Plant Physiol. (1988) 87, 41–45
0032-0889/88/87/0041/05/$01.00/0

Purification and Characterization of a Specific Nucleoside Diphosphatase from Soybean Root Nodules[1]

Received for publication October 23, 1987 and in revised form January 20, 1988

HOLLY D. DOREMUS[2] AND DALE G. BLEVINS*
Interdisciplinary Plant Physiology and Biochemistry Group, 204 Curtis Hall, University of Missouri-Columbia, Columbia, Missouri 65211

ABSTRACT

A specific nucleoside diphosphatase was purified from the plant portion of soybean (*Glycine max* L.) root nodules. This enzyme is highly specific for nucleotide diphosphates; it is unable to hydrolyze nucleotide tri- and monophosphates or a variety of other phosphorylated compounds. It will, however, hydrolyze any nucleotide disphosphate tested. The pH optimum of the enzyme is about 7.5; it requires a divalent cation for activity; and it is neither inhibited nor activated by any of the metabolites tested. It appears that *in vivo* this enzyme would be very active, but its function is not clear.

Nearly all reports of specific nucleoside diphosphatases, whether from animal (8, 9, 12), algal (2), or higher plant (3, 10) sources, describe membrane-bound enzymes which are essentially unable to hydrolyze ADP. The one exception is a brief report of the detection in soybean and alfalfa nodule extracts of an NDPase[3] which could hydrolyze any NDP tested (11). In the previous paper (5), we reported confirmation of this work and assayed a variety of tissues for this NDPase activity. Soybean nodules were found to have by far the highest levels of this activity. Here, we report the purification and characterization of the soybean nodule NDPase.

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Plant Material. Soybeans (*Glycine max* L. Merr cv Williams 82) were germinated 2 d prior to planting and treated with commercial inoculum (Urbana Laboratories, Urbana, IL) at the time of planting. Plants were grown in a greenhouse under natural light and watered with a nitrogen-free nutrient solution (22).

NDPase Purification. Soybean nodules were harvested 5 to 7 weeks after inoculation and stored frozen until use. Nodules were homogenized in 30 mM Hepes-KOH (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$ (buffer A) at a ratio of about 1 ml buffer per g fresh weight. After filtration through cheesecloth, the homogenate was centrifuged at 10,000*g* for 20 min.

Ammonium Sulfate Fractionation. The supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and allowed to stand on ice for 20 min. The precipitate was removed by centrifugation and discarded. The supernatant was then adjusted to 85% saturation and again allowed to stand. After centrifugation at 12,000*g* for 20 min, the supernatant was discarded. The pellet was resuspended in a small volume of 30 mM Hepes-KOH (pH 7.5) (buffer B) and dialyzed at 4°C overnight against the same buffer.

Affigel Blue Chromatography. The dialyzed extract was loaded on a 10 ml column of Affigel Blue (100–200 mesh, Bio-Rad Laboratories) equilibrated with the dialysis buffer. The column was eluted stepwise with four 10-ml aliquots of 30 mM Hepes-KOH (pH 7.5), followed by three 10-ml aliquots of 30 mM Hepes-KOH (pH 7.5), 1 M NaCl, 1 M AMP. NDPase activity was recovered primarily (80%) in the first two high-salt fractions; these fractions contained about 5% of the protein loaded on the column. They were pooled and dialyzed overnight against buffer B. The dialyzed enzyme was then concentrated by filtration (Minicon, Amicon Corporation).

DEAE-Sepharose Chromatography. The concentrate was loaded onto a 40 ml column of DEAE-Sepharose CL6B equilibrated with buffer B. The column was eluted first with 30 ml of buffer B, followed by a 150 ml gradient of 0 to 500 mM NaCL in buffer B. Fractions of 3 ml were collected and assayed for protein and NDPase activity.

Gel Filtration Chromatography. A Superose-12 column (Pharmacia) attached to an FPLC system was equilibrated with 30 mM Hepes-KOH (pH 7.5), 100 mM NaCl. A 200 μl aliquot of the NDPase peak from DEAE-Sepharose chromatography was loaded onto the column, which was eluted isocratically at a flow rate of 0.2 ml/min. Fractions (0.4 ml) were collected and assayed for NDPase activity. A standard curve for mol wt estimation was produced by injecting and running standards in a similar manner, with detection by UV absorbance.

SDS Gel Electrophoresis. Fractions were analyzed by SDS-PAGE on 13% acrylamide gels using the discontinuous buffer system of Laemmli (13).

Enzyme Assays. Routine NDPase assays were as given in the preceding paper (5); specific details are given in the test where appropriate.

For product identification studies, the reaction mixture was applied to a 0.3-ml column of PEI-cellulose, and the nucleotides were separated by elution with increasing concentrations of LiCl (15). The reaction mixture contained: 30 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 10 mM ADP and NDPase in a total volume of 0.5 ml. At 0, 30, and 60 min, 100-μl aliquots were removed to 0.3-ml PEI-cellulose columns which were eluted successively with three 1-ml aliquots of water, three 1-ml aliquots of 50 mM

[1] Supported by the Missouri Agricultural Experiment Station and by a grant from the United States Department of Agriculture, Science and Education Administration, Competitive Grants Office, Grant 85-CRCR-1-1638. This research is a contribution of the Missouri Agricultural Experiment Station Journal Series No. 10412.

[2] Supported by a Food for the 21st Century Postdoctoral Fellowship.

[3] Abbreviations: NDPase, nucleoside diphosphatase, EC 3.6.1.6; PEI, polyethyleneimine; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamio)ethanesulfonic acid; Caps, 3-cyclohexylamino-1-propanesulfonic acid.

LiCl, and three 1-ml aliquots of 250 mM LiCl. Nucleosides or nucleotides in each fraction were detected by absorbance at 254 nm. Adenosine was eluted with water, cAMP with 50 mM LiCl, AMP with 250 mM LiCl, while ADP and ATP were retained under these conditions. Protein was determined by the method of Smith (20).

RESULTS AND DISCUSSION

Purification of NDPase from Soybean Nodule Extract. Table I summarizes the purification scheme used. The final yield was calculated as about 13% with about 400-fold purification. However, the initial total activity is probably somewhat overestimated due to the presence of substantial 5′-nucleotidase activity in the extract. The true yield and fold purification are thus probably somewhat higher than the numbers given in Table I.

The 5′-nucleotidase activity co-purified with the NDPase through the ammonium sulfate precipitation step and Affigel Blue chromatography steps. Figure 1 shows that these two activities were completely resolved by DEAE-Sepharose chromatography. The 5′-nucleotidase was not retained on the column and could be eluted with the starting buffer. Leghemoglobin which had not been removed by the Affigel step eluted with starting buffer, as did a total of 40% of the protein loaded. No evidence for multiple 5′-nucleotidases was found, contrary to the report of two isozymes of 5′-nucleotidase separable by DEAE-cellulose chromatography in pigeon pea nodule extracts (1). Those authors used a step gradient of NaCl to elute their column, a procedure which can give rise to artifactual multiple peaks (18).

The soybean NDPase was recovered after DEAE-Sepharose chromatography in a highly purified form and free of interfering activities. Figure 2 shows the results of SDS-PAGE on the fractions obtained at each step of the purification. After ion-exchange chromatography, about 90% of the protein was found in a single band of mol wt approximately 51,000 D. This band correlated well with NDPase activity when successive fractions off the ion-exchange column were electrophoresed (data not shown). This band was not detectable in the crude nodule extract or 50 to 85% ammonium sulfate fraction, but it became apparent after Affigel Blue chromatography. Fractions 42, 43, and 44 from the DEAE-Sepharose column had the highest specific NDPase activity. These fractions were pooled, divided into aliquots, and frozen at −40°C; the purified NDPase retained 100% of its original activity. This preparation was used for all subsequent experiments.

Identification of Reaction Products. The colorimetric assay for Pi production used routinely to assay NDPase activity establishes Pi as one product of the reaction. PEI-cellulose chromatography was used to confirm that the other product of ADP degradation was AMP. Adenine and adenosine eluted in the water washes, cAMP in the 50 mM LiCl, and AMP in the 250 mM LiCl washes, while ADP was retained on the columns. Reaction mixtures without extract were incubated alongside and aliquots removed to identical columns at 0, 30, and 60 min. The absorbance of fractions eluted from these blank columns was subtracted from that in the complete reaction eluates to give the enzyme-dependent increase in absorbance. No adenine, adenosine, or cAMP was produced, while the absorbance attributable to AMP in-

Table I. *Purification of NDPase from Soybean Nodules*

Procedures were as given in "Materials and Methods." NDPase assays were carried out at pH 7.5 using 5 mM ADP as the substrate.

Fraction	Protein	Total Activity	Specific Activity
	mg	$\mu mol \cdot min^{-1}$	$\mu mol \cdot mg^{-1} \cdot min^{-1}$
Crude extract	2330	2633	1.1
50 to 85% $(NH_4)_2SO_4$ pellet, dialyzed	745	1724	2.3
Affigel Blue peak	39	1270	32.6
DEAE-Sepharose peak	0.8	341	437.2

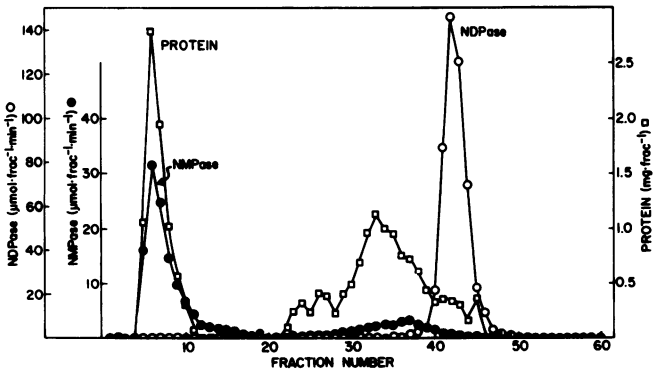

FIG. 1. DEAE-Sepharose chromatography of soybean nodule NDPase. The column was run at 1 ml per min, and 3 ml fractions were collected. The column was eluted with 30 ml 30 mM Hepes-KOH followed by a gradient of 0 to 500 mM NaCl in 150 ml. The first protein peak and the 5′-nucleotidase activity eluted with no NaCl. (○), NDPase activity; (●) 5′-nucleotidase activity; (□), protein.

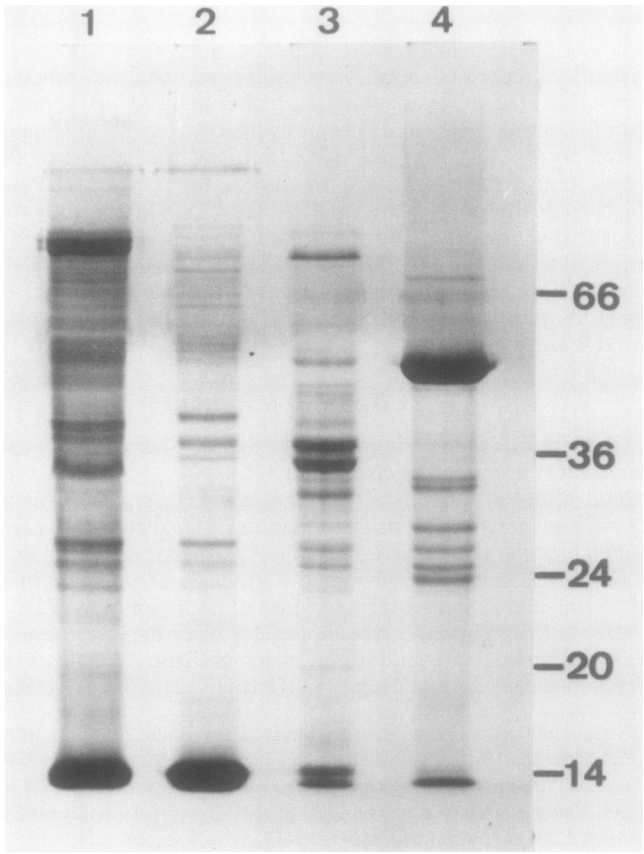

FIG. 2. SDS-PAGE showing steps in the purification of the soybean nodule NDPase. Lane 1, crude nodule extract (20,000*g* supernatant); lane 2, 50 to 85% $(NH_4)_2SO_4$ precipitate; lane 3, NDPase activity peak following Affigel Blue chromatography; lane 4, pooled NDPase peak following DEAE-Sepharose chromatography. Mol wt (D × 10^{-3}) of standard proteins is shown at right.

creased dramatically over the time course of the reaction (data not shown). The products of the enzymatic reaction with ADP were therefore confirmed as AMP and Pi.

Substrate Specificity of NDPase. The activity of the purified NDPase assayed by Pi release, with various substrates is shown in Table II. The enzyme hydrolyzed 2'-deoxyADP and ethenoADP (modified in the purine ring system) about half as rapidly as unmodified ADP. Essentially no activity was detected with ATP or cAMP as the substrate; the low level of activity shown in Table II is probably due to the presence of small quantities of contaminating ADP in the substrate. All NDPs tested were hydrolyzed to some extent, though none, under these conditions, was used as efficiently as ADP. The purified NDPase showed no activity (by Pi release) with the following substrates at concentrations up to 5 mM: *p*-nitrophenyl phosphate; ribose-5-phosphate; phosphoserine; pyrophosphate; phosphoribosylpyrophosphate; thiamine pyrophosphate; AMP; UMP; IMP; and periodate-oxidized, borohydride-reduced ADP (in which the ribose has been cleaved between the 2' and 3' carbons). Activity equivalent to 0.7% of the activity with 5 mM ADP would have been detected.

Conversion of cAMP to 5'-AMP by the enzyme was not detectable using PEI cellulose columns to separate cAMP and AMP. Another reaction this enzyme could theoretically catalyze is the ATP pyrophosphorylase reaction, which produces AMP and PPi from ATP. This reaction would not have been detected by the routine assay procedure used. ATP pyrophosphorylase activity has been identified in maize scutella, but the enzyme catalyzing it does not degrade ADP (7). Using PEI-cellulose columns, AMP production from ATP was found to proceed at 1.3% of the rate of AMP production from ADP. This low rate can be accounted for by contaminating ADP in the ATP preparation. The NDPase was also tested for ability to hydrolyze UDP-Glc to UMP and glucose 1-P, a reaction which can apparently be catalyzed by a tobacco phosphodiesterase (19). Glucose-1-phosphate production from UDP-Glc was not detectable using a coupled system including phosphoglucomutase and glucose 6-P dehydrogenase. Activity equivalent to 0.1% of the level of Pi production from ADP would have been detectable. Nor was UDP-Glc hydrolyzed to UDP and glucose, as Pi production was not observed using UDP-Glc as the substrate, but was readily produced from UDP.

The soybean nodule NDPase, then, is quite specific for nucleoside 5'-diphosphates. Only phosphate-phosphate bonds are cleaved, and one phosphate must have a nucleoside substituent, while the other must be terminal. Specificity is low with regard to the base portion of the nucleoside, but an intact ribose or deoxyribose ring appears to be required.

Table II. *Substrate Specificity of Soybean Nodule NDPase*

The assay mixture included 30 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, enzyme, and substrate as shown in a total volume of 0.5 ml. All substrates were adjusted to a pH between 6.5 and 7.5 prior to use. Assays were run for 10 min at room temperature.

Substrate	ADPase
	%
2'-DeoxyADP, 1 mM	47.4[a]
EthenoADP, 5 mM	47.5
cAMP, 5 mM	1.0
ATP, 5 mM	1.5
CDP, 5 mM	28.6
UDP, 1 mM	80.8
GDP, 1 mM	32.3
IDP, 1 mM	24.0
XDP, 1 mM	8.2

[a] ADPase activity was 400 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ at 5 mM ADP, 378 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ at 1 mM ADP.

Kinetic Constants for Various NDPs. The enzyme showed simple hyperbolic kinetics with ADP as the substrate (Fig. 3). The K_m and V_{max} with various substrates was calculated by nonlinear regression (6) (Table III). Observed K_ms ranged from 19 μM to nearly 900 μM. In general, pyrimidine nucleotides show higher K_ms than purine nucleotides, although the highest K_m is for the purine XDP. ADP, GDP, and IDP show similar K_m values, indicating that the enzyme has little specificity with respect to substitution at the 2 or 6 carbons of the purine ring system. However, the K_m for XDP is close to 20 times higher than for the others, suggesting that reduction of the double bond between carbons 2 and 3 reduces binding affinity. Maximum velocities for all the tested substrates were within a factor of three.

Other Properties of the Purified Enzyme. The native mol wt of the NDPase was estimated by gel filtration chromatography as 92,000 ± 10,000 D (Fig. 4). Combined with the SDS-PAGE data, this implies that the NDPase exists as a dimer of 50,000 D subunits *in vivo*.

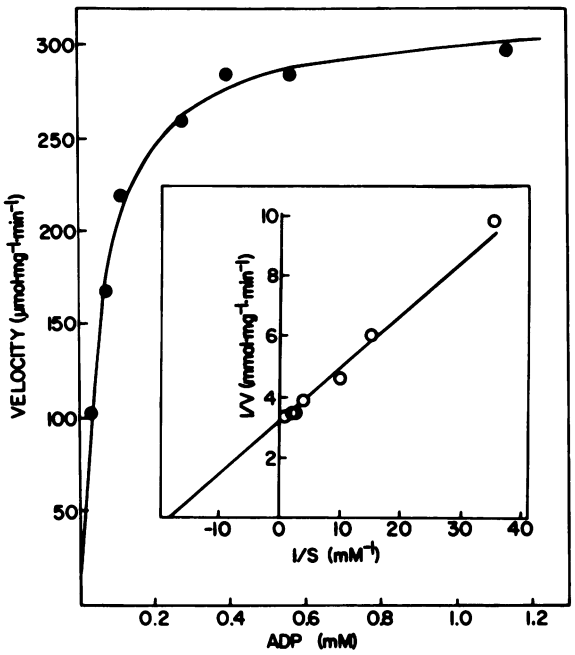

FIG. 3. Velocity *versus* substrate concentration curve for nodule NDPase. Experimental points were fitted by nonlinear regression to a hyperbolic curve; the best fit curve is shown by the solid line. Inset shows the same data in a double-reciprocal plot.

Table III. *Kinetic Constants Determined for Purified Soybean Nodule NDPase*

The reaction mixture contained 30 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, enzyme and substrate. The substrate was added from stocks containing equimolar substrate and $MgCl_2$, and substrate concentrations were checked by UV absorbance. The substrate concentration at each point was taken as the mean of the initial and final concentrations. The points were fitted to a hyperbolic curve by nonlinear regression (6) for determination of K_m and V_{max}.

Substrate	K_m	V_{max}
	μM	$\mu mol \cdot mg^{-1} \cdot min^{-1}$
ADP	54.0 ± 4.9	315.3 ± 6.4
UDP	142.6 ± 38.0	482.9 ± 41.1
CDP	220.3 ± 41.4	425.0 ± 28.9
GDP	31.0 ± 2.3	216.0 ± 3.6
IDP	19.1 ± 1.4	174.8 ± 2.4
XDP	876.6 ± 20.9	486.1 ± 5.3

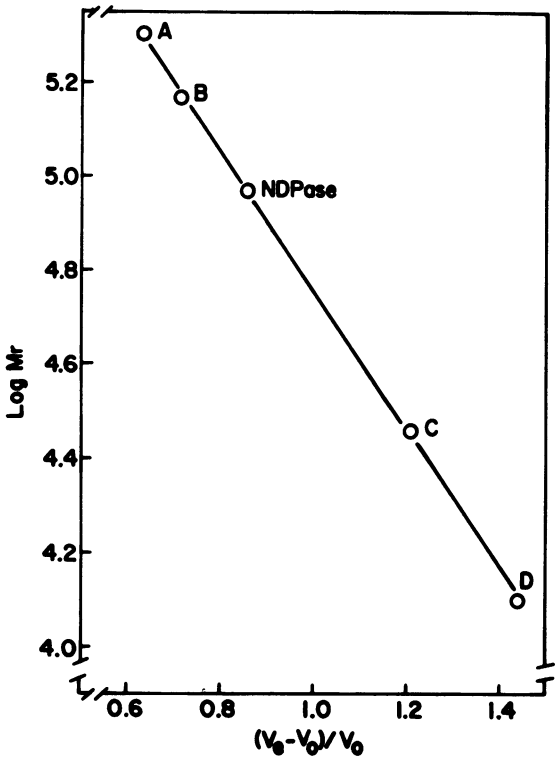

FIG. 4. Native mol wt determination. Standards used were: A, β-amylase (200,000 D); B, yeast alcohol dehydrogenase (150,000 D); C, carbonic anhydrase (29,000 D); D, Cyt *c* (12,500 D).

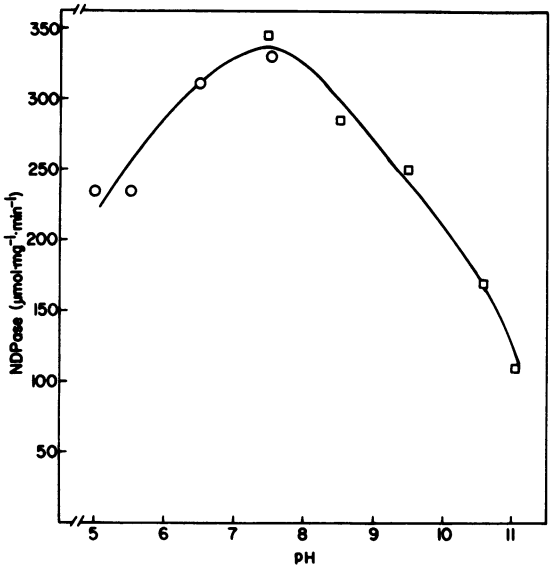

FIG. 5. Response of NDPase activity to pH. Two buffers were used: (○), a mixture of 20 mM (final concentration) each Mes, Mops, Hepes; (□), a mixture of 20 mM each Hepes, Taps, Ches, Caps. The substrate was 5 mM ADP, and assays were run for 5 min at room temperature.

The pH optimum for activity was relatively broad, with the peak at roughly pH 7.5 (Fig. 5). At pH 5.5 or pH 9.5, activity was between 70 and 75% of the activity at pH 7.5.

NDPase activity was completely inhibited by inclusion of 50 μM EDTA and omission of Mg^{2+} from the reaction mixture. This inhibition was apparently due to removal of cations from the solution and not to some direct effect of EDTA on the enzyme, since no inhibition was observed when 1 mM EDTA was added with 1 mM $MgCl_2$ (data not shown). Figure 6 shows

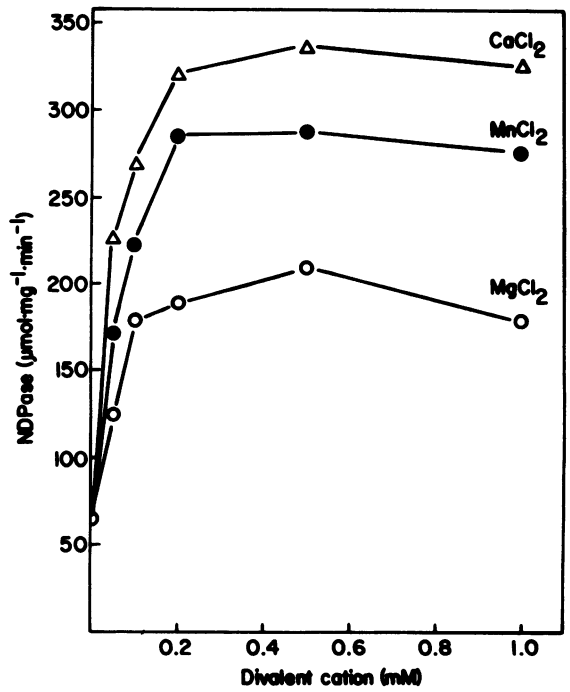

FIG. 6. Divalent cation response of NDPase. The substrate was 0.5 mM ADP. Assays were run from 10 min at room temperature. NaCl added at up to 5 mM had no effect on activity.

the effect on NDPase activity of varying divalent cation concentrations. Mg^{2+}, Mn^{2+}, and Ca^{2+} all stimulated activity; activity with Ca^{2+} was nearly twice as high as with Mg^{2+}, with Mn^{2+} giving intermediate activity. In the absence of added cation, the activity was low but not zero, perhaps because traces of divalent cations were still present. The $K_{1/2}$ for cation stimulation was about 30 μM for each of the ions tested, with either 0.5 mM ADP (Fig. 6) or 5.0 mM ADP (data not shown) as the substrate. These data do not allow us to distinguish between the possibilities that divalent cations are binding directly to the enzyme to cause activation or that the substrate of the enzyme is the ADP-cation complex. Other specific NDPases also require divalent cations for activity (2, 9, 17), but the mechanism of cation stimulation has not been shown for these enzymes either. Nonspecific phosphatases and phosphodiesterases are able to hydrolyze NDPs in the absence of cations (19, 21).

A number of metabolites were tested for possible effects on NDPase activity (Table IV). No significant stimulation or inhibition by any of the substances tested was noted. Even AMP, one product of the NDPase reaction, did not inhibit activity detectably when added at equimolar concentrations with ADP, implying that AMP has a much lower affinity for the enzyme than ADP.

Some known inhibitors of other phosphatases were tested for their ability to inhibit the soybean nodule NDPase. No inhibition was detected with 10 mM NaF; 100 μM vanadate; 1 mM ascorbate, an irreversible inhibitor of alkaline phosphatase (16); 5 mM molybdate, an inhibitor of some acid phosphatases (4); or 10 μM P^1,P^5-di(adenosine-5′)-pentaphosphate, an adenylate kinase inhibitor (14). Treatment with 25 μM $CuSO_4$ resulted in essentially no inhibition, suggesting that no readily accessible thiol is required for activity. This is consistent with the observation that NDPase activity was identical in extracts prepared in buffer with or without 5 mM DTT and 1 mM EDTA (data not shown).

Overall, the broad pH curve, low cation requirement and lack of any obvious inhibitor suggest that this NDPase is highly active *in vivo*. The fact that activity is essentially equivalent in desalted

Table IV. *Metabolite Effects on Purified Soybean Nodule NDPase*

The reaction mixture contained 30 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM ADP, enzyme and additions as noted. The control activity was 329 $\mu mol \cdot mg^{-1} \cdot min^{-1}$.

	Activity
	% of control
Control	100
+ 4 mM P-enolpyruvate	99.0
+ 50 mM sucrose	89.3
+ 2 mM glucose-1-P	97.1
+ 2 mM glucose-6-P	92.4
+ 2 mM fructose	100.7
+ 2 mM fructose-6-P	97.2
+ 2 mM fructose-1,6-bisP	101.8
+ 10 μM fructose-2,6-bisP	83.3
+ 2 mM ribose-5-P	99.6
+ 1 mM PPi	99.9
+ 1 mM UTP	98.9
+ 1 mM UDPGlucose	103.6
+ 0.5 mM NAD	100.3
+ 1 mM ATP	89.9
+ 1 mM AMP	99.7

and nondesalted crude nodule extracts (data not shown) supports this contention. The biological function of this enzyme remains unknown.

Acknowledgments—We thank Nila Emerich for preparing the figures.

LITERATURE CITED

1. Amarjit R Singh 1986 Properties of 5'-nucleotidase from nodules of pigeonpea (*Cajanus cajan*). Phytochemistry 25: 2267–2270
2. Coughlan S, LV Evans 1978 Isolation and characterization of Golgi bodies from vegetative tissue of the brown alga *Fucus serratus*. J Exp Bot 29: 55–68
3. Dauwalder M, WG Whaley, JE Kephart 1969 Phosphatases and differentiation of the Golgi apparatus. J Cell Sci 4: 455–497
4. D'Auzac J 1975 Caracterisation d'une ATPase membranaire en presence d'une phosphatase acide dans les lutoides du latex d'*Hevea brasiliensis*. Phytochemistry 14: 671–675
5. Doremus HD, DG Blevins 1988 Nucleoside diphosphatase and 5'-nucleotidase activities of soybean root nodules and other tissues. Plant Physiol 87: 36–40
6. Dugglesby RG 1981 A nonlinear regression program for small computers. Anal Biochem 110: 9–18
7. Echeverria E, T Humphreys 1984 Involvement of sucrose synthase in sucrose catabolism. Phytochemistry 23: 2173–2178
8. Ernster L, LC Jones 1962 A study of the nucleoside tri- and diphosphate activities of rat liver microsomes. J Cell Biol 15: 563–578
9. Gibson DM, P Ayengar, DR Sanadi 1955 A phosphatase specific for nucleoside diphosphates. Biochim Biophys Acta 16: 536–538
10. Green JR, DH Northcote 1979 Polyprenyl phosphate sugars synthesized during slime-polysaccharide production by membranes of the root-cap cells of maize (*Zea mays*). Biochem J 178: 661–671
11. Hunt RE, JL Haynes, JR Cowles 1977 A possible new nucleoside diphosphatase from the cytosol of soybean and alfalfa nodules. Plant Physiol 59: 773–774
12. Kuriyama Y 1972 Studies on microsomal nucleoside diphosphatase of rat hepatocytes. Its purification, intramembranous localization and turnover. J Biol Chem 247: 2979–2988
13. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685
14. Lienhard GE, II Sacemski 1973 P^1, P^5-di(adenosine-5')-pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. J Biol Chem 248: 1121–1123
15. Magnusson RP, AR Portis Jr, RE McCarty 1976 Quantitative analytical separation of adenine nucleotides by column chromatography on polyethyleneimine-coated cellulose. Anal Biochem 72: 653–657
16. Miggiano GAD, A Mordente, GE Martorana, E Meucci, A Castelli 1984 Characterization of alkaline phosphatase inactivation by ascorbic acid. Biochim Biophys Acta 789: 343–346
17. Plaut GWE 1955 An inosine diphosphatase from mammalian liver. J Biol Chem 217: 235–245
18. Scopes RK 1982 Protein Purification: Principles and Practice. Springer-Verlag, New York, pp 99–100
19. Shinshi H, M Miwa, K Kato, M Noguchi, T Matsushima 1976 A novel phophodiesterase from cultured tobacco cells. Biochemistry 15: 2185–2190
20. Smith PK, RI Krohn, GT Hermanson, AK Mallia, FH Gartner, MD Provenzano, EK Fujimoto, NM Goeke, BJ Olson, DC Klenk 1985 Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85
21. Szabo-Nagy A, Z Olah, L Erdei 1987 Phosphatase induction in winter wheat roots during adaptation to phosphorous deficiency. Physiol Plant 70: 544–552
22. Triplett EW, DG Blevins, DD Randall 1980 Allantoic acid synthesis in soybean root nodule cytosol via xanthine dehydrogenase. Plant Physiol 65: 1203–1206