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Studies on glutamate synthase from the roots of maize.

Effects of nicotinamide-adenine dinucleotides and inorganic salts on its activity

by

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RESUMO

A actividade da glutamato sintase de raízes de milho foi medida na presença de diferentes concentrações de NAD(P)H. A glutamato sintase dependente do NADH (EC 1.4.1.14) não exibiu actividade quando se utilizou o NADPH como única fonte de equivalentes redutores, mas a presença desta coenzima no meio da reacção (utilizando NADH como agente redutor) inibiu a sua actividade. Também foram observados efeitos inibitórios na presença de concentrações elevadas de NADH. A representação gráfica de v_o em função da concentração de NADH mostrou um aumento de actividade enzimática até 100 μM, decrescendo posteriormente de maneira gradual até se tornar indetectável para concentrações de NADH próximas de 600 μM. Foram examinados os efeitos de alguns catiões na actividade da glutamato sintase, tendo-se observado um aumento de actividade na presença de cálcio. A estimulação máxima da actividade (até 126%) foi obtida para concentrações de cálcio entre 0,25 e

2,5 mM, o que dependia das condições de ensaio. Foram observados idênticos efeitos estimulatórios com o estrôncio.

SYNOPSIS

Maize root glutamate synthase activity was assayed with varying concentrations of NAD(P)H. The NADH-dependent glutamate synthase (EC 1.4.1.14) showed no activity when NADPH was used as the only source of reducing equivalents, but the presence of this coenzyme in the reaction medium (with NADH as reductant) inhibited its activity. Inhibitory effects were also observed with high concentrations of NADH. Saturation curves of v_o versus NADH concentration showed an increase in activity up to 100 μM, decreasing thereafter gradually until it became undetectable when NADH concentration was about 600 μM. The effects of a number of cations on the activity of glutamate synthase have been examined. A rather strong stimulatory effect was observed with calcium. The concentration of calcium for maximum activations (up to 126%) varied between 0.25 and 2.5 mM, depending on the assay conditions. Identical stimulatory effects were found with strontium.

1. INTRODUCTION

The electron donor specificity of glutamate synthase appears to vary with the origin of the plant tissue (1). It has been considered for some time that ferredoxin-dependent glutamate synthase was typically found in green leaves, while the enzyme present in non-green tissues was specific for NAD(P)H. However, glutamate synthase activity which is able to use either NAD(P)H or ferredoxin as the source of reducing equivalents has been detected in pea roots (2), pea cotyledons (3), maize roots (4), and roots of halophytic plants (5). On the other hand, it has been reported that glutamate synthases from some non-photosynthetic tissues are unable to use either NADH or NADPH, their activities depending on reduced ferredoxin (6, 7). The physiological role of ferredoxin-specific glutamate synthase in roots was doubtful at the time since ferredoxin had not yet been identificed in these tissues. In many non-photosynthetic tissue extracts in which glutamate synthase is active with both NADH and NADPH the rate of oxidation of NADH is greater than that of NADPH (8-10). However, in some

cases, a similar eficiency of NADH and NADPH as electron donors has been reported (11-13). The functioning of glutamate synthase with both coenzymes has been initially attributed to a lack of specificity of the enzyme for the reduced nicotinamide-adenine nucleotides (14, 15). It has also been suggested that *in vivo* the enzyme is specific for NADH, its *in vitro* activity with NADPH being an artifact due to the presence of an endogenous nucleotide phosphatase which converts NADPH to NADH (8, 9). On the other hand, the available data do not permit to exclude the possible existence of two different forms of glutamate synthase, one specific for NADH, the other for NADPH.

Previous work (16) reported that the activity of maize root glutamate synthase could not be detected with NADPH and that the saturation curve of the enzyme towards NADH was not a quadratic hyperbole as a result of the inhibitory effects observed with NADH concentrations higher than 100 μM. In this study we examine in more detail the specificity of the root maize glutamate synthase towards the nicotinamide-adenine dinucleotides and inhibitory effects of high concentrations of these coenzymes on the activity of the enzyme. We also report the action of several inorganic salts on the *in vitro* activity of the enzyme.

2. MATERIALS AND METHODS

MATERIALS

All the experiments were carried out with the maize hybrid ACCO 597. Seeds were germinated and the root material obtained as described previously (16). All reagents used were of grade for biochemical analysis.

ENZYME PREPARATION AND ASSAY. PROTEIN DETERMINATION

The preparation of the extract, the measurement of its protein content and of the glutamate synthase activity were performed as

previously described (16).

A unit of enzyme is defined as the rate of oxidation of 1 μmol of NADH per min. Specific activity is expressed in units per mg of protein.

3. RESULTS AND DISCUSSION

COENZYME SPECIFICITY

Table I shows the enzyme activity when NADH or/and NADPH were present in the reaction medium as sources of reducing equivalents.

Contrary to the data reported by Oaks *et al.* (11) for the glutamate synthase of maize roots, we did not detect any enzyme activity when NADPH was used as the only source of reducing equivalents. The lower specific activity obtained in the presence of both coenzymes (NADH+NADPH) is the result of an inhibitory effect promoted by the higher concentration of the pyridine nucleotides in the reaction medium, as explained in the next sections.

TABLE I

Activities of glutamate synthase from maize roots with NADH and NADPH

Assay conditions	Specific activity (units/mg of protein)
Complete with NADH	0.017
Glutamine	0.000
Complete with NADH	0.000
Complete with NADH + NADPH	0.011

The standard enzyme assay was carried out at pH 7.5 and 30°C by measuring the decrease in A_{340} associated with NADH oxidation. The assay mixture contained L-glutamine ($10mM$), 2-oxoglutarate ($4mM$), NADH ($0.2mM$) or NADPH ($0.2mM$), or NADH + NADPH ($0.2mM+0.2mM$), 2-mercaptoethanol ($14mM$), enzyme extract ($0.1ml$) and phosphate buffer ($100mM$, $pH7.5$), in a volume of $3ml$ in silica cells of $1cm$ light path.

INHIBITORY EFFECTS OF NICOTINAMIDE - ADENINE DINUCLEOTIDES

The results in Fig. 1 show that the enzyme is inhibited by concentrations of NADH higher than $100\mu M$. Increasing concentrations of NADH lead to a gradual but steady decrease of the enzyme activity which becomes undetectable when the coenzyme reaches a concentration of about $600\mu M$.

Though our enzyme preparation was inactive with NADPH, the presence of this nucleotide in the standard reaction mixture (containing NADH as reductant) strongly affected the NADH-dependent activity. This effect is illustrated in Fig. 2.

The increased inhibitory effects observed with rising concentrations of NADPH, for a fixed level of NADH, suggests that the degree of inhibition may depend on the total concentration of pyridine nucleotides in the reaction mixture and not to one of them in particular. This was confirmed by the results obtained when different concentrations of both nucleotides were present in the reaction mixture (Table II and Fig. 3).

FIGURE 1

Effect of NADH concentrations on the initial velocity of the reaction catalysed by glutamate synthase. Enzyme assays were carried out as described in Table I. The relative reaction rate is expressed as the percentage of the initial velocity obtained when [NADH] = $100\mu M$.

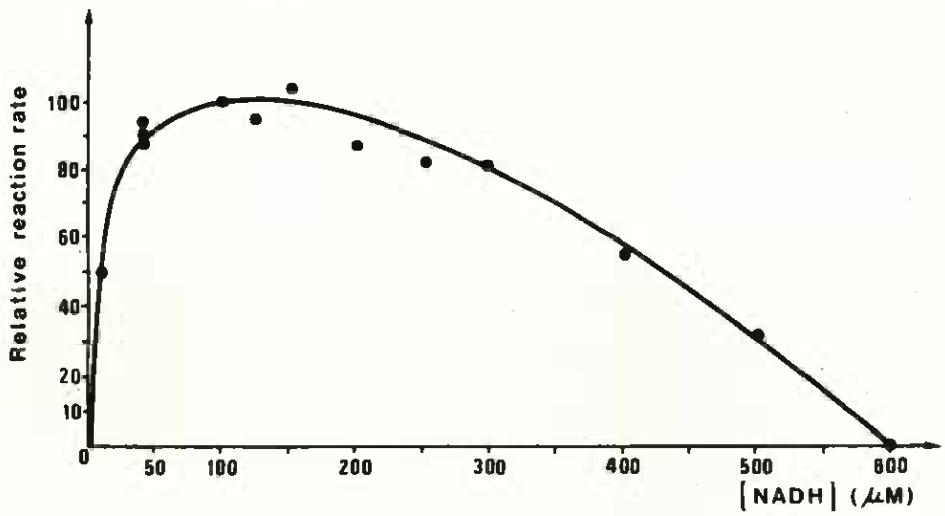


FIGURE 2

Effect of NADPH concentration on the initial velocity of the reaction. Assay conditions as specified in Table I except NADH concentration (100μM). Reaction rates are expressed as a percentage of the activity obtained when [NADH]= 100μM and in the absence of NADPH.

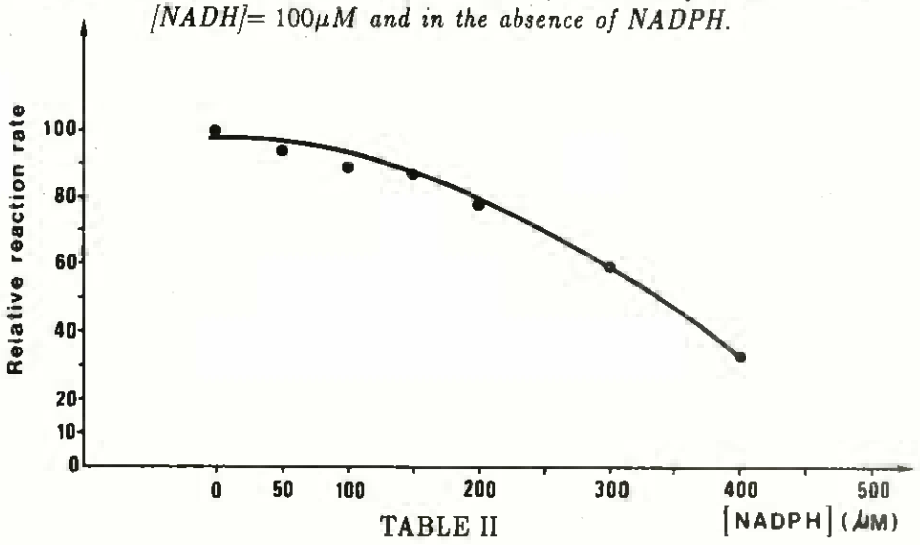


TABLE II

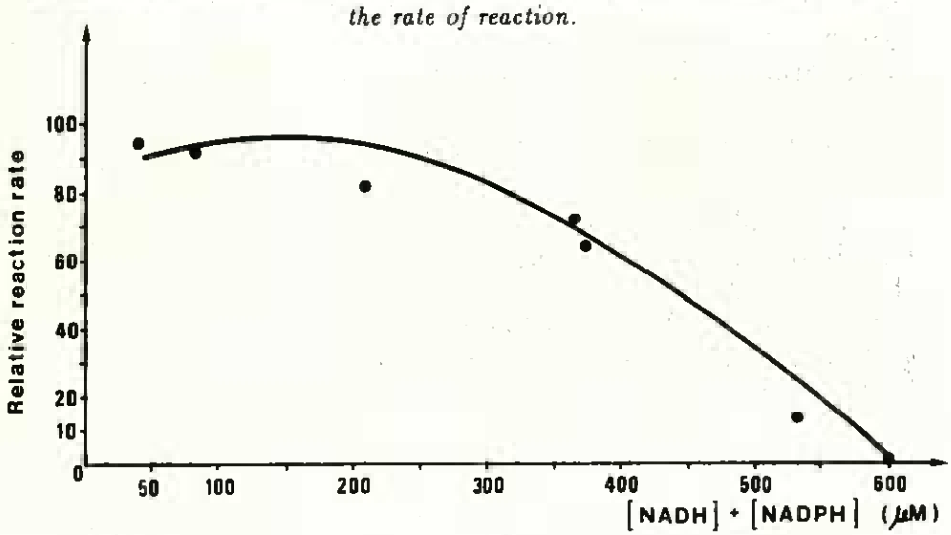
Effects of different concentrations of NADH and NADPH on the activity of glutamate synthase

Concentration (μM)			Relative rate
NADH	NADPH	NADH+NADPH	
100	0	100	100
40	0	40	93.9
40	41.7	81.7	91.3
40	167	207	82.2
200	167	367	72.9
40	334	374	64.2
200	334	534	13.0
300	300	600	0.0

Assay mixture as described in Table I except the concentrations of NADH and NADPH.

FIGURE 3

Effects of different concentrations of NADH and NADPH (see Table II) on the rate of reaction.



NADPH inhibition of bacterial glutamate synthase has been reported by Mantsala and Zalkin (17) and by Geary and Meister (18), respectively in *Escherichia coli* and *Aerobacter aerogenes*. Glutamate synthase has been obtained in higly purified form from a number of bacteria (19-12) and it has been found to be an iron--sulphide flavoprotein. The inhibition of the bacterial enzyme by NADPH has been attributed to reduction of the enzyme-bound flavin that occurs in the absence of other substrates; the subsequent metal-catalysed autooxidation of reduced flavin by molecular oxygen generates hydrogen peroxide which may oxidize one or more groups at the active site of the enzyme which are required for the binding and/or reaction of glutamine (17, 18). The validity of this hypothesis has been confirmed by determining the effects of catalase and EDTA on the enzyme inactivation produced by NADPH, which was virtually abolished by catalase (17, 18) and partially prevented by EDTA (17).

Glutamate synthase from higher plants is not characterized as well as that from bacterial origin. However, the NADH-dependent enzymes present in the nodules of *Lupinus* (22) and in pea cotyledons (23) have been described as flavoproteins, while the ferredoxin-dependent glutamate synthase does not appear to possess either flavin or non-heme iron moieties (24, 25). The possibility that

the inhibition by pyridine nucleotides observed in our experiments may folow a mechanism identical to that suggested for the bacterial enzyme (17, 18), has been examined by studying the effects of hydrogen peroxide, catalase and EDTA on the NAD(P)H-promoted incativation of the maize root enzyme.

EFFECT OF HYDROGEN PEROXIDE

The addition of hydrogen peroxide to the reaction mixture inhibited the activity of glutamate synthase from maize roots, as shown in Table III. These results agree with those obtained by Trotta *et al.* (26) with carbamyl phosphate synthetase, another glutamine amidotransferase which exhibits some properties similar to those of glutamate synthase.

TABLE III

Effect of hydrogen peroxide on the activity of glutamate synthase

Addition	Specific activity (Units/mg protein)	%
None	0.0152	100
$H_2O_2(0.3mM)$	0.0127	84
$H_2O_2(0.9mM)$	0.0103	68
$H_2O_2(15.0mM)$	0.0000	0

Standard reaction mixture as indicated in Table I, using NADH ($0.2mM$) as reductant.

EFFECT OF CATALASE

During the preliminary assays we realized that the commercial preparation of bovine liver catalase was contaminated by some substance(s) that caused a rapid oxidation of both NADH and NADPH even in the absence of glutamine. It appeared that the main contaminant was ammonia which, in the presence of 2-oxoglutarate, triggered the functioning of glutamate dehydrogenase present in the enzymic extract. To get rid of such contaminant, 0.5 ml of the commercial preparation of catalase was passed

through a column ($2.5 \times 30cm$) of Sephadex G-200. The collected catalase solution (10 ml) was directly used in the experiments.

As shown in Table IV, when catalase was added to the reaction mixture virtually no activity was lost in the presence of high concentrations of pyridine nucleotides.

TABLE IV

Protective effect of catalase on the glutamate synthase inhibition by pyridine nucleotides

Experimental conditions	Specific activity	%
1. NADH	0.0137	100
2. NADH+ NADPH	0.0063	46
3. NADH+ NADPH +catalase (0.1ml)	0.0102	74
4. NADH + NADPH +catalase (0.3ml)	0.0129	94
5. NADH + NADPH + catalase (0.5ml)	0.0160	117
6. As Exp.5-glutamine	0.0054	39
7. NADPH (0.4mM) + catalase (0.5ml)	0.0066	48
8. Exp.5-Exp.6	0.0106	78
9. NADH + NADPH + catalase (1ml)	0.0242	176
10. As Exp.9-glutamine	0.0098	72
11. As Exp.9-Exp.10	0.0143	104
12. NADH + NADPH + boiled catalase (1ml)	0.0087	64

Standard assay conditions as specified in Table I except in 7 where the NADPH concentration was $0.4mM$. The catalase solution was obtained by filtering $0.5ml$ of a commercial preparation of bovine liver catalase through a G-200 Sephadex column as described in the text.

The experiments 3, 4, 5 and 9 show that catalase prevents the inhibition of glutamate synthase by high concentrations of pyridine nucleotides. However, the presence of catalase increases the residual oxidation of NAD(P)H (as shown by experiments 5, 6, 7 and 9). The rate of NADH oxidation by glutamate synthase was obtained by subtracting the activity in the absence of glutamine

from that obtained with all substrates present (experiments 8 and 11).

EFFECTS OF EDTA AND CALCIUM NITRATE ON THE INHIBITION BY PYRIDINE NUCLEOTIDES

As shown in Table V the addition of EDTA (50mM) led to the recovery of about 30% of the original activity, when the enzyme was either partially (experiments 2 and 3) or totally (experiments 4 and 5) inhibited by the pyridine nucleotides. The presence of calcium (experiment 6), which activates the glutamate synthase from maize roots (as reported in the latter sections of this paper), did not affect the inhibition caused by pyridine nucleotides.

The results shown in Figures 1-3 and Tables II-V agree with the data presented by Trotta *et al.* (26) for carbamyl phosphate synthetase and by Mantsala and Zalkin (17) and by Geary and Meister (18) for bacterial glutamate synthase. So the possibility that the mechanism involved in the inhibition of maize root glutamate synthase by NAD(P)H may be similar to that proposed for the enzyme of bacterial origin cannot be excluded.

TABLE V

Effects of EDTA and calcium nitrate on the inhibition of glutamate synthase activity by pyridine nucleotides

Experimental conditions	Specific activity (units/mg protein)	%
1. NADH	0.0150	100
2. NADH + NADPH (0.2 mM)	0.0078	52
3. NADH + NADPH (0.2 mM) + EDTA	0.0123	82
4. NADH + NADPH (0.4 mM)	0.0000	0
5. NADH + NADPH (0.4 mM) + EDTA	0.0045	30
6. NADH + NADPH (0.4 mM) + $Ca(NO_3)_2$	0.0000	0

Assay mixtures as specified in Table I except NADPH concentrations (which are shown) and the addition of EDTA (50 mM) and of calcium nitrate (2.5 mM).

We found, in addition, that the enzyme inhibition by NADPH was relieved as the reaction progressed (Table VI). This suggests that the inhibition process is reversible (at least partially), that is, as NADH is being oxidized the consequent decrease in the concentration of its reduced form leads to a lower degree of inhibition.

TABLE VI

Effect of reaction time on the initial velocity in conditions of inhibition of glutamate synthase by NADPH

Conditions	Specific activity (units/mg protein)	%
Standard (with NADH only)	0.0152	100
+ NADPH (at zero time)	0.0034	23
+ NADPH (8 min after starting the reaction)	0.0065	43

Assay conditions as in Table I except NADPH concentrations (0.4 mM)

EFFECTS OF CATIONS ON THE ACTIVITY OF THE ENZYME

The effect of inorganic ions on the activity of glutamate synthase from maize roots is shown in Table VII.

Monovalent cations have no significant effect on the enzyme activity as was already observed by Wallsgrove *et al.* (24). Our results do not confirm the strong stimulatory effect of KCl on the enzyme activity as reported by Oaks *et al.* (11) for the glutamate synthase of maize roots and endosperms.

Amongst the divalent cations examined, calcium behaved as a potent activator of the enzyme. This stimulatory effect was maximum in the concentration range 0.25-2.5mM decreasing gradually with increasing concentrations (see Fig. 4). It appears that strontium may substitute calcium as an activator (see Table VII and

TABLE VII

Effect of inorganic cations on glutamate synthase activity

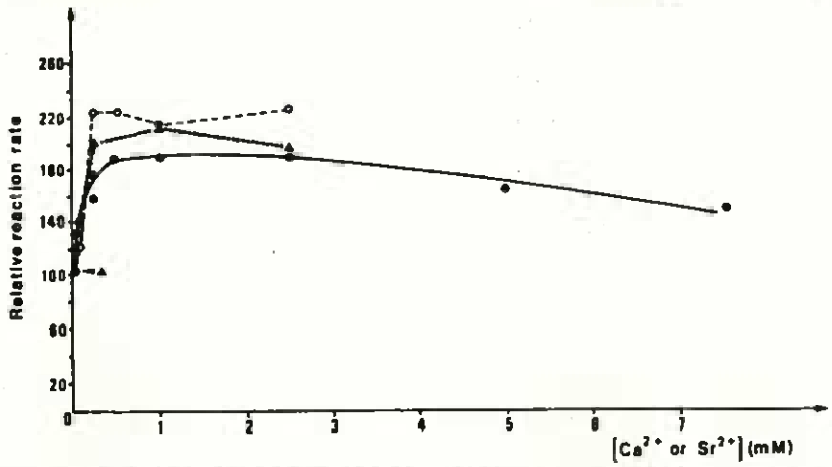
Compound added	Buffer	Concentration (mM)	Relative activity
None	P	0	100
KCL	P	0.05	103.0
KCL	P	0.25	97.4
KCL	P	2.50	94.8
NaCl	P	0.05	92.9
NaCl	P	0.25	93.5
NaCl	P	2.50	94.2
$CaCl_2$	P	0.05	126.6
$CaCl_2$	P	0.25	137.0
$CaCl_2$	P	2.50	-
$CaCl_2$	T	0.05	123.8
$CaCl_2$	T	0.25	223.8
$CaCl_2$	T	2.50	226.2
$Ca(NO_3)_2$	P	0.05	132.5
$Ca(NO_3)_2$	P	0.25	158.4
$Ca(NO_3)_2$	P	2.50	-
$Ca(NO_3)_2$	T	0.05	104.6
$Ca(NO_3)_2$	T	0.10	143.6
$Ca(NO_3)_2$	T	0.20	205.6
$Ca(NO_3)_2$	T	2.50	189.7
$ZnSO_4$	P	0.01	90.9
$ZnSO_4$	P	0.05	63.0
$ZnSO_4$	P	0.25	45.5
$ZnSO_4$	P	2.50	-
$MnCl_2$	P	0.05	130.0
$MnCl_2$	P	0.25	66.2
$MnCl_2$	P	2.50	-
$MgCl_2$	P	0.05	100.7
$MgCl_2$	P	0.25	113.6
$MgCl_2$	P	2.50	119.5
$CdSO_4$	P	0.05	74.0
$CdSO_4$	P	0.25	70.0
$CdSO_4$	P	2.50	32.0
$NiSO_4$	P	0.025	110.7
$NiSO_4$	P	0.05	117.8
$NiSO_4$	P	0.25	105.9
$NiSO_4$	P	2.50	-
$CuCl_2$	P	0.025	84.2
$CuCl_2$	P	0.05	70.0
$CuCl_2$	P	0.25	34.6
$CuCl_2$	P	2.50	-
$SrCl_2$	T	0.10	142.9
$SrCl_2$	T	0.25	200.0
$SrCl_2$	T	2.50	195.2

Reaction mixture L-glutamine (5 mM), 2-oxoglutarate (2 mM), NADH (0.04 mM), 0.2ml of ensymic extract and 0.1M of sodium phosphate buffer (P) or Tricin buffer (T), pH 7.5, containing 14mM mercaptoethanol, in a final volume of 3ml. The omission of some results at the highest salt concentration (2.5mM) was due to the occurence of salt precipitation.

Fig. 4). Other divalent cations, namely cadmium, copper, manganese and zinc, behaved as inhibitors. However, magnesium and low concentrations of manganese had a slight stimulatory effect on the enzyme activity.

FIGURE 4

Effect of calcium and strontium on the activity of glutamate synthase. Assay conditions as referred in Table VII. •—• $Ca(NO_3)_2$; ○- - -○ $CaCl_2$; ▲...▲ $SrCl_2$.



EFFECT OF EDTA ON THE ENZYME ACTIVITY IN THE PRESENCE OF CALCIUM SALTS

The effect of various concentrations of EDTA on the glutamate synthase activity is presented in Table VIII. The slight inhibition observed may result from indirect effects associated with the quelation of some ions. However, they are almost negligible probably because EDTA (1 mM) had already been added to the buffer utilized in the extraction procedure.

To confirm that the activation of glutamate synthase by $CaCl_2$ and by $Ca(NO_3)_2$ was effected by calcium ions, we examined the stimulatory efficacy of the same salts in the presence of EDTA. The results shown in Table IX strengthen the conclusion that calcium ions are strong activators of the NADH-dependent glutamate synthase from maize roots. Suzuki and Gadal (27) reported that calcium at a concentration of 1 mM produces a slight enhancement of the activity of the ferredoxin-dependent glutamate synthase from rice leaves.

TABLE VIII

Effect of EDTA on the activity of glutamate synthase

EDTA Concentration (mM)	Relative activity
0.00	100
0.02	96.75
0.05	95.45
0.10	98.05
0.20	95.75
0.40	98.47

Standard reaction as specified in Table I.

TABLE IX

Effect of EDTA on the stimulation of glutamate synthase activity by calcium salts

Compounds added	Relative activity
None	100
$Ca(NO_3)_2$	177.3
$Ca(NO_3)_2$ + EDTA (0.1 mM)	140.0
$Ca(NO_3)_2$ + EDTA (0.2 mM)	114.7
$CaCl_2$	225.3
$CaCl_2$ + EDTA (0.2 mM)	112.5
$CaCl_2$ + EDTA (0.4 mM)	97.1

Assay conditions as specified in Table VII, using phosphate buffer and calcium salts concentrations of 0.25mM.

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