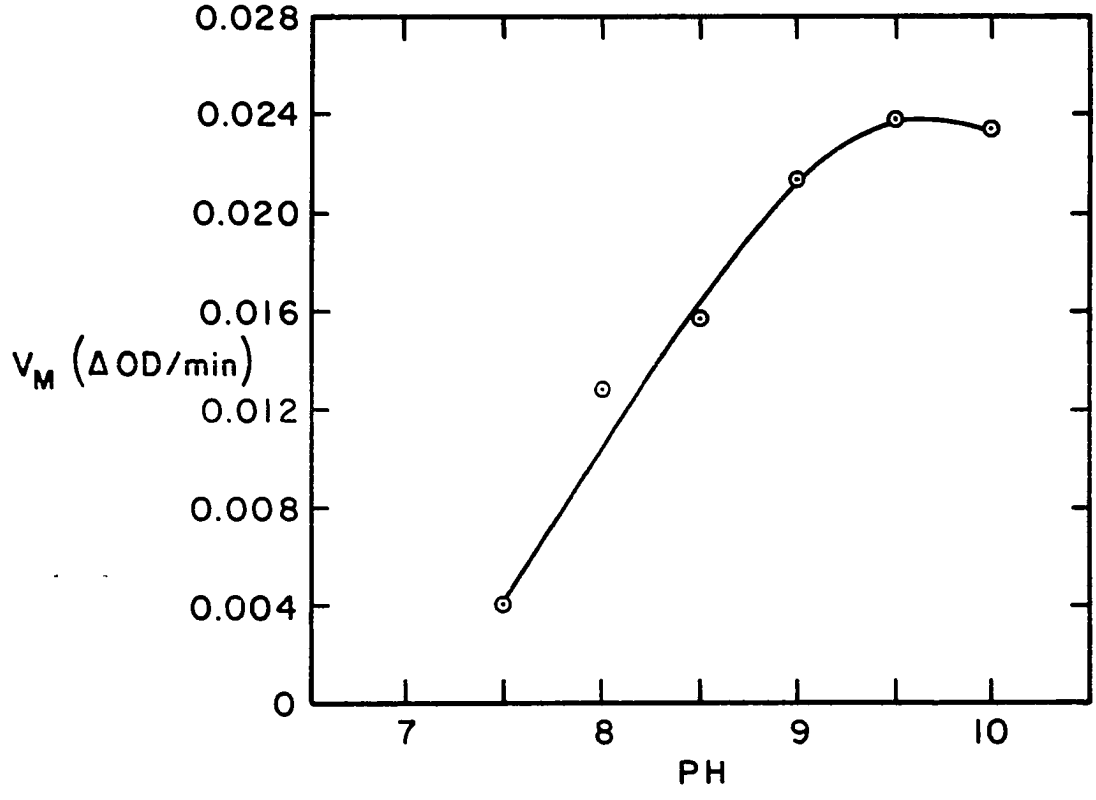
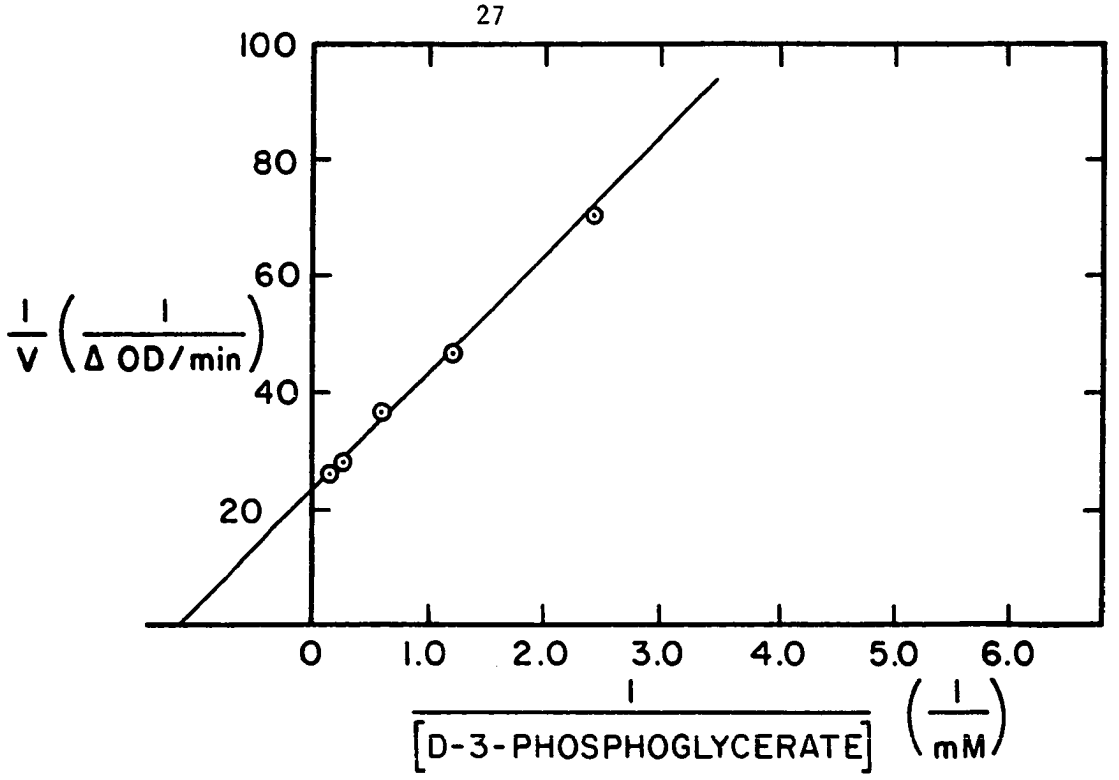


Fig. 2-1. The pH profile for D-3-phosphoglycerate dehydrogenase from N. corallina. Tris·HCl and hydrazine-acetate were used for buffer. Reaction velocity is given in optical density units. All points are experimental.



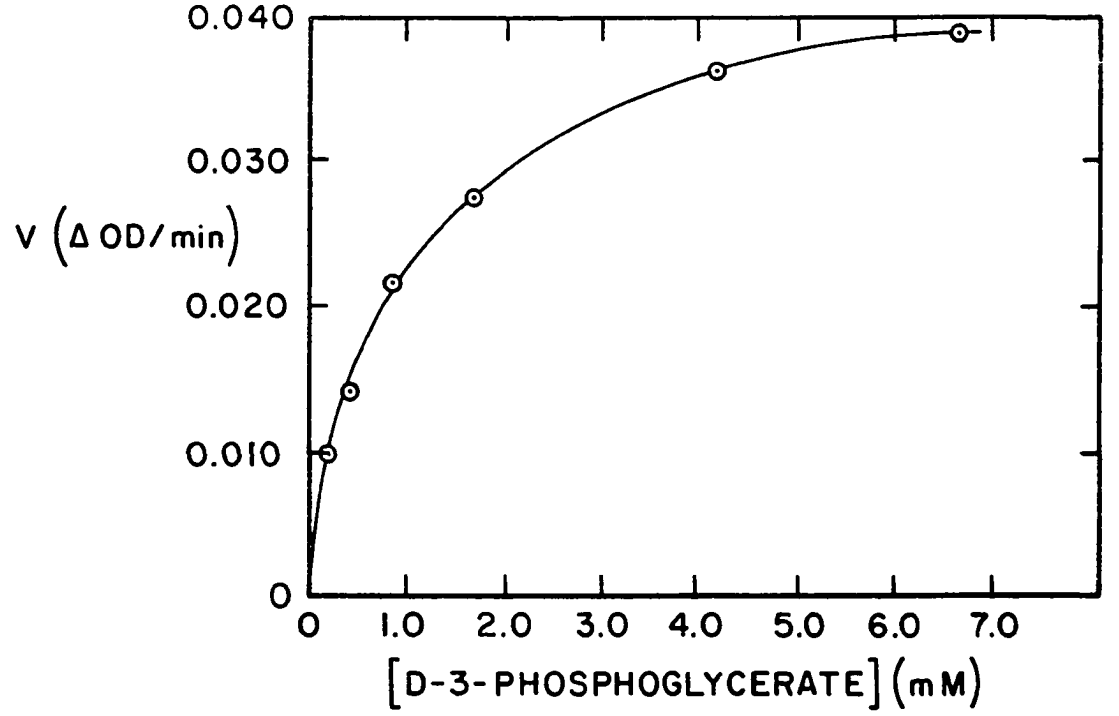


Fig. 2-2. (upper) A double reciprocal plot of reaction velocity vs. D-3-phosphoglycerate concentration for D-3-phosphoglycerate dehydrogenase from N. corallina.

Fig. 2-3. (lower) The saturation function of D-3-phosphoglycerate for D-3-phosphoglycerate dehydrogenase from N. corallina.

Since D-3-phosphoglycerate dehydrogenase from E. coli and S. typhimurium is known to be sensitive to the feedback inhibition by L-serine, the enzyme from Nocardia corallina, also a bacterium, was tested for inhibition by L-serine. Even a high concentration of serine (6.6 mM) had no effect on the enzyme, compared with the fact that 4×10^{-5} M serine causes a 50% inhibition of the E. coli enzyme.

To check the possibility of inhibition by other metabolic compounds, glycine and L-alanine were tested for their effects on the enzyme. Glycine and L-alanine were chosen because L-serine is known to be converted to glycine and to L-alanine. Neither 3.3 m M glycine nor 3.3 mM alanine had any effect on the enzyme from N. corallina.

3-phosphoglycerate dehydrogenase from the fresh enzyme preparation was also studied for its regulatory properties with the compounds L-serine, L-alanine and glycine. Slaughter and Davies (1967) found that 3-phosphoglycerate dehydrogenase loses the sensitivity to feedback inhibition as time elapses. Even the fresh enzyme from N. corallina was not affected by any of these three compounds, indicating the possibility that the enzyme activity may be controlled by some other means in this organism.

Finally, the occurrence of D-3-glycerate dehydrogenase in N. corallina was investigated. No activity of this enzyme was detected at the substrate concentration of 0.83 mM glycerate, while at the same concentration of phosphoglycerate, D-3-phosphoglycerate dehydrogenase showed an activity of 0.020 Δ OD/min. At a concentration ten times greater (8.3 mM glycerate), activity of D-3-glycerate dehydrogenase was slight or nonexistent, implying that this enzyme is lacking or in small quantities

in this organism compared to D-3-phosphoglycerate dehydrogenase and that serine biosynthesis of this bacterium may be accomplished mainly through the phosphorylated pathway rather than the alternative non-phosphorylated pathway.

Discussion

It is quite remarkable that D-3-phosphoglycerate dehydrogenase from the mold-like bacterium Nocardia corallina is not regulated by L-serine, whereas the same enzyme from bacteria E. coli (Pizer, 1963) and S. typhimurium (Umbarger and Umbarger, 1962) is sensitive to the feedback inhibition by serine. It has been reported that the animal phosphoglycerate dehydrogenase is not inhibited by L-serine (Willis and Sallach, 1964). If inhibition of the enzyme by L-serine is a general property of bacteria, the N. corallina enzyme is unique.

For N. corallina and animals where D-3-phosphoglycerate dehydrogenase is not regulated by the end product, serine, a question arises as to how the rate of serine biosynthesis may be controlled in the cells. Besides being utilized directly for protein synthesis, serine may be converted to pyruvate, alanine or glycine. Assuming that physiological concentrations of serine within the cells should be kept at a constant, optimal level for the survival of the organism, one can speculate that when the amount of serine exceeds this optimal level, serine could act as a repressor against the synthesis of D-3-phosphoglycerate dehydrogenase in order to reduce the rate of serine synthesis and at the same time act also as derepressor for the synthesis of the enzymes related to the serine catabolism; namely, enzymes which catalyze the reactions of serine to pyruvate, alanine and glycine. This

reduces the amount of serine to the optimal level. When the serine concentration is lower than the optimal level, the reverse process may take place; serine increases the amount of D-3-phosphoglycerate dehydrogenase through derepression and decreases the catabolic enzymes through repression. It is also possible that in the future some other metabolite may be found which controls the rate of serine biosynthesis.

The major pathway of serine biosynthesis in E. coli and S. typhimurium is considered to be the phosphorylated pathway. This also seems to be the case with the N. corallina enzyme, for it is in good agreement with experimental evidence that D-glycerate dehydrogenase, which is involved in the non-phosphorylated pathway, was found to be absent or nearly so in N. corallina, whereas D-3-phosphoglycerate dehydrogenase, which catalyzes the first step of the phosphorylated pathway, was detected in great quantities.

Summary

D-3-phosphoglycerate dehydrogenase from Nocardia corallina was partially purified and the properties of the enzyme were studied. The pH profile of this enzyme was drawn and the pH optimum was determined to be around 9.5. The plot of the reaction rates vs. the substrate D-3-phosphoglycerate showed a non-sigmoidal, simple Michaelis-Menten saturation curve. The Michaelis constant K_m was found to be 0.87 mM.

The enzyme was not sensitive to feedback inhibition by the end product, serine, unlike other bacterial (E. coli and S. typhimurium) enzymes. Even the freshly prepared enzyme was not inhibited by 6.6 mM L-serine.

With the idea that some other metabolic compounds may inhibit the enzyme, 3.3 mM glycine and 3.3 mM alanine were studied for their effect on the enzyme. Neither compound had any effect.

The activity of D-glycerate dehydrogenase from N. corallina was detected as very low or absent, whereas under the same conditions great activity of D-3-phosphoglycerate dehydrogenase was found, indicating that the phosphorylated pathway, rather than the non-phosphorylated one, is the major route of serine biosynthesis in this organism.

CHAPTER III

GLUTAMINE SYNTHETASE FROM TOBACCO TISSUE

Introduction

Glutamine synthetase from various microorganisms, especially E. coli, has been extensively studied. The regulatory properties of the enzyme from microorganisms are quite diverse and complex. The importance of this enzyme for biosynthesis of various amino acids and nucleotides cannot be overemphasized. Metabolic control of glutamine synthetase in higher plants, however, has not yet been investigated.

The properties and possible metabolic regulation of glutamine synthetase from tobacco tissue were studied with the assumption that glutamine synthetase from the higher plant may be significantly different from that of the microorganisms investigated so far. Purification of the enzyme was attempted by various methods.

Tobacco wildfire toxin, produced by the phytopathogenic bacterium Pseudomonas tabaci and methionine sulphoximine (MSO) induce chlorosis in leaves of higher plants (Braun, 1955). In aminals, the wildfire toxin causes convulsions identical to those produced by MSO (Sinden, Durbin, Uchytil and Lamar, 1969). It was found that these two compounds also

inhibit cerebral glutamine synthetase (Lamar, Sinden and Durbin, 1968; Sellinger and Weiler, 1963). The mechanism of inhibition of glutamine synthetase from sheep brain by MSO has been studied in detail (Ronzio and Meister, 1968; Ronzio, Rowe and Meister, 1969). Glutamine synthetase from sheep brain is markedly inhibited by MSO and this inhibition requires the presence of ATP and Mg^{++} or Mn^{++} and is irreversible. The inhibited enzyme has been isolated by gel filtration and found to contain about 8 moles each of a phosphorylated MSO and ADP, which are tightly bound to the enzyme.

The effects of MSO and wildfire toxin isolated from P. tabaci on glutamine synthetase from peas and on intact tobacco leaves have also been investigated by Sinden and Durbin (1968). MSO and wildfire toxin inhibit strongly the pea glutamine synthetase and cause chlorosis in tobacco leaves. Because of the similarities exhibited by the two compounds in plants and animals, and the observation of inhibition of glutamine synthetase, a study of the effect of MSO on glutamine synthetase from tobacco tissue was undertaken. The experiment was stimulated by Phil Schafer, who is studying the effect of MSO on the growth of tobacco tissue as a graduate student at Oklahoma University.

Materials and Methods

Growth of Cultures

The tobacco tissue culture used for the following experiments was a suspension culture line WR-132 (Nicotiana tabacum L., var. Xanthi) obtained from Dr. A. C. Olson, Plant Enzyme Pioneering Research Laboratory, United States Department of Agriculture, Albany, California. The cells (1.6 - 2g) were aseptically transferred to 50 ml medium in a 125 ml flask. Transfers were made in a laminar flow hood (Agnew-Higgins). The cells in the flask were grown in constant agitation on a reciprocal shaker (Eberback; 95 - 105 reciprocations/min) for 10 days at room temperature.

The composition of the medium used to grow the plant cells was as follows (Linsmaier and Skoog, 1965):

Compound	mg/l	Compound	mg/l
NH_4NO_3	1650	$Na_2MoO_4 \cdot 2H_2O$	0.25
KNO_3	1900	$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440	$CoCl_2 \cdot 6H_2O$	0.025
$MgSO_4 \cdot 7H_2O$	370	Sucrose	30000
KH_2PO_4	170	myo-Inositol	100
Na_2EDTA	37.3	Glycine	20
$FeSO_4 \cdot 7H_2O$	27.8	Thiamine·HCl	1
H_3BO_3	6.2	Niacin	5
$MnSO_4 \cdot 4H_2O$	22.3	Pyridoxine·HCl	5
$ZnSO_4 \cdot 4H_2O$	8.6	2,4,-Dichlorophenoxy-acetic acid	0.5
KI	0.83		

After 10 days of growth, the cells were collected by suction filters and thoroughly washed with 0.1 M imidazole-0.03 M β-mercaptoethanol buffer (pH 6.5). Then the cells were used for preparation of the enzyme solution or were frozen for future use.

Enzyme Preparation

Forty grams of tobacco tissue cells was mixed with 40 g glass beads, 25 g polyclar AT which had been hydrated with deionized water overnight, 0.8 ml of 200 mM EDTA and 80 ml of cold 0.1 M imidazole-0.03 M β-mercaptoethanol buffer (pH 6.5). This mixture was blended for 10 min in a blender (Sorvall Omnimixer) at 5000 rpm. The mixer cup was immersed

in ice water during the blending. This crude enzyme solution was centrifuged at 34,800 X g for 10 min to remove the unwanted glass beads, polyclar and cell debris. All operations concerned with the preparation of the enzyme were performed at approximately 4°C unless otherwise indicated.

To the supernatant thus obtained, solid $(NH_4)_2SO_4$ (17 g per 100 ml of preparation) was slowly added with constant stirring. The stirring was continued for 5 min and the precipitated protein was collected by refrigerated centrifugation at 34,800 X g for 10 min and discarded. The remaining supernatant solution was brought to about 70% saturation in $(NH_4)_2SO_4$ by slowly adding additional $(NH_4)_2SO_4$ to the level of 46.8 g per 100 ml of preparation. The precipitated protein was collected by refrigerated centrifugation at 34,800 X g for 10 min and resuspended in 5 ml of 0.1 M imidazole HCl-0.03 M β-mercaptoethanol buffer (pH 6.5) and dialyzed overnight against 500 ml of the same buffer. After dialysis the solution was diluted with an appropriate volume of buffer to obtain a final concentration of 1.5 mg of protein per ml of preparation. This solution was used for all the assays presented in this dissertation.

The polyclar AT used was washed with various solvents. First, 250 g of the polyclar was placed in 1000 ml of distilled water for 1 hr and suction-filtered. It was then placed in the following solvents consecutively to be soaked for 30 min and washed and filtered: 385 ml dimethylformamide, 770 ml acetic acid, 2310 ml water, 1540 ml methanol, and finally, 2000 ml water. The wet polyclar AT was air-dried for three days. This polyclar was placed in deionized water overnight to be hydrated before use. Polyclar AT was not used for the attempts at enzyme purification,

because it was found that it was not necessary for preparation of the enzyme solution.

Enzyme Assay

The transferase activity of glutamine synthetase was determined by following the formation of γ-glutamylhydroxamate from L-glutamine and hydroxylamine in the presence of other cofactors and substrates. γ-glutamylhydroxamate forms a colored complex with ferric ion and the color intensity was measured spectrophotometrically.

The 3 ml standard reaction mixture contained (Stumpf, Loomis and Michelson, 1951): 5×10^{-4} M $MnCl_2$; 0.01 M sodium arsenate; 0.09 M L-glutamine (Sigma); 1×10^{-4} M ADP (Sigma); 0.01 M NH_2OH; and the enzyme.

The reaction mixture was incubated at room temperature for 20 min. Under this condition the reaction rate was linear for at least 30 min. The reaction was stopped and the color was developed by adding 0.75 ml of a solution containing equal volumes of 24% trichloracetic acid, 6 N HCl, and 10% $FeCl_3 \cdot 6H_2O$ in 0.02 N HCl. The optical density of the solution was measured at 540 mμ.

For the attempts at purification of glutamine synthetase, the forward assay was used. The reaction mixture for a typical assay consisted of the following (Woolfolk, Shapiro and Stadtman, 1966): 0.1 M L-glutamate; 7.6 mM ATP; 0.05 M NH_4Cl; 0.05 M $MgCl_2$; 0.1 M imidazole buffer (pH 6.5); and 0.1 ml of the enzyme solution. The total volume of the solution was 0.4 ml. The reaction was initiated by the addition of the enzyme. The reaction mixture was incubated and the reaction was stopped. Then the color was developed in the same manner as described for the enzyme in

Chapter I.

For the above standard reaction mixture without the substrate L-glutamate, the color intensity observed was considered a function of ATPase activity. The color intensity observed for the standard reaction mixture was considered a function of the sum of ATPase and glutamine synthetase activities. The ratio of ATPase activity to the sum of ATPase and glutamine synthetase activities as well as the specific activity was used as an indicator of the degree of enzyme purity during processes of purification attempts of glutamine synthetase.

Enzyme Purification Attempts

All operations concerned with the preparation and purification attempts of the enzyme were performed at approximately $4^{o}C$ in order to keep denaturation of the enzyme to a minimum. The buffer used for the following processes was 0.1 mM imidazole buffer unless otherwise indicated.

Fractional $(NH_4)_2SO_4$ precipitation was done by addition of solid $(NH_4)_2SO_4$ to the enzyme solution with constant stirring to the desired levels of saturation. Protamine sulfate treatment was accomplished by addition of solid protamine sulfate to the enzyme solution in such a way as to obtain 0.3% solution in protamine sulfate. The precipitate formed was eliminated by centrifugation. Heat treatment was performed by first immersing a small 5 ml test tube of the enzyme solution for the desired time periods in water at the specified temperature. This was then followed by cooling of the solution in tap water to room temperature. Then the solution was immediately assayed. For isoelectric pH precipitation, the pH of the enzyme solution was adjusted to 5.0 with 0.2 N

acetic acid and the precipitate formed was collected by centrifugation and dissolved in the buffer and assayed (Levintow and Meister, 1954). It was assumed that glutamine synthetase from tobacco tissue has an isoelectric point similar to that from peas. For batch-wise DEAE cellulose treatment, 0.1 ml of the enzyme solution (26 mg protein/ml) was added to each test tube containing 3 ml DEAE-cellulose in suspension in buffer at different pH's. The test tube was shaken occasionally for 5 min. The suspension was centrifuged and the supernatant was assayed. Batch-wise cellulose phosphate treatment was performed by addition of 0.5 ml of enzyme solution (1.5 mg protein/ml) to a test tube containing 5 ml of cellulose-phosphate suspension in buffer adjusted to pH 5. The test tube was shaken occasionally for 5 min. The suspension was centrifuged and the supernatant was assayed. The centrifuged cellulose-phosphate in the test tube was washed with 2.5 ml of fresh buffer at different pH's consecutively, in increasing order of pH values. The test tube was shaken each time for 5 min, then centrifuged and the supernatant was assayed. For the Sephadex G-150 column, a reverse-flow type column was used with a column bed volume of about 200 ml. The flow rate was adjusted to approximately 5 ml/hr and 3 - 4 ml of the enzyme solution (28 mg protein/ml) was placed in the column. For elution, 0.1 M imidazole buffer (pH 6.5) was used and the eluting solution was collected in 5 ml aliquots by the fractional collector (Gilson) and then assayed.

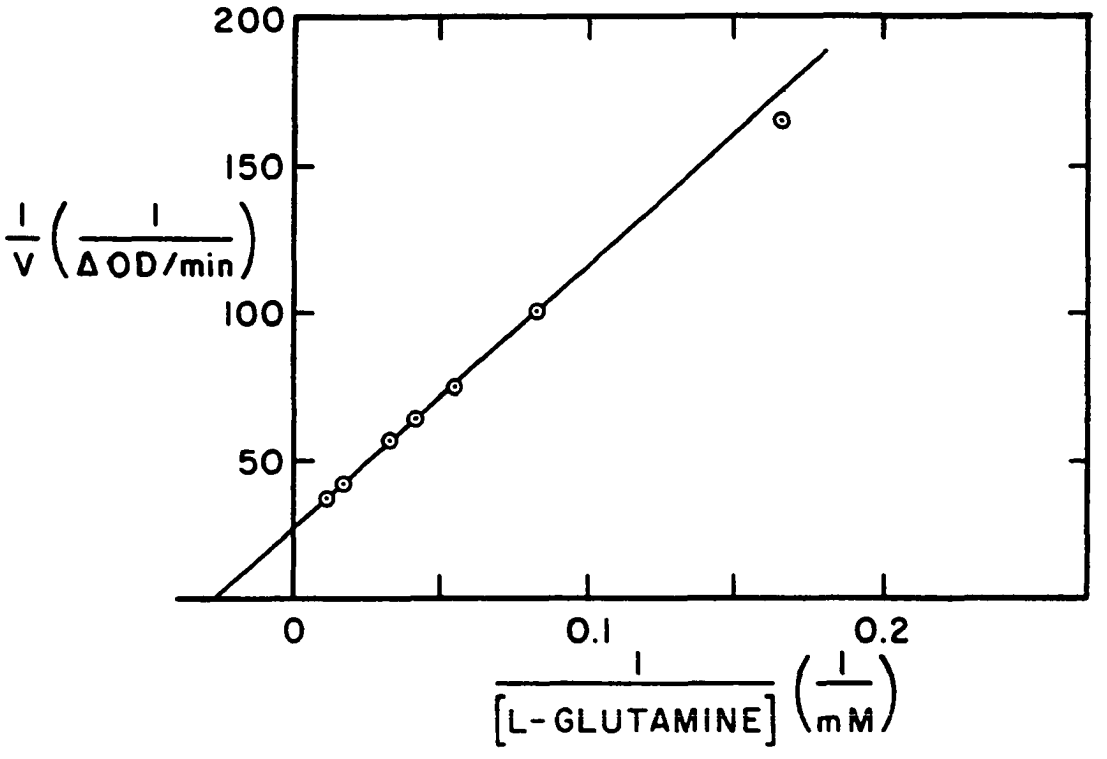
Protein Determination

Protein concentrations were estimated by the micro-biuret method (Itzhaki and Gill, 1964) using bovine serum albumin as a standard.

Results

The pH optimum of the enzymic reaction was determined to be at 6.0 - 6.5. The plot of the substrate (L-glutamine) concentration vs. initial velocity was hyperbolic, indicating a non-sigmoidal, simple Michaelis-Menten saturation curve as shown in Fig. 3-2. The Michaelis constant K_m was found to be 33 mM from the reciprocal plot of substrate concentration vs. reaction rate (Fig. 3-1).

In an attempt to determine the significance of glutamine synthetase in the plant tobacco tissue, thirteen metabolic compounds that may be related to glutamine metabolism were studied for their effects on the transferase activity of glutamine synthetase from the plant. The results are summarized in Table 3-1, which indicates the percentage of inhibition by these compounds at different concentrations. The physiological concentrations of these compounds in tobacco tissue are not known but are expected to be not much higher than 5 mM since this is the concentration of some metabolic compounds found in various plants. For example, the concentrations of amino acids, glutamic acid and aspartic acid in various plant tissues are about 4 mM to 0.5 mM as shown in Table 3-2 (MacLennan, Beevers and Harley, 1963), assuming that 90% of plant tissue consists of water and compartmentation of the compounds in plant tissue would not change the concentrations drastically. The content of glucose in pea cotyledon tissue is about 2 mM (Brown and Wray, 1968). Therefore, it is much more proper to discuss the possible regulatory properties of the enzyme at 5 mM concentrations of those compounds tested than at 10 mM or 20 mM, except the purine and pyrimidine nucleoside triphosphates CTP, GTP, ITP and ATP that inhibited the enzyme completely at their concentration of 5 mM (100% inhibition).



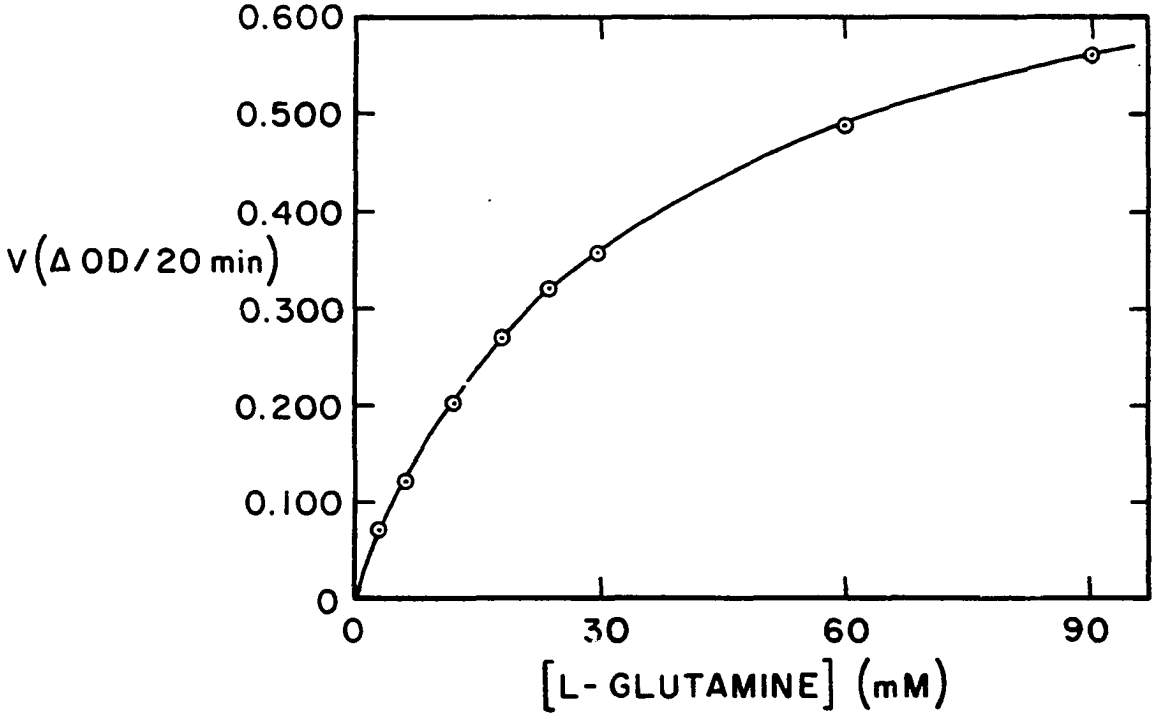


Fig. 3-1. (upper) A double reciprocal plot of reaction velocity vs. glutamine concentration for transferase activity of glutamine synthetase from tobacco tissue.

Fig. 3-2. (lower) The saturation function of L-glutamine for transferase activity of glutamine synthetase from tobacco tissue.

TABLE 3-1

PERCENTAGE OF INHIBITION OF GLUTAMINE TRANSFERASE ACTIVITY OF GLUTAMINE SYNTHETASE FROM TOBACCO TISSUE

Compounds	% Inhibition		
	20 mM	10 mM	5 mM
L-alanine	79%	65%	52%
	20 mM	10 mM	5 mM
Glycine	57	33	19
	20 mM	10 mM	5 mM
L-tryptophan	13	12	0
	20 mM	10 mM	5 mM
L-histidine	49	32	20
	20 mM	10 mM	5 mM
Glucosamine-6-P	37	31	20
	20 mM	10 mM	5 mM
Carbamyl-P	86	72	55
	20 mM	10 mM	5 mM
AMP	31	14	0
	20 mM	10 mM	5 mM
NAD^+	35	20	3
	20 mM	10 mM	5 mM
$NADP^+$	80	35	18
	5 mM	0.5 mM	
CTP	100	32	
	5 mM	0.5 mM	
GTP	100	17	
	5 mM	0.5 mM	
ITP	100	30	
	5 mM	0.5 mM	
ATP	100	33	

Note. The different concentrations for each inhibitor used are underlined. Standard conditions of the transfer assay were employed, except that 0.03 M glutamine was used.

TABLE 3-2

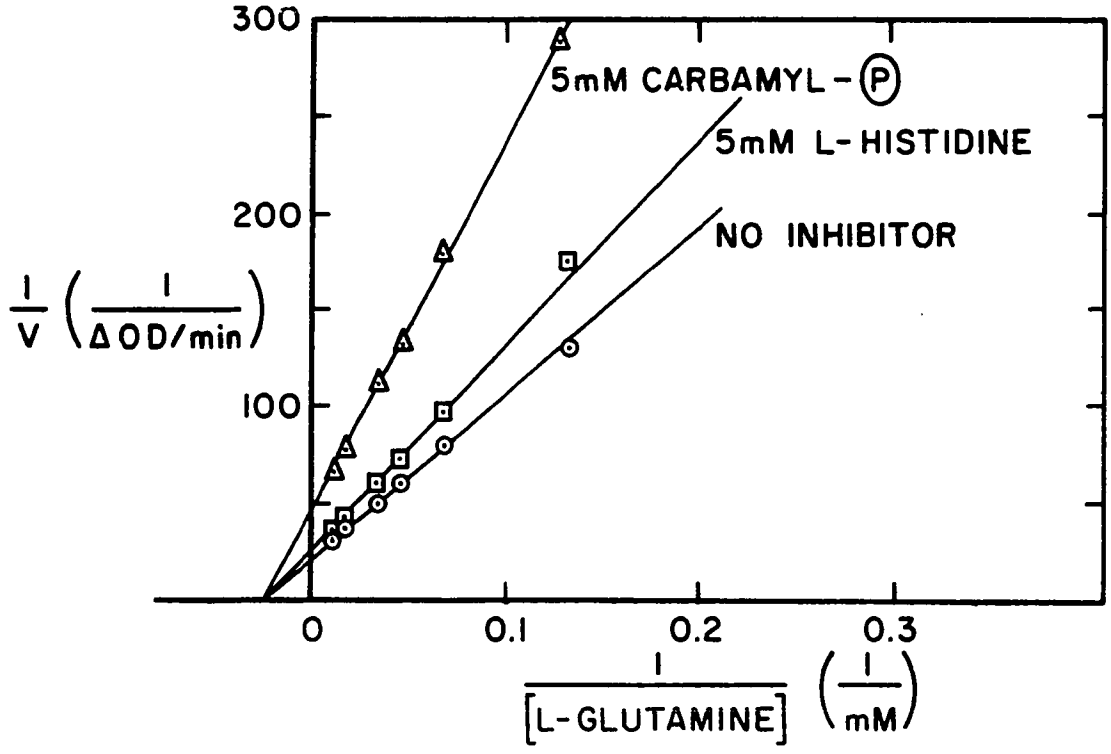
THE CONTENTS OF GLUTAMIC ACID AND ASPARTIC ACID IN VARIOUS PLANT TISSUES (μ moles/g fresh wt)

	Maize Coleoptile	Carrot Root	Beet Root	Wheat Leaf
Glutamic Acid	1.9	2.3	1.5	2.6
Aspartic Acid	2.3	4.1	0.5	1.5

Note. Data were taken from MacLennan, D. H., Beevers, H., and Harley, J. L., Biochem. J. 89, 316 (1963).

The percentage of inhibition at 10 mM and 20 mM of the compounds were obtained to determine if inhibition for each compound at 5 mM was saturating or not. Higher concentrations than 20 mM were not used because they are outside the expected range of physiological concentration. It is clear from Table 3-1 that inhibitions caused by the compounds tested, except the nucleoside triphosphates, were not saturating at 5 mM and increased at higher concentrations. The concentration of 0.5 mM for purine and pyrimidine nucleoside triphosphates was chosen because 5 mM of these compounds showed 100% inhibition and was not suitable for study of regulation and because it seems to be close to the expected physiological concentration: the concentration of ATP in pea cotyledon tissue is about 0.3 mM. Consequently, attention was paid mainly to the results at 0.5 mM for the purine and pyrimidine nucleoside triphosphates and at 5 mM for other compounds.

The reciprocal plots for those compounds that inhibited the enzyme definitely at 5 mM are shown in Fig. 3-3 to Fig. 3-7. Since NAD^+



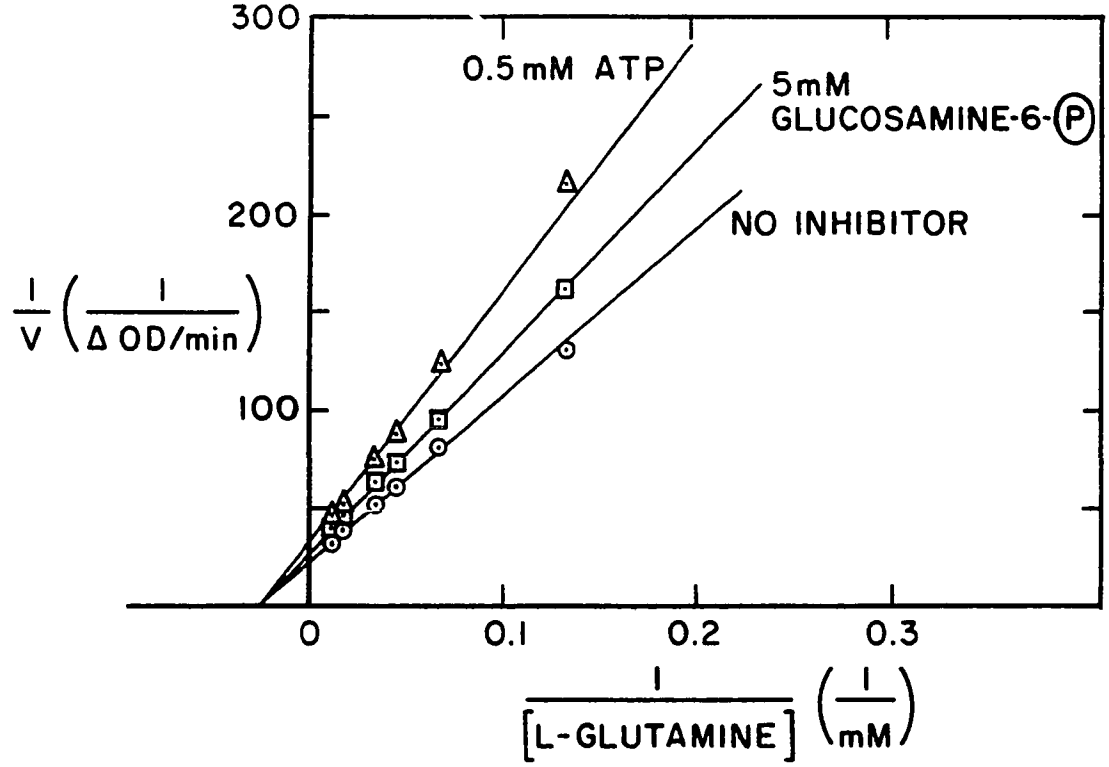
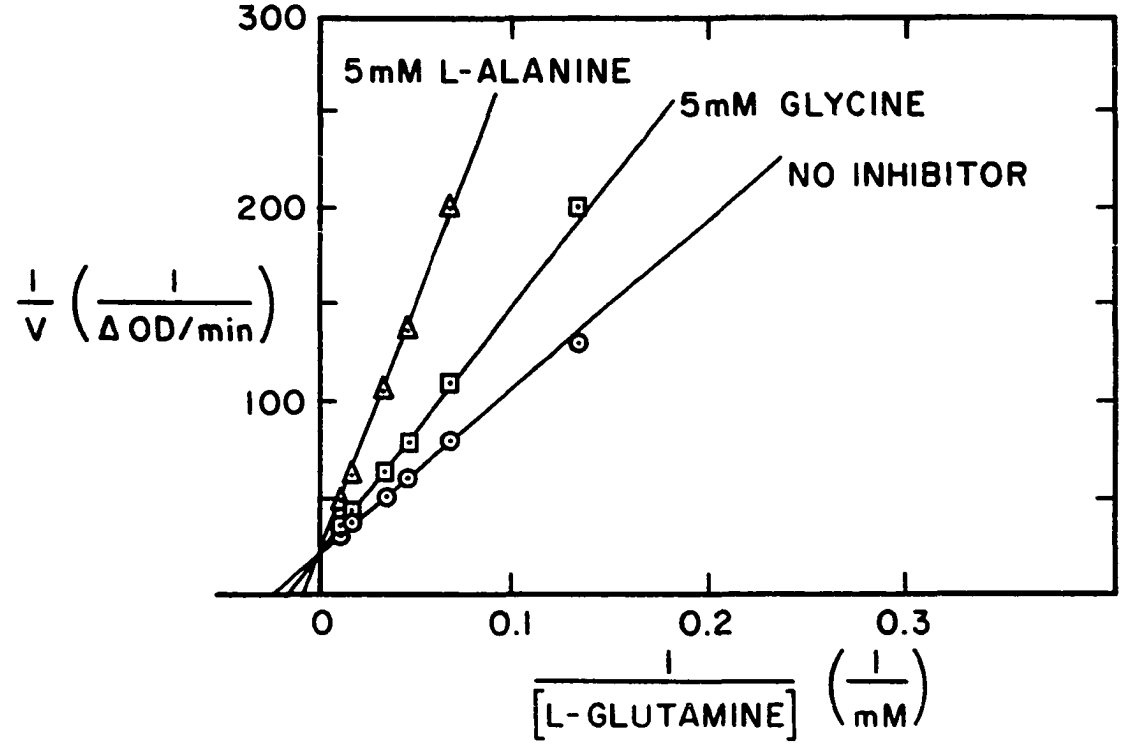


Fig. 3-3. (upper) Double reciprocal plots of reaction velocity vs. glutamine concentration in the presence of fixed concentrations of carbamyl phosphate and L-histidine for transferase activity of glutamine synthetase from tobacco tissue.

Fig. 3-4. (lower) Double reciprocal plots of reaction velocity vs. glutamine concentration in the presence of fixed concentrations of ATP and glucosamine-6-phosphate for transferase activity of glutamine synthetase from tobacco tissue.



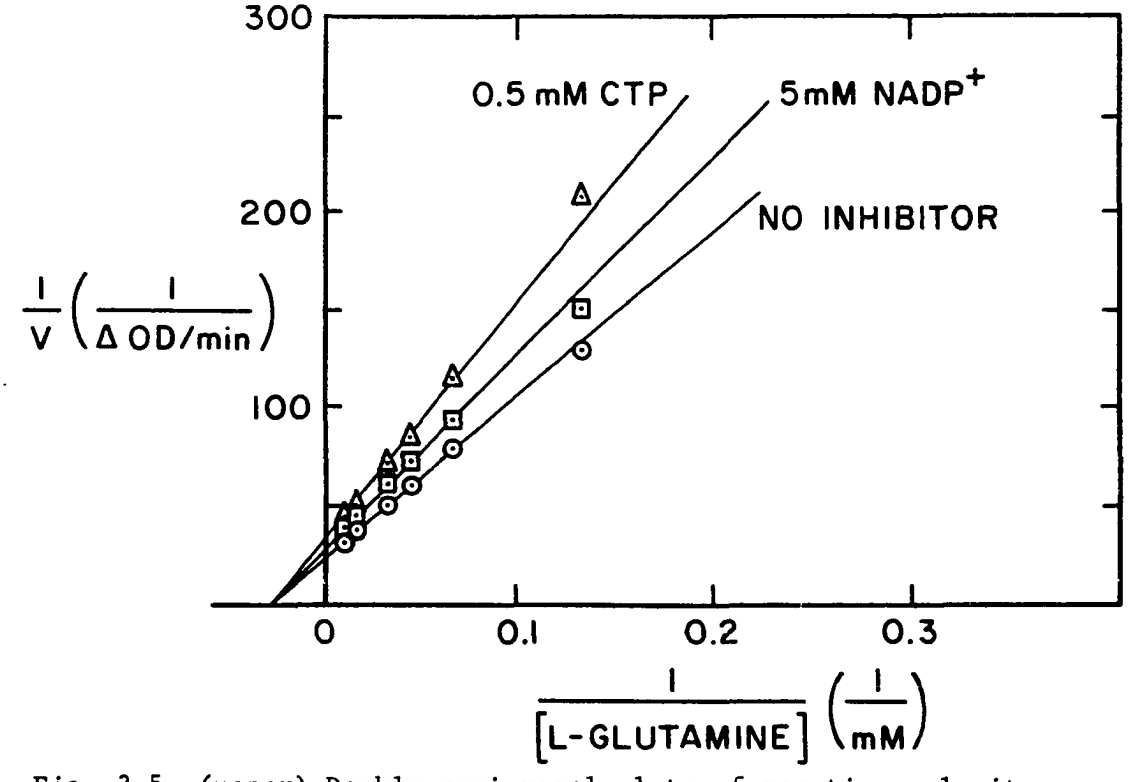


Fig. 3-5. (upper) Double reciprocal plots of reaction velocity vs. glutamine concentration in the presence of fixed concentrations of L-alanine and glycine for transferase activity of glutamine synthetase from tobacco tissue.

Fig. 3-6. (lower) Double reciprocal plots of reaction velocity vs. glutamine concentration in the presence of fixed concentrations of CTP and $NADP^+$ for transferase activity of glutamine synthetase from tobacco tissue.

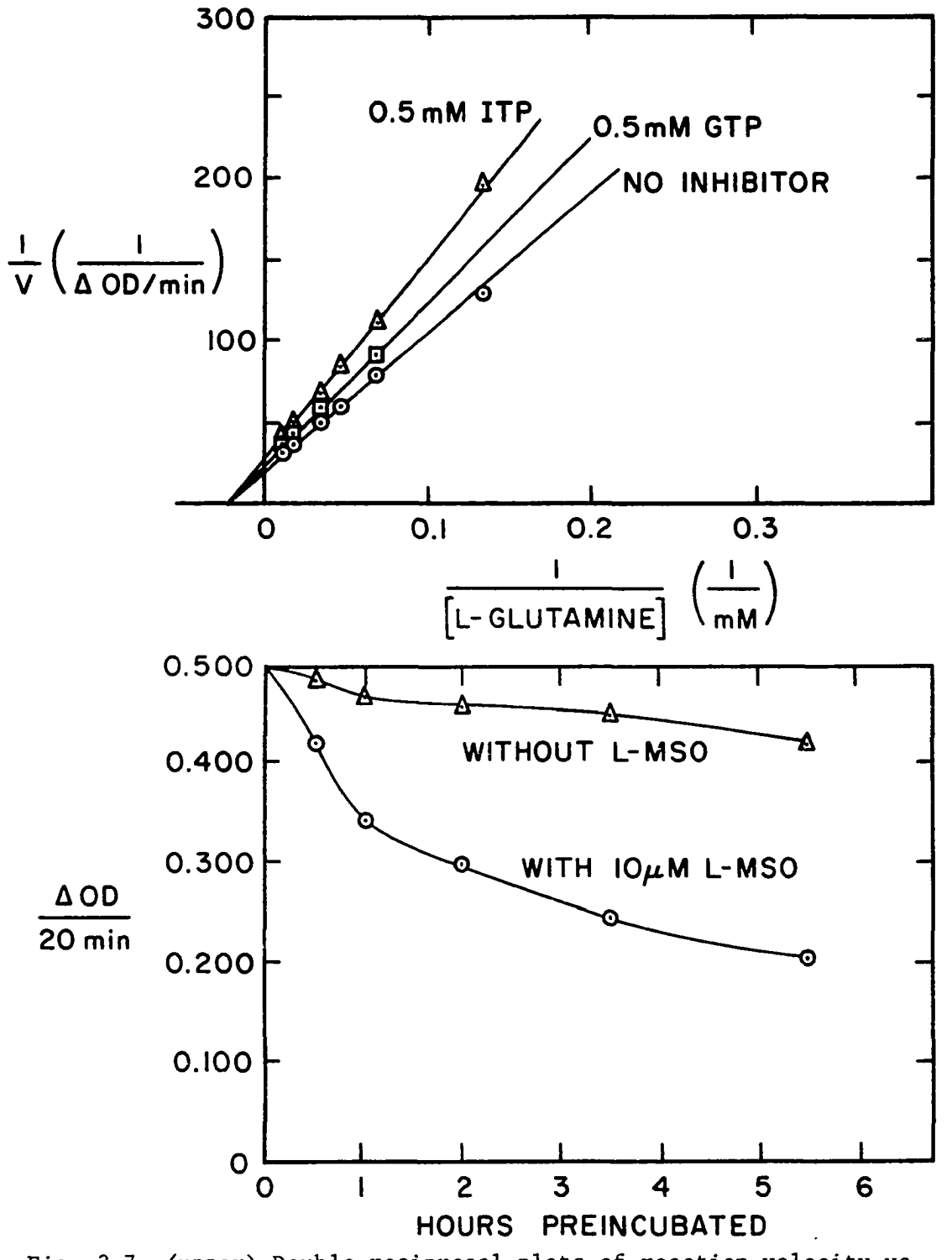


Fig. 3-7. (upper) Double reciprocal plots of reaction velocity vs. glutamine concentration in the presence of fixed concentrations of ITP and GTP for transferase activity of glutamine synthetase from tobacco tissue.

Fig. 3-8. (lower) Transferase activity of glutamine synthetase from tobacco tissue at different time periods of preincubation with and without L-methionine sulphoximine (L-MSO).

demonstrated very little inhibition at the concentration of 5 mM, the reciprocal plot for it was not included in those figures.

Among thirteen compounds tested, two compounds, AMP and L-tryptophan, did not display any effect on the enzyme at concentrations of 5 m M.

It has been reported that the same enzyme from the mold N. crassa is not inhibited by L-tryptophan (Kapoor and Bray, 1968), whereas the enzyme from the bacterium E. coli is inhibited (Woolfolk and Stadtman, 1964). It is quite unique that AMP did not show any effect on the enzyme at 5 mM from the plant, because the enzyme from microorganisms is known to be sensitive to AMP.

The strongest inhibitors among the compounds tested were four purine and pyrimidine nucleoside triphosphates, CTP, ITP, GTP, and ATP. In fact, they showed 100% inhibition of the enzyme when 5 mM was used. Even at the 0.5 mM level, these four compounds demonstrated a definite effect on the enzyme, as can be seen from Table 3-1.

L-alanine and glycine, which inhibit the E. coli enzyme at 5 mM, also displayed a definite effect on the enzyme from the plant at a concentration of 5 mM. The reciprocal plot for glycine showed a straight line, unlike the dimodal line found for E. coli and N. corallina enzymes. These two compounds were the only ones that inhibited the enzyme competitively.

L-histidine and glucosamine-6-phosphate were found to be moderate inhibitors of the plant enzyme.

Carbamyl phosphate at 5 mM demonstrated 54% inhibition and was revealed as the strongest inhibitor after the four purine and pyrimidine

nucleoside triphosphates.

It is interesting that both N. corallina and tobacco tissue enzymes were found to be sensitive to NAD^+ and $NADP^+$, although NAD^+ did not have much effect on the tobacco tissue enzyme at 5 mM. However, NAD^+ inhibited the bacterial enzyme to a greater extent than did $NADP^+$, while it inhibited the plant enzyme much less than $NADP^+$.

In order to investigate and elucidate the possible mode of action of wildfire toxin, the influence of L-methionine sulphoximine (MSO) on glutamine synthetase from tobacco tissue was studied.

The transferase activity of the plant glutamine synthetase was determined at different concentrations of L-methionine sulphoximine. The same experiment was also done preincubating the enzyme solution for 30 min at room temperature with MSO, all cofactors and substrates, except L-glutamine. The results are shown in Table 3-3, which indicates the percentage of inhibition of the enzyme by MSO.

TABLE 3-3

EFFECT OF METHIONINE SULPHOXIMINE (MSO) ON GLUTAMINE TRANSFERASE ACTIVITY OF GLUTAMINE SYNTHETASE FROM TOBACCO TISSUE

MSO	Preincubated (30 min at room temp)	Not Preincubated
5 μM	11%	0%
10 μM	14%	0%
25 μM	27%	0%
1 mM	87%	9%

Note. Standard conditions of the transfer assay were employed. Preincubation of the enzyme was done in the standard reaction mixture without L-glutamine for 30 min at room temperature.

The transferase activity of glutamine synthetase from tobacco tissue was definitely inhibited by L-MSO. Preincubation of the enzyme with MSO increased inhibition. With the preincubated enzyme, inhibition was apparent at as low a concentration as 5 μM of MSO, whereas it became apparent at the MSO concentration of 1 mM with the non-preincubated enzyme.

The mode of binding of MSO with the enzyme was also investigated. The enzyme solution was preincubated with 10 μM MSO for different periods of time and the enzyme activity was determined and compared with activity of the enzyme solution preincubated for the same periods of time without MSO as a control. It was found that the longer the enzyme solution was preincubated with MSO, the greater the inhibition of the enzyme, implying irreversible binding of MSO to the enzyme: 30 min preincubation, 14% inhibition, 1 hr, 27%; 2 hr, 36%; 3.5 hr, 47%; 5.5 hr, 52%. These results are also graphically shown in Fig. 3-5. Similar results were obtained for glutamine synthetase from sheep brain by Ronzio, Rowe and Meister (1969), except that the enzyme was preincubated with a higher concentration (1 mM) of MSO for shorter periods of time (32 min).

In order to confirm the observation that MSO binds to the enzyme irreversibly and inhibits it, the enzyme solution which had been preincubated with 10 μM MSO for 5.5 hr was dialyzed overnight against 0.05 M imidazole buffer (pH 6.5) and was assayed for its activity. When it was compared with the control it was found that 70% inhibition of the enzyme by MSO occurred, revealing that MSO does, in fact, bind with the enzyme irreversibly, causing inhibition of the enzyme. If it had not been irreversibly binding, all activity of the enzyme should have been regained after dialysis.

For the purpose of confirming all the results obtained above by transfer assay, the use of the forward assay for the enzyme was attempted. Because of the great amount of ATPase present in the enzyme solution, accurate measurement of glutamine synthetase activity was unsuccessful by this method. Therefore, purification of glutamine synthetase was attempted by various methods. The results are shown in Table 3-4, indicating procedures used and corresponding ratios of ATPase activity to the sum of ATPase and glutamine synthetase activities determined by the forward assay. The ratios never became smaller than that for the original extract, revealing that none of the methods were successful for the removal of ATPase activity. Besides, the specific activity of the enzyme during all the purification processes was always lower than that of the original extract.

TABLE 3-4

SUMMARY OF PURIFICATION ATTEMPTS

Procedure	ATPase/ATPase + G.S.*
Crude extract	0.80 - 0.90
90% $(NH_4)_2SO_4$ ppt.	0.82 - 0.90
40% $(NH_4)_2SO_4$ ppt.	0.81 - 0.88
pH precipitation (pH 5.0)	0.94
Heat treatment (2 min at 50°C)	0.98
Heat treatment (4 min at 50°C)	1.04
Batch-wise cellulose-phosphate treatment	0.85 - 0.95
Batch-wise DEAE-cellulose treatment	0.85 - 0.96
Sephadex G-150 treatment	0.84 - 0.94

Note. *G.S. stands for glutamine synthetase.

Discussion

There are two main assay methods commonly used for glutamine synthetase; forward assay and transfer assay. The E. coli enzyme has been studied with both assay methods by Stadtman et al. (1967). Their study reveals that the results obtained by these two assay methods parallel each other quite well. For example, the degree of inhibition of the enzyme by 5 mM alanine was 30% by both forward and transfer assay; inhibition by 5 mM tryptophan, 15% by both assays; inhibition by 3 mM AMP, 26% by forward and 30% by transfer assay. This means that either assay method can be used to study the properties of glutamine synthetase, although the forward assay is preferable because all the substrates used for it are metabolic compounds, whereas hydroxylamine used for the transfer assay is a non-metabolic compound.

In this experiment the transfer assay was used because the great amount of ATPase activity present compared to glutamine synthetase activity (Table 3-4) in the enzyme solution made an accurate forward assay impossible. The forward assay of this enzyme must await the further purification of the enzyme, although the results of various purification attempts indicate that in order for further attempts at purification of this enzyme to be successful other methods or different conditions will be necessary. It may also be possible that ATPase and glutamine synthetase may be parts of one enzyme and that the two cannot be separated.

The forward assay for glutamine synthetase from N. corallina in Chapter I was possible because very little ATPase was present in the enzyme preparation from that microorganism.

The regulatory properties of glutamine synthetase from various microorganisms have been explored in much detail by other investigators, but those of the plant enzyme have not been reported so far. The findings on the regulatory properties of glutamine synthetase from tobacco tissue imply that the plant enzyme is unique in more than one way, compared with the microbial enzymes.

Glutamine synthetase from tobacco tissue is unique in that it was not inhibited by AMP at 5 mM, while AMP has been revealed to inhibit the enzyme from the bacterium E. coli, the mold N. crassa and other sources. The enzyme is one of a kind, also, in that very low concentrations (0.5 mM) of the purine and pyrimidine nucleoside triphosphates, CTP, GTP, ITP and ATP have a definite effect on the enzyme and that these compounds at 5 mM levels display complete inhibition of the enzyme. The transferase activity of E. coli glutamine synthetase has been reported (Woolfolk and Stadtman, 1967) to be inhibited 25% by 3 mM CTP, whereas the enzyme activity of the plant was inhibited 32%, but by a much lower concentration (0.5 mM) of CTP. In the cumulative inhibition found for the E. coli enzyme, none of eight metabolic compounds tested alone inhibited the enzyme, but inhibition of the enzyme was exhibited when all the inhibitors were present together. Since the purine and pyrimidine nucleoside triphosphates each inhibited the plant enzyme activity completely at 5 mM, the enzyme is not sensitive to cumulative inhibition.

The plant enzyme showed similarity to the mold N. crassa enzyme in that it was not inhibited by L-tryptophan at the 5 mM level, whereas bacterial enzymes were inhibited.

The plant enzyme also revealed similarity to the enzyme from microorganisms in its sensitivity to L-alanine, glycine, L-histidine, glucosamine-6-phosphate and carbamyl phosphate, but the degree of sensitivity to these compounds differs from the enzyme from other sources.

The significance of inhibition by these compounds with respect to glutamine metabolism was discussed in Chapter I on glutamine synthetase from N. corallina. Therefore, the discussion of it for the plant enzyme is omitted here.

Investigations on the influence of the wildfire toxin on glutamine synthetase from peas has been studied in vitro by Sinden and Durbin (1968). They found that the wildfire toxin inhibited the transferase activity of glutamine synthetase from peas.

The fact that the transferase activity of glutamine synthetase from tobacco tissue was irreversibly inhibited by MSO suggests that inhibition of glutamine synthetase may be the primary site of action of wildfire toxin within the plant cell. The great similarity between the activities of L-MSO and wildfire toxin in plants (Braun, 1955) implies that these two compounds may be acting on the same metabolic pathway, causing chlorosis in plants. Inhibition of glutamine synthetase in higher plants would result both in a depletion of glutamine and in a build-up of reduced nitrogeneous intermediates from nitrate to ammonia. It has been reported (Puritch and Barker, 1967; Krogmann, Jagendorf and Avron, 1959) that when the ammonium levels present in leaves of plants is high, uncoupling of photophosphorylation occurs, consequently reducing the energy available for chlorophyll, protein and RNA syntheses. In tomato leaves excess ammonia is toxic and rapidly causes structural mod-

ifications of the chloroplasts, resulting in chlorosis (Puritch and Barker, 1967). Thus, the chlorosis caused by MSO or wildfire toxin may be due to the build-up of toxic intermediates in nitrate metabolism rather than to a depletion of glutamine.

Summary

Glutamine synthetase from tobacco tissue was studied for its properties by transfer assay. The pH optimum of this enzymic reaction was determined to be 6.0 - 6.5. The plot of the substrate (L-glutamine) vs. reaction rate was a non-sigmoidal, simple Michaelis-Menten saturation curve. The Michaelis constant K_m was 33 mM.

In an attempt to elucidate the significance of glutamine synthetase in the plant tissue, thirteen metabolic compounds that may be related to glutamine metabolism were tested for their effects on the enzyme. Only two compounds, AMP and L-tryptophan, did not display any effect on the enzyme at the 5 mM level. The strongest inhibitors were the purine and pyrimidine nucleoside triphosphates CTP, ITP, GTP and ATP. In fact, ATP and CTP at 5 mM showed 100% inhibition of the enzyme. Comparison of the regulatory properties of the tobacco tissue enzyme with those of the microbial enzyme indicates that the plant enzyme is quite unique and significantly different from the enzyme of the microorganisms studied by other investigators.

In order to elucidate the possible mode of action of wildfire toxin, the influence of L-methionine sulphoximine (MSO) on the glutamine synthetase from tobacco tissue was studied. It was found that MSO inhibited the enzyme at as low a concentration as 5 μM and that MSO bound to

the enzyme irreversibly. The results suggest that inhibition of glutamine synthetase may be the primary site of action of the wildfire toxin within the plant cell.

CHAPTER IV

NADPH PRODUCING DEHYDROGENASES FROM TOBACCO TISSUE

Introduction

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, E C 1.1.1.49) is an important enzyme that catalyzes the reaction of the first step of the pentose phosphate pathway. The enzyme from yeast has been reported to be inhibited by such metabolic compounds as phosphate ion, NADPH, D-glucosamine-6-phosphate (Glaser and Brown, 1955), long chain acyl-coenzyme A like stearyl-Co A, palmityl-Co A, lauryl-Co A, (Eger-Neufeldt, Teinzer, Weiss and Wieland, 1965) and pyridoxal 5'-phosphate (Engel, Domschke, Alberti and Domagk, 1969).

For the results reported in this chapter glucose-6-phosphate dehydrogenase from tobacco tissue was studied for its properties and regulations. In order to initiate the investigations of the metabolic significance of the phenolic compounds scopoletin and scopolin in plants, the effects of these compounds on the four NADPH producing enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, $NADP^+$ specific isocitrate dehydrogenase and $NADP^+$ specific malate dehydrogenase (or malic enzyme) were studied.

Materials and Methods

The growth of cultures, enzyme preparation and protein determination were all done in the same manner as described in Chapter III.

Enzyme Assay

The glucose-6-phosphate dehydrogenase standard assay solution contained (Brown and Wray, 1968): 5 mM glucose-6-phosphate (Sigma); 5 mM $MgCl_2$; 1 mM $NADP^+$ (Sigma); 100 mM Tris·HCl (pH 8.0) and the enzyme solution. The total volume of the assay solution was 3 ml.

For 6-phosphogluconate dehydrogenase, the standard assay solution contained: 5 mM 6-phosphogluconic acid (Sigma); 5 mM $MgCl_2$; 1 mM $NADP^+$; 100 mM Tris·HCl (pH 8.0) and the enzyme solution. The total volume was 3 ml.

The standard assay solution for $NADP^+$ specific isocitrate dehydrogenase contained (Yamamoto, 1969): 1.3 mM isocitric acid (Sigma); 2.5 mM $MgCl_2$; 0.066 mM $NADP^+$; 100 mM imidazole·HCl (pH 7.0) and the enzyme solution. The total volume was 3 ml.

For $NADP^+$ specific malate dehydrogenase or malic enzyme the standard assay solution contained (Hsu, Lardy and Cleland, 1967): 0.5 mM malic acid (Sigma); 4 mM $Mg(CH_3COO)_2$; 0.011 mM $NADP^+$; 100 mM imidazole·HCl (pH 6.8) and the enzyme solution. The total volume was 3 ml. For this experiment it was not determined whether the enzyme assayed was malate dehydrogenase or malic enzyme.

For each assay mentioned above, the increase in optical density was measured at 340 mμ after 5 min preincubation without the substrate (glucose-6-phosphate, 6-phosphogluconic acid, isocitric acid or malic acid). The reaction was started by the addition of the substrate.

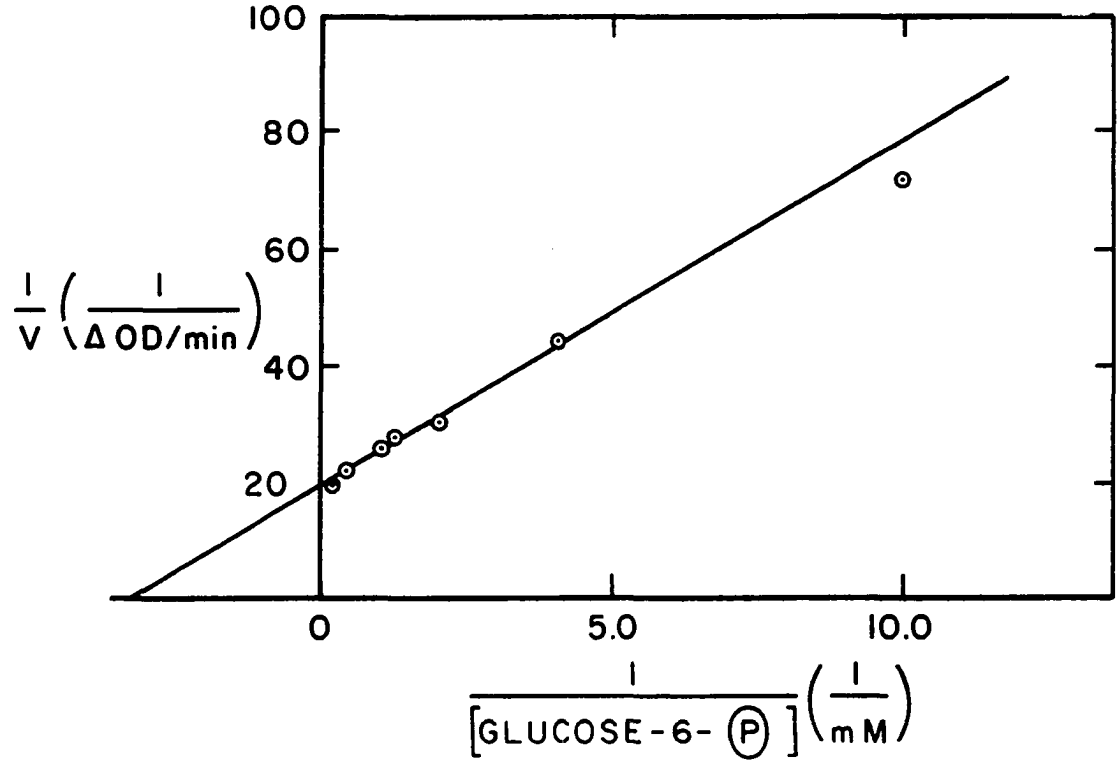
Results

The pH optimum for glucose-6-phosphate dehydrogenase was determined to be 8 to 9. The plot of the reaction velocity vs. glucose-6-phosphate concentration showed a non-sigmoidal, hyperbolic saturation

curve (Fig. 4-2) and the corresponding double reciprocal plot is shown in Fig. 4-1. The Michaelis constant K_m was determined to be 3×10^{-4} M. The plot of the reaction velocity vs. $NADP^+$ concentration displayed a similar hyperbolic saturation curve (Fig. 4-4) and the Michaelis constant K_m for $NADP^+$ was found from the corresponding double reciprocal plot to be 4.5×10^{-5} M (Fig. 4-3). These two Michaelis constants are very similar to those for crystalline glucose-6-phosphate dehydrogenase from yeast (Engel, Domschke, Alberti and Domagk, 1969); $K_m = 2.3 \times 10^{-4}$ M for glucose-6-phosphate, $K_m = 6.7 \times 10^{-5}$ M for $NADP^+$. It was assumed that the pH optimum and Michaelis constant K_m for 6-phosphogluconate dehydrogenase are similar to those for glucose-6-phosphate dehydrogenase and that pH optima and Michaelis constant K_m's for the other two dehydrogenases, $NADP^+$ specific isocitrate and malate dehydrogenases, are similar to those for the same enzymes from other sources; $NADP^+$ specific isocitrate dehydrogenase from bean, pH 6.8, $K_m = 1.3 \times 10^{-4}$ M (Yamamoto, 1969); $NADP^+$ specific malate dehydrogenase from pigeon liver, pH 7.0, $K_m = 7.7 \times 10^{-5}$ M (Hsu, Lardy and Cleland, 1967).

To investigate the significance of glucose-6-phosphate dehydrogenase for the metabolism of tobacco tissue, several metabolic compounds that may be related to glucose-6-phosphate metabolism were tested for their effect on the enzyme. Each of the following compounds was tested at a concentration of 2 mM: ATP, ADP, AMP, phosphate, glyoxylic acid, ribose-5-phosphate, L-phenylalanine, L-tryptophan and glyceraldehyde-3-phosphate. None of the compounds above had any effect on the enzyme.

In order to elucidate the possible metabolic significance of scopoletin, scopolin and some other phenolic compounds, the effects of these



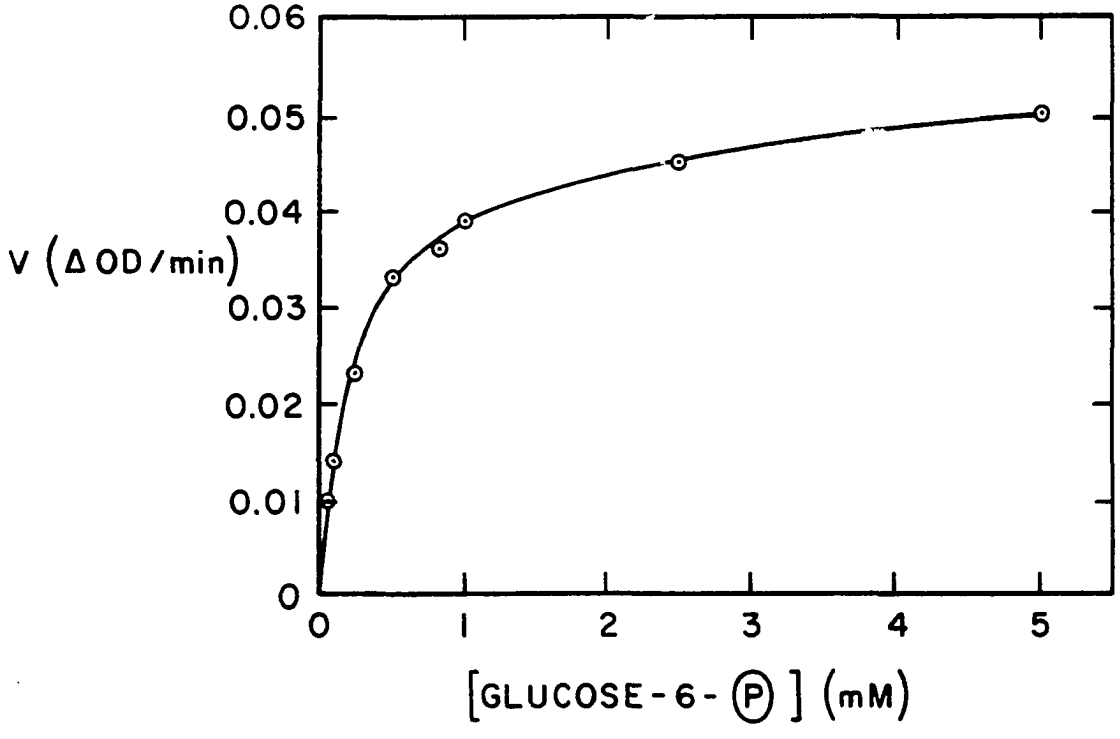
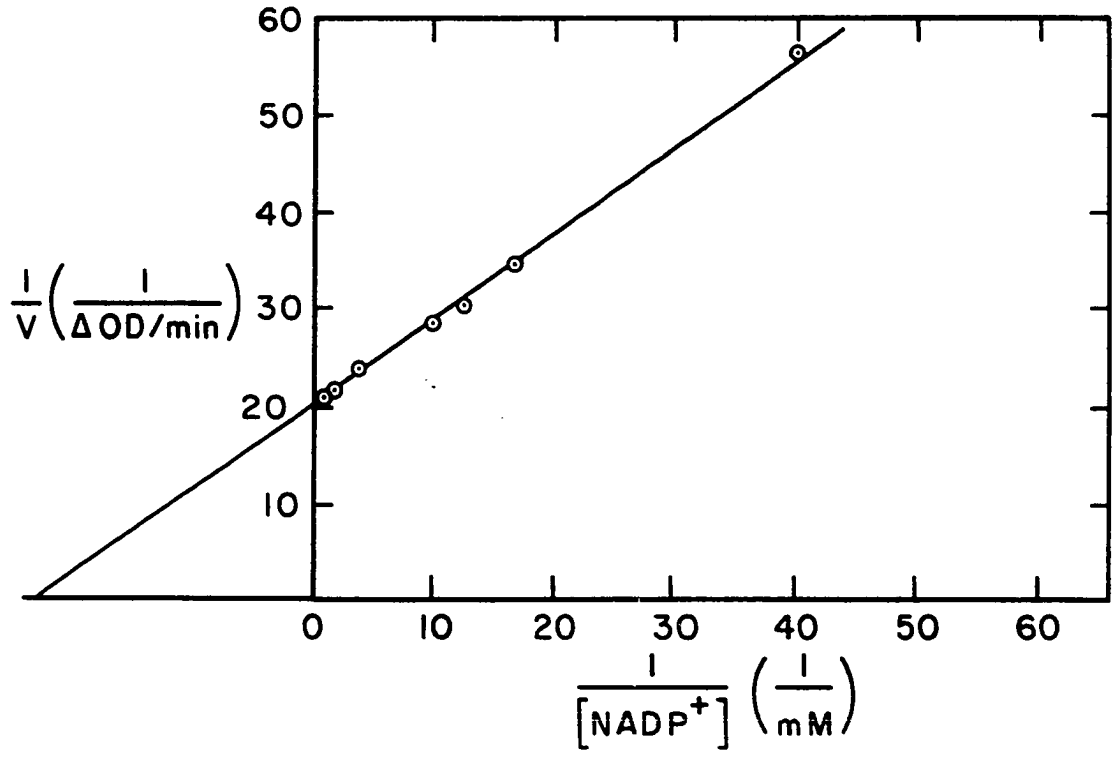


Fig. 4-1. (upper) A double reciprocal plot of reaction velocity vs. glucose-6-phosphate concentration for glucose-6-phosphate dehydrogenase from tobacco tissue.

Fig. 4-2. (lower) The saturation function of glucose-6-phosphate for glucose-6-phosphate dehydrogenase from tobacco tissue.



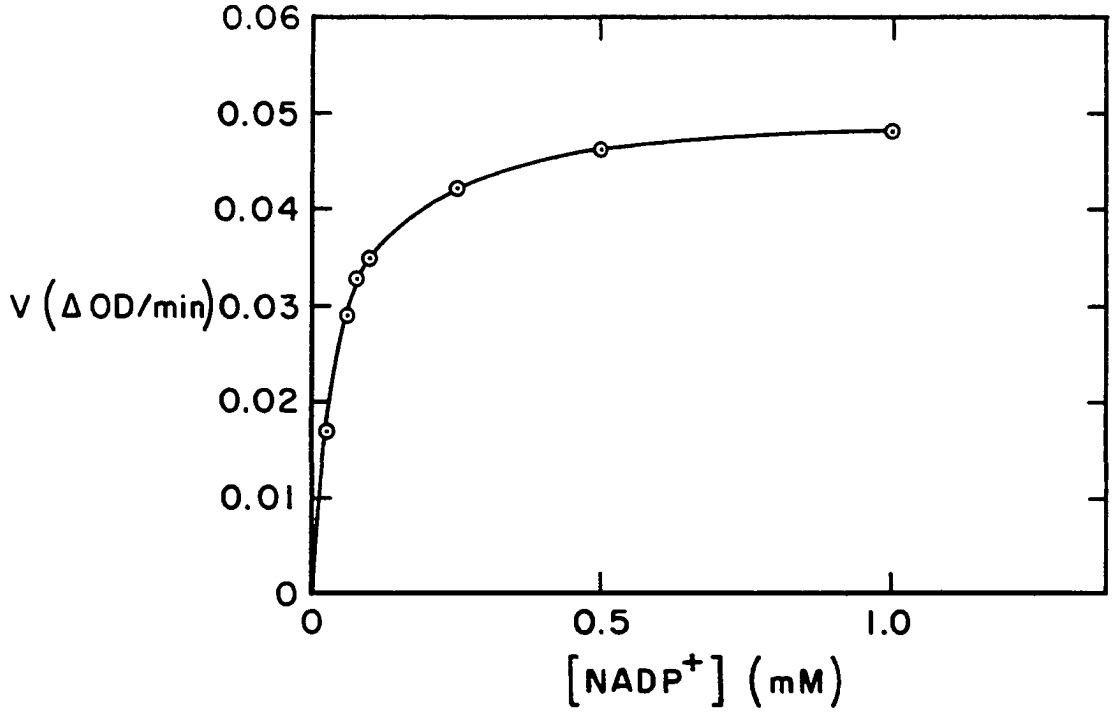


Fig. 4-3. (upper) A double reciprocal plot of reaction velocity vs. $NADP^+$ concentration for glucose-6-phosphate dehydrogenase from tobacco tissue.

Fig. 4-4. (lower) The saturation function of $NADP^+$ for glucose-6-phosphate dehydrogenase from tobacco tissue.

compounds on glucose-6-phosphate dehydrogenase from tobacco tissue were investigated. The results are shown in Table 4-1, indicating the percentage of inhibition by these compounds. The effects of esculin and ferulic

TABLE 4-1

PERCENTAGE OF INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO TISSUE BY PHENOLIC COMPOUNDS

Compounds	% Inhibition
0.1 mM Scopoletin	25%
0.1 mM Scopolin	9
0.1 mM Esculin	30
0.1 mM Ferulic acid	15

Note. Standard Assay conditions were used.

acid on the enzyme were tested in order to show whether the inhibitions by scopoletin or scopolin were typical of inhibitions of the enzyme caused by phenolic compounds.

As can be seen from Table 4-1, a very low concentration of the phenolic compounds inhibited the enzyme. A concentration higher than 0.1 mM of these compounds was not used because the high absorption by these compounds at 340 m μ made accurate assay impossible. To prove that the observed inhibitions by these compounds were not caused by artifacts, the following questions were examined: whether the absorption of NADPH and the absorption of scopoletin or scopolin at 340 mμ are additive to insure

that the optical density change observed is caused solely by the amount of NADPH production and whether $NADP^+$, glucose-6-phosphate, or NADPH produced by the enzyme reaction are reacting somehow with scopoletin or scopolin with or without the enzyme solution causing a change in absorption at 340 mμ. It was found that absorptions of NADPH and scopoletin were, in fact, additive and no reaction among the substrates and scopoletin or scopolin occurred in such a way as to change the optical density at 340 mμ. Therefore, the inhibitions observed were, in fact, inhibitions caused by the phenolic compounds. Glutamine synthetase from the same tobacco tissue was affected by neither scopoletin nor scopolin.

Scopolin, which is the 7-glucosylated form of scopoletin, was much less inhibitory to glucose-6-phosphate dehydrogenase than was scopoletin (6-methoxy-7-hydroxy coumarin).

A double reciprocal plot of reaction velocity vs. glucose-6-phosphate concentration at 0.1 mM of scopoletin for glucose-6-phosphate dehydrogenase was drawn and it was found that scopoletin inhibited the enzyme non-competitively. Since three other phenolic compounds inhibited the enzyme at the substrate concentration of V_M, they were also non-competitive inhibitors.

It is interesting to observe that scopoletin and scopolin also were found to have similar effects on three other enzymes from tobacco tissue: 6-phosphogluconate dehydrogenase, $NADP^+$ specific isocitrate dehydrogenase and $NADP^+$ specific malate dehydrogenase. The results are shown in Table 4-2, indicating the percentage of inhibition by these compounds at different concentrations. The effects of these

TABLE 4-2

PERCENTAGE OF INHIBITION OF NADPH PRODUCING DEHYDROGENASES FROM TOBACCO TISSUE BY SCOPOLETIN AND SCOPOLIN

Enzyme		% Inhibition		
Glucose-6-phosphate dehydrogenase	Scopoletin	0.1 mM	0.05 mM	0.01 mM
		25%	12%	0%
	Scopolin	0.1 mM	0.05 mM	0.01 mM
		9	6	0
6-phosphogluconate dehydrogenase	Scopoletin	0.1 mM	0.05 mM	0.01 mM
		22	13	0
	Scopolin	0.1 mM	0.05 mM	0.01 mM
		12	7	0
$NADP^+$ specific isocitrate dehydrogenase	Scopoletin	0.1 mM	0.05 mM	0.01 mM
		19	8	0
	Scopolin	0.1 mM	0.05 mM	0.01 mM
		7	3	0
$NADP^+$ specific malate dehydrogenase (or malic enzyme)	Scopoletin	0.1 mM	0.05 mM	0.01 mM
		31	18	2
	Scopolin	0.1 mM	0.05 mM	0.01 mM
		6	0	0

Note. The different concentrations of scopoletin and scopolin used are underlined. Standard assay conditions were used.

compounds on glucose-6-phosphate dehydrogenase are also included in the table. Scopolin consistently inhibited all the enzymes less than scopoletin. The four dehydrogenases that were inhibited by scopoletin and scopolin catalyze the reactions that produce NADPH.

In order to establish the mode of binding of the phenolic compounds to the dehydrogenases, glucose-6-phosphate dehydrogenase was preincubated with 0.1 mM scopoletin for different periods of time and assayed. The results, summarized in Table 4-3, indicate the percentage of inhibition for different periods of preincubation.

TABLE 4-3

PERCENTAGE OF INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO TISSUE FOR DIFFERENT TIME PERIODS OF PREINCUBATION WITH 0.1 mM SCOPOLETIN

Time of Preincubation	% Inhibition
5 min	21%
20 min	20
40 min	22
60 min	20
12 hr	23

Note. Standard assay conditions were used.

As can be seen from Table 4-3, the percentage of inhibition was independent of preincubation time at least for 12 hr, suggesting the reversible binding of scopoletin to the enzyme. The enzyme solution that had been preincubated with 0.1 mM scopoletin for 60 min was dialyzed overnight and assayed. The enzyme activity was all regained after the dialysis, indicating that scopoletin binds to the enzyme reversibly.

Discussion

The results show that some phenolic compounds inhibited glucose-6-phosphate dehydrogenase, which catalyzes the initiating reaction for the pentose phosphate pathway. The pentose phosphate pathway contributes not only to the formation of NADPH but also to the formation of shikimic acid, which is synthesized by condensation of erythrose-4-phosphate and phosphoenolpyruvate. Shikimic acid is an important precursor of aromatic amino acids and phenolic compounds such as flavonoids and lignins. The possibility for a feedback type of metabolic control is suggested by the results.

Since NADPH producing enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, $NADP^+$ specific isocitrate dehydrogenase, $NADP^+$ specific malate dehydrogenase were similarly inhibited by the phenolic compounds, a second type of control may be possible. The amount of NADPH production may be controlled in such a way as to maintain a fairly constant pool of NADPH available for metabolism of phenolic compounds as well as for the fatty acid synthesis in the cells of this plant. The exact nature of the involvement of NADPH in the metabolism of phenolic compounds is unknown, but NADPH may play a role in keeping the phenolic compounds

from being oxidized to quinones which may be toxic to the cells. Havir and Hanson (1968) stated on the basis of their study of the active site of phenylalanine ammonia-lyase that the enzyme activity could be controlled through the action of a specific dehydrogenase or transaminase for the formation of the necessary reactive carbonyl group of the active site. If the necessary carbonyl group comes from the reduction of a carboxyl residue of the enzyme, the dehydrogenase which is involved in the reduction process may require NADPH. Then, the phenolic compounds would effectively have two regulation points along the long biosynthetic pathway from glucose-6-phosphate to phenolic compounds; the first step of the pentose phosphate pathway catalyzed by glucose-6-phosphate dehydrogenase and the first step of biosynthesis of phenolic compounds catalyzed by phenylalanine ammonia-lyase. When phenolic compounds are accumulated within cells, they would inhibit glucose-6-phosphate dehydrogenase, with a resulting reduction of NADPH production. This would in turn reduce the activity of phenylalanine ammonia-lyase. When the phenolic compounds are a low concentration, the reverse process would occur. This type of regulation may be possible.

It is interesting that scopolin showed much less inhibition of all the four dehydrogenases than did scopoletin. Scopolin is the glucosylated form of scopoletin and it is quite possible that these two compounds are enzymatically interconvertible within the cell. The metabolic significance of this glucosylation is not clear, but the fact that the glucosylated form, scopolin, inhibited NADPH producing dehydrogenase much less than the non-glucosylated form, scopoletin, suggests that the glucosylation may be permitting storage of the phenolic compound for some purpose (i.e. lignification in the plant) without reduction of the biosyn-

thetic rate of the compound, or simply permitting the reduction of the inhibitory effect of the compound by detoxification.

Summary

The pH optimum for glucose-6-phosphate dehydrogenase from tobacco tissue was determined to be at 8 to 9. The plot of the reaction velocity vs. glucose-6-phosphate concentration showed a non-sigmoidal, hyperbolic saturation curve. A similar curve was obtained for the $NADP^+$ saturation function. The Michaelis constant K_m for glucose-6-phosphate was revealed to be 3×10^{-4} M and for $NADP^+$, 4.5×10^{-5} M.

Some metabolic compounds that may be related to glucose-6-phosphate metabolism were tested for their effects on the enzyme. It was found that the phenolic compounds, scopoletin, scopolin, esculin and ferulic acid, inhibited the enzyme. Similar inhibitions were observed for three other NADPH producing dehydrogenases from the same plant cells; namely, 6-phosphogluconate dehydrogenase, $NADP^+$ specific isocitrate dehydrogenase and $NADP^+$ specific malate dehydrogenase. The possibility of metabolic control by these compounds was suggested by these results.

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