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PURIFICATION AND PROPERTIES OF NADP PYROPHOSPHATASE FROM *PROTEUS VULGARIS*

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SUMMARY

1. NADP pyrophosphatase was purified about 700-fold from *Proteus vulgaris* by a procedure consisting of solubilization by detergents, fractionation by ammonium sulfate and cold ethanol, and column chromatography using DEAE-cellulose, hydroxylapatite and phosphocellulose.

2. The purified enzyme was slightly activated by Mg^{2+} and Mn^{2+} and inhibited by Co^{2+} and Zn^{2+}. When the enzyme was treated with 5 mM EDTA and dialyzed against 10 mM Tris–HCl buffer, pH 7.0, the activity almost disappeared and the enzyme was reactivated strongly by Co^{2+} and slightly by Mn^{2+}.

3. The optimum pH is 7.0. K_m value for $NADP^+$ in Tris–HCl buffer at pH 7.0 was about 25 μM.

4. Both $NADP^+$ and NADPH were cleaved rapidly at almost the same rate. NADH was also cleaved at half the rate of $NADP^+$ cleavage, while NAD^+ was cleaved at only 7% of this rate. ADP and UDP were also cleaved to release inorganic orthophosphate.

5. $NADP^+$ cleavage by the enzyme was competitively inhibited by CoA, FAD, ATP, GTP, UTP and CTP. Among them CoA, GTP and CTP were very effective inhibitors. RNA, DNA and denatured DNA also inhibited NADP pyrophosphatase activity as well as boiled extracts of *P. vulgaris*, but these inhibitions were not competitive with $NADP^+$.

INTRODUCTION

Enzymatic cleavage of the pyrophosphate linkage of NAD and NADP to produce NMN and AMP or 2′,5′-ADP has been found in a number of organisms[1–11]. Many investigators reported that the enzyme seemed to be associated with cell particles or membranes[12–17]. Although the enzyme was highly purified from potatoes and characterized by Kornberg and Pricer, Jr[2], most of the other reports were based on experiments with crude or partially purified preparations. Difficulty in purifying

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this enzyme may be attributed to the fact that the enzyme is associated with the subcellular structure and is hard to solubilize[14].

One of the properties of this enzyme is a broad substrate specificity. In respect of pyridine coenzymes as substrates, NAD seems to be cleaved faster than NADP by most of the enzymes, while enzymes from pigeon liver[5] and rat liver[18] split NADH and NADPH but not their oxidized forms. At all events, a nucleotide pyrophosphatase seems to have an important role in the metabolism of nucleotide coenzymes and nucleotide sugars.

In our laboratory, during studies on citrate metabolism by *Proteus vulgaris*, it was assumed that NADP might be absent or present in very low concentration in the extracts[19], and it was found that $NADP^+$ was degraded very rapidly by the extracts. In this report, procedures for the purification of NADP pyrophosphatase and some characteristics of the enzyme are described and its physiological significance is discussed.

MATERIALS AND METHODS

Chemicals

$NADP^+$, NADPH and NADH were obtained from Boehringer, Mannheim. NAD^+ was a product of the Sigma Chemical Co. CoA and FAD were generously given by the Daiichi Pure Chemicals Co., Ltd, and the Toa Eiyo Chemical Ind. Co., respectively. 2'-AMP containing 3'-AMP was a product of the Nutritional Biochemical Corp. Brij-58 was purchased from Nakarai Chemicals, Ltd. Yeast RNA was purchased from Wako Pure Chemical Ind., Ltd. DNA was prepared from wheat germs by the method described by Marmur[20]. All other reagents were of analytical grade, and all bivalent cations were used as chlorides. Hydroxylapatite was prepared by the method described by Levin[21].

Enzymes

Proteus vulgaris was grown at 37 °C for 18 h in a liquid medium containing 1% each of peptone and meat extract by vigorous shaking. The cells which were harvested and washed twice with deionized water by centrifugation were used as the source of the enzyme preparation. Isocitrate dehydrogenase (EC 1.1.1.42) was prepared as follows: The washed cells (about 7 g) were suspended in 20 ml of 80 mM phosphate buffer (pH 8.0), containing 0.5% sodium deoxycholate, disrupted by sonic oscillation at 20 kcycles for 7 min in a Umeda Sonor by chilling with ice–water, and centrifuged at 12 000 × *g* for 30 min at 4 °C. To the supernatant was added 1.4 vol. of $(NH_4)_2SO_4$ solution which had been saturated at 4 °C. After standing at 4 °C for 1 h, the precipitate was removed by centrifugation. Powdered $(NH_4)_2SO_4$ was added to the supernatant to 90% saturation. The precipitate obtained by centrifugation was dissolved in deionized water and dialyzed against running water for about 4 h at about 12 °C. After addition of $MnCl_2$ to about 0.5 mM, the dialyzed solution was stored at −20 °C until use as isocitrate dehydrogenase. The frozen solution was almost free from NADP-degradating activity.

Glyoxylate reductase (EC 1.1.1.26) was prepared from *P. vulgaris* by the following method: A sonic extract was prepared as described above except that deoxycholate was omitted. The fraction precipitated by between 30 and 50% satu-

ration of $(NH_4)_2SO_4$ was collected and stored at 4 °C. Before use, the stored precipitate was dissolved in 10 mM Tris–HCl buffer (pH 7.0), and dialyzed against the same buffer at 4 °C for several hours. The glyoxylate reductase solution was almost free from NADP-degradating activity.

Alcohol dehydrogenase (EC 1.1.1.1) was prepared from baker's yeast by the method described by Racker[22].

Assay of cleavage activity

In the following assays of cleavage activities for pyridine coenzymes and the others, the amount of the enzyme used was decided so as to make the reaction proceed linearly for about the initial 30 min.

$NADP^+$ cleavage activity was assayed by the following method according to the principle of the isocitrate dehydrogenase system described by Kornberg *et al.*[2]. The standard reaction mixture consisted of 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), and a suitable amount of the enzyme in a final volume of 3.0 ml. The reaction was initiated by addition of $NADP^+$ and carried out at 30 °C. After 20 min of incubation, 0.1 ml of the isocitrate dehydrogenase solution and 0.1 ml of 50 mM sodium isocitrate were added to the reaction mixture and the reduction of residual $NADP^+$ was determined by measuring the absorbance change at 340 nm in a Hitachi Perkin–Elmer spectrophotometer Type 139. The amount of $NADP^+$ cleaved during the incubation was calculated from the difference of the concentration of reducible $NADP^+$ before and after incubation based on an extinction coefficient of $6.22 \cdot 10^6$ cm^2/mole (see ref. 23). It was necessary to check that the isocitrate dehydrogenase had enough activity to reduce $NADP^+$ completely within 40 s after the reaction had been started.

NADPH cleavage activity was assayed by the glyoxylate reductase and sodium glyoxylate system, and those of NAD^+ and NADH by the alcohol dehydrogenase and ethanol system and the alcohol dehydrogenase and acetaldehyde system, respectively. The details of these systems are described in the legend to Table VI.

The cleavage activity with respect to ADP, UDP, thiamine pyrophosphate and inorganic pyrophosphate was measured by the determination of the amount of inorganic orthophosphate released during incubation with the enzyme. Details of the reaction systems for these cleavage reactions are described later (see the legend to Table VI). The concentration of inorganic orthophosphate was determined by the method of Allen[24] as modified by Nakamura[25].

Assay of protein

The concentration of protein was measured by the method of Warburg and Christian[26].

Paper chromatography

Paper chromatography of some nucleotides, coenzymes and the products of the enzyme reaction was carried out with Toyo filter paper No. 51A in three solvent systems: System 1, 1 M ammonium acetate–ethanol (3:7, v/v, pH was adjusted to 5.0 with 3 M HCl)[27]; System 2, pyridine–water (2:1, v/v)[28]; System 3, 5% Na_2HPO_4–isoamyl alcohol (isoamyl alcohol was overlayed on the 5% Na_2HPO_4 solution to a

thickness of 0.5 cm. The end of a filter paper was submerged through the layer of isoamyl alcohol to penetrate the 5% Na_2HPO_4 solution to a depth of 1 cm)[29].

The development was carried out at room temperature by the ascending technique until the solvent front moved about 25 cm from the origin. The spots were determined by quenching under ultraviolet light and NMN and related compounds were detected as fluorescent spots after spraying with 1 M KCN under ultraviolet light through a Toshiba UV-D2 glass filter.

RESULTS

Purification of the pyrophosphatase

Step 1. The washed cells (about 28 g) of *P. vulgaris* obtained from 4 l of the culture medium were suspended in 80 ml of 83 mM phosphate buffer (pH 8.0) containing 0.5% of sodium deoxycholate. Each 24 ml of the suspension was exposed to sonic vibration for 7 min in an ice bath, and collected. After centrifugation at 12 000 $\times$ *g* for 30 min, 69 ml of the supernatant solution was obtained. To the supernatant solution, 97 ml of neutralized, saturated $(NH_4)_2SO_4$ prepared at 4 °C was added by stirring and occasionally the pH was adjusted to neutral by dropwise addition of 1 M NH_4OH. After standing for more than 1 h, the precipitate was harvested by centrifugation and chilled at −20 °C. The insoluble materials harvested by centrifugation at 12 000 $\times$ *g* for 20 min were again homogenized with cold ethanol and harvested in the same manner. After chilling at −20 °C for about 2 h, the insoluble precipitate was suspended in 100 ml of 20 mM phosphate buffer (pH 7.4), by stirring and dialyzed against 3 l of the same buffer for about 10 h with several changes of buffer. To about 150 ml of very cloudy suspension obtained after dialysis was added sodium deoxycholate to 0.2%. Each 50 ml of the suspension was exposed to sonic oscillation for about 5 min in an ice bath. After collection, it was centrifuged at 12 000 $\times$ *g* for 1 h, and a yellowish clear supernatant was obtained. The supernatant solution was concentrated to 30–40 ml by polyethylene glycol No. 6000, and 1.4 vol. of saturated $(NH_4)_2SO_4$ solution was added. The precipitate was harvested and treated again with chilled ethanol as described above. The precipitate was collected, chilled at −20 °C, suspended in 50 ml of 20 mM phosphate buffer (pH 7.4), and dialyzed against the same buffer. To about 70 ml of the dialyzed solution, sodium deoxycholate was added to 0.2%. Sonic oscillation, $(NH_4)_2SO_4$ fractionation and ethanol treatment were repeated as described above. The resultant precipitate was collected, chilled at −20 °C for 2 h, suspended in 25 ml of 20 mM phosphate buffer (pH 7.4) containing 0.2% of Brij-58 and dialyzed overnight against 500 ml of 20 mM phosphate buffer, with two changes of buffer. After dialysis, Brij-58 was added to 0.4%, then the solution was again exposed to sonic oscillation for about 5 min and centrifuged at 100 000 $\times$ *g* for 1 h. About 30 ml of a yellowish clear supernatant was obtained and it was designated as the third ethanol fraction.

Step 2. The third ethanol fraction was concentrated by polyethylene glycol No. 6000 to about 10 ml and exposed to sonic oscillation for about 5 min, then it was applied to a DEAE-cellulose column (15 mm $\times$ 200 mm) previously equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.2% Brij-58. Elution was carried out stepwise with 50 ml of 20 mM phosphate buffer (pH 7.4) containing 0.2% Brij-58 and with 100 ml of the same buffer containing 0.2 M NaCl. Fractions of 5 ml were

collected and the protein concentration and the enzyme activity in each fraction were determined. The enzyme activity was found in the regions eluted both with 20 mM phosphate buffer and with the buffer containing 0.2 M NaCl. These fractions were combined.

Step 3. The combined enzyme solution eluted from a DEAE-cellulose column was concentrated to about 10 ml and exposed to sonic oscillation. The concentrated solution was applied onto a hydroxylapatite column (15 mm × 150 mm) equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.2% Brij-58. Fractions of 5 ml were collected. Enzyme activity was found mostly in the region eluted with 20 mM phosphate buffer and slightly with 42 mM buffer. These fractions were combined.

Step 4. The enzyme solution was concentrated to about 10 ml and dialyzed overnight against 500 ml of 20 mM phosphate buffer (pH 7.4) containing 0.2% Brij-58. After exposure to sonic oscillation for several minutes, the enzyme solution was loaded on a phosphocellulose column (15 mm × 350 mm) equilibrated thoroughly with 20 mM phosphate buffer (pH 7.4) containing 0.2% of Brij-58. The column was then washed with 120 ml of the equilibrating buffer and the enzyme was eluted with 120 ml of 20 mM phosphate buffer (pH 7.4) containing 0.2% Brij-58 and 0.2 M NaCl. Fractions of 4 ml were collected, and most of the enzyme activity appeared in the fractions eluted with the buffer containing 0.2 M NaCl. The resultant enzyme solution had a volume of about 10 ml and was stored at 4 °C. The purification of the enzyme from a typical preparation is summarized in Table I.

TABLE I

PURIFICATION OF NADP PYROPHOSPHATASE

The reactions were carried out under the standard assay method described in Materials and Methods. In the case of the crude extracts, 1 ml of the crude extracts was dialyzed against 500 ml of 20 mM phosphate buffer (pH 7.4) at 4 °C overnight, then the enzyme activity and the protein concentration were assayed. The units are μmoles of $NADP^+$ cleaved per h.

Fraction	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification*	*Yield (%)*
Crude extracts	69.0	2360	130.5	0.0552	1.0	100
3rd ethanol	39.0	126.5	110.2	0.875	15.7	86.5
DEAE-cellulose	95.0	69.0	70.6	1.021	18.5	56.2
Hydroxylapatite	99.0	43.2	73.8	1.71	31.0	56.5
Phosphocellulose	8.0	0.92	34.0	37.4	675.0	26.2

A part of the most purified enzyme solution was treated with 5 mM EDTA for several hours, and dialyzed overnight against 10 mM Tris–HCl buffer, pH 7.0. This preparation was designated as the EDTA-treated enzyme and stored at 4 °C.

Requirement for bivalent cations

The purified enzyme was slightly activated by 1.0 mM Mg^{2+} or Mn^{2+} but inhibited strongly by 0.33 mM Zn^{2+}, Co^{2+} or Ca^{2+}. In the EDTA-treated enzyme, much of the activity was lost, only 13% remaining as compared with the untreated enzyme. However, the EDTA-treated enzyme was reactivated by 1.0 mM Mn^{2+} to the original

level and by 0.33 mM Co^{2+} to about twenty times the original level. Reactivation by Zn^{2+} was very slight and Mg^{2+} and Ca^{2+} could not reactivate the EDTA-treated enzyme. These results are represented in Table II. The striking activation by Co^{2+} was depressed by preincubation of the EDTA-treated enzyme with 0.33 mM Mg^{2+}, Mn^{2+} or Zn^{2+}. When the enzyme preincubated with these bivalent cations was dialyzed against 10 mM Tris–HCl buffer (pH 7.0) for 24 h at 4 °C, the suppressive effects of these cations were retained. These effects were, however, considerably reduced by treatment with 5 mM EDTA (see Table III). During this treatment about 70% of the original activity was lost.

TABLE II

EFFECT OF BIVALENT METAL IONS AND EDTA ON NADP PYROPHOSPHATASE

The EDTA-treated enzyme used here was prepared as described in the text. The reaction mixtures contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), 4.77 μg of the enzyme and the indicated amounts of metals. The reaction mixture without $NADP^+$ was preincubated for 3 min at 30 °C, then the reaction was started by addition of 0.2 ml of $NADP^+$ solution.

Metal	*Concentration (mM)*	*Purified enzyme*		*EDTA-treated enzyme*	
		Specific activity (units/mg protein)	*Relative activity (%)*	*Specific activity (units/mg protein)*	*Relative activity (%)*
None	—	32.0	100	4.29	13.4
$ZnCl_2$	0.33	9.2	28.8	8.8	27.5
$CoCl_2$	0.33	18.7	58.4	668.0*	2080.0
$CaCl_2$	0.33	25.6	80.0	4.29	13.4
$MgCl_2$	0.33	26.6	83.1	4.29	13.4
$MgCl_2$	1.00	42.6	133.1	4.29	13.4
$MnCl_2$	0.33	36.2	113.1	16.5	56.5
$MnCl_2$	1.00	47.0	147.0	26.2	84.0

* 0.32 μg of the enzyme was used.

TABLE III

EFFECT OF METAL IONS ON THE ACTIVATION BY Co^{2+} OF NADP PYROPHOSPHATASE

The EDTA-treated enzyme (3.8 μg/ml) was incubated with 0.33 mM of each metal ion at room temperature for 2 h (A), then dialyzed against 10 mM Tris–HCl buffer (pH 7.0) at 4 °C overnight (B). The dialyzed enzyme solution was again treated with 5 mM EDTA for 2 h at 4 °C and dialyzed in the same manner as above (C). The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), 1 μmole of $CoCl_2$ and 0.51 μg of one of the above three kinds of enzyme solution, in a final volume of 3.0 ml.

Preincubation with	*(A) Before dialysis*		*(B) After dialysis*		*(C) After treatment with EDTA*	
	Specific activity (units/mg protein)	*Relative activity (%)*	*Specific activity (units/mg protein)*	*Relative activity (%)*	*Specific activity (units/mg protein)*	*Relative activity (%)*
$MnCl_2$	45	8.5	0	0	125	80
$ZnCl_2$	45	8.5	39	13	118	75
$MgCl_2$	230	43	39	13	45	29
$CoCl_2$	533	100	302	100	157	100

TABLE IV

EFFECT OF METAL IONS ON THE $NADP^+$ SPLITTING BY THE CRUDE EXTRACTS TREATED AND UNTREATED WITH EDTA

The crude extract was dialyzed against 10 mM Tris–HCl buffer (pH 7.0) overnight at 4 °C, then used for the assay of the enzyme activity. A part of the dialyzed extract was treated with 5 mM EDTA for 2 h at 4 °C, and dialyzed in the same manner as above described. After dialysis, the volume was adjusted with deionized water to be 2-fold of the original volume. The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), 1 μmole of metal ions and 0.1 ml of the crude extract (or 0.2 ml of the EDTA-treated extract) in a final volume of 3.0 ml.

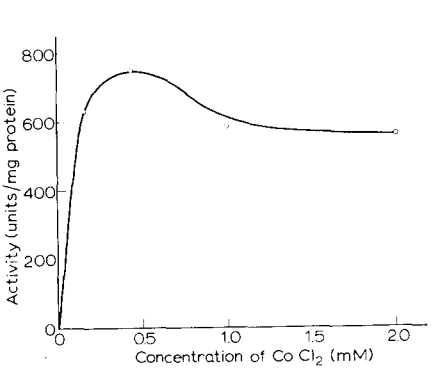
Metal (0.33 mM)	*Before EDTA treatment*		*After EDTA treatment*	
	Activity (units)	*Relative activity (%)*	*Activity (units)*	*Relative activity (%)*
None	0.325	100	0.107	33
$MnCl_2$	0.402	124	0.575	177
$MgCl_2$	0.404	125	0.275	85
$ZnCl_2$	0.111	34	0.138	42
$CoCl_2$	0.355	109	1.762*	542

* 0.02 ml of the EDTA-treated enzyme was used.

In the case of the crude extracts, the activity of $NADP^+$ degradation was not influenced by Co^{2+}. After treatment with EDTA, however, the activity was enhanced by Co^{2+} to five times as high as the original level (Table IV).

Kinetics

The effect of the concentration of Co^{2+} on the enzyme activity was examined. As shown in Fig. 1, the maximum activity was observed at 0.33 mM of Co^{2+}.



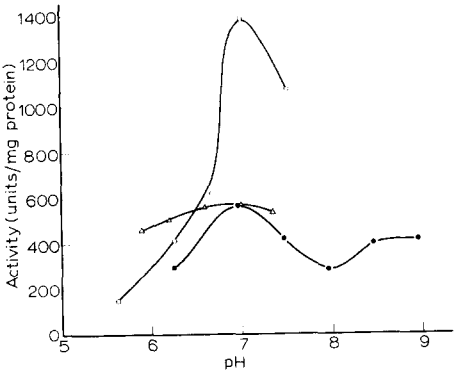


Fig. 1. Effect of the concentration of Co^{2+} on NADP pyrophosphatase activity. The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), 0.57 μg of the EDTA-treated enzyme and indicated amount of $CoCl_2$ in a final volume of 3.0 ml. The reaction was carried out at 30 °C for 20 min after addition of $NADP^+$.

Fig. 2. Effect of pH on NADP pyrophosphatase activity. Activity was assayed in the reaction mixture containing 0.2 μmole of $NADP^+$, 0.47 μg of the EDTA-treated enzyme, 1.0 μmole of $CoCl_2$, and 170 μmoles of Tris–HCl buffer (●), citrate buffer (○), or *N*-ethylmorpholine buffer △), in a final volume of 3.0 ml.

The activity of the EDTA-treated enzyme was estimated at a variety of pH values in the presence of 0.33 mM Co^{2+}. As shown in Fig. 2, optimum pH of the reaction was found to be at 7.0. In the region of higher pH than 7.8, a slight increase in the enzyme activity was observed.

$NADP^+$ cleavage activities of the EDTA-treated enzyme as a function of $NADP^+$ concentration are plotted in Fig. 3. The K_m value was calculated to be approximately 25 μM.

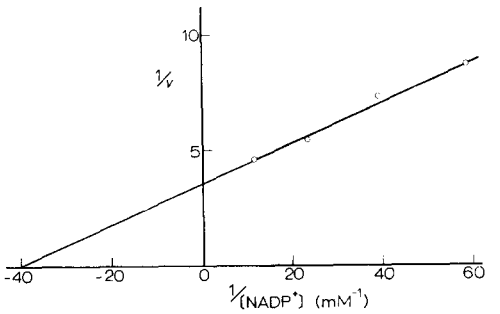


Fig. 3. K_m value for $NADP^+$. The reaction mixture contained 170 μmoles of Tris–HCl buffer (pH 7.0), 0.57 μg of the EDTA-treated enzyme, 1.0 μmole of $CoCl_2$ and various concns of $NADP^+$. Velocity, v, is given in μmoles $NADP^+$ cleaved per h.

Identification of the products of the $NADP^+$ cleavage reaction

The products of the reaction were identified by paper chromatography using three different solvent systems as described under Materials and Methods. After enzymatic cleavage of $NADP^+$, the reaction mixture was deproteinized by perchloric acid, centrifuged, and neutralized with 5 M KOH. The sample was subjected to paper chromatography. Only two spots were detectable, one of which was detected as a quenching spot with the same R_F value as the authentic 2′-AMP (3′-AMP), and another was a fluorescent spot with the same R_F value as NMN in all three solvent systems (see Table V). As a product of the reaction, 2′,5′-ADP was not detected unexpectedly. However, 2′,5′-ADP was cleaved by this enzyme preparation to release inorganic orthophosphate as mentioned in the next section. These results, therefore, suggested that $NADP^+$ was cleaved at the pyrophosphate bond to 2′,5′-ADP and NMN, and 2′,5′-ADP once formed might be further converted to 2′-AMP by the monoesterase present in the enzyme preparation.

Activity of the enzyme preparation on various substrates

Eleven substrates such as pyridine coenzymes and adenosine phosphates were examined under pH 7.0, and 0.33 mM Co^{2+}. As seen in Table VI, $NADP^+$ and NADPH were cleaved at the same rate. The cleavage rate of NADH was about half that of NADP, while that of NAD^+ was about 15% of NADP. ADP and UDP were also cleaved to release inorganic orthophosphate, but thiamine pyrophosphate and inorganic pyrophosphate were not cleaved. The apparent rates of ADP and UDP cleavages were probably estimated higher than the net cleavages at their pyrophosphate bonds, since this enzyme preparation contains a considerable amount of monoesterase activity and a part of the AMP (or UMP) produced from ADP (or UDP) by the pyrophos-

TABLE V

PAPER CHROMATOGRAPHY OF THE PRODUCTS OF THE ENZYMATIC $NADP^+$ CLEAVAGE

The reaction mixture containing 2 μmoles of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0), 1.0 μmole of $CoCl_2$ and 1.7 μg of the EDTA-treated enzyme in a final volume of 2.0 ml was incubated for 10 min at 30 °C, then the reaction was terminated by addition of perchloric acid to a final concentration of 6%, deproteinized by centrifugation at 12 000 × *g* for 30 min and neutralized by 5 M KOH with stirring in an ice bath. The neutralized sample was spotted onto a filter paper (Toyo No. 51A). As the control, the reaction mixture without $NADP^+$ was incubated and treated as described above. The authentic samples were dissolved in the control solution, and spotted on a paper. The procedure of development and detection were carried out as described in Materials and Methods. Figures in the table represent R_F values.

Sample	*Solvent system*		
	1 *Ammonium acetate–ethanol (3:7)*	*2* *Pyridine–water (2:1)*	*3* *5% Na_2HPO_4–isoamyl alcohol*
Product I	0.45	0.39	0.87
Product II	0.44	0.53	0.72
NMN	0.45	0.40	0.87
2′,5′-ADP	0.27	0.37	0.87
2′(3′)-AMP	0.44	0.52	0.64; 0.72
5′-AMP	0.44	0.60	0.73
5′-ADP	0.27	0.44	0.79
$NADP^+$	0.10	0.33	0.88
NAD^+	0.20	0.59	0.71

phatase might be further split to adenosine (or uridine) and inorganic orthophosphate by the monoesterase, thus in addition to the β-phosphate of ADP (or UDP) the α-phosphate might also be estimated.

Inhibition of $NADP^+$ cleavage reaction

Experimental results on the effects of various compounds on the activity of $NADP^+$ cleavage reaction indicated that such nucleotides as CoA, GTP and CTP inhibited the activity strongly, and especially CoA was a very effective inhibitor (Table VII). FAD, ADP, UDP, ATP and UTP inhibited the activity to some extent. 2′,5′-ADP supposed to be a product of the reaction also inhibited the activity, but NMN, another product, could not inhibit at a concentration of 0.13 mM. NAD^+, AMP and inorganic pyrophosphate had no effect. Inhibitions by CoA, ATP, FAD, UTP, GTP and CTP seemed to be competitive with $NADP^+$ according to the double reciprocal plots represented in Fig. 4. These results suggest that CoA, FAD and four nucleoside triphosphates are possibly substrates for the NADP pyrophosphatase.

Inhibition by boiled extracts, RNA, DNA and denatured DNA

During the preparation of the enzyme, we found that an endogenous inhibitor might be present in this bacteria. The inhibitor was partially purified as follows: The sonic extract was prepared without detergent as described previously. The extract was heated in a boiling water bath for 10 min, and after chilling centrifuged at 12 000 × *g* for 30 min. The supernatant was fractionated by $(NH_4)_2SO_4$ between 30 and 50% saturation. The precipitate was collected by centrifugation, dissolved in 10 mM

TABLE VI

ACTIVITY OF NADP PYROPHOSPHATASE PREPARATION ON VARIOUS SUBSTRATES

The reaction mixture A contained 2 μmoles of each substrate, 170 μmoles of Tris–HCl buffer (pH 7.0), 0.7 μmole of $CoCl_2$ and 1.1 μg of the EDTA-treated enzyme in a final volume of 2.0 ml. The reaction was initiated by addition of each substrate at 30 °C. At zero time and 20 min intervals, a 0.2-ml portion of the reaction mixture was transferred into each assay system for pyridine coenzymes which contained the following components in a volume of 2.8 ml: for $NADP^+$ reduction, 5 μmoles of isocitrate, 170 μmoles of Tris–HCl buffer (pH 7.0) and an appropriate amount of the isocitrate dehydrogenase; for NADPH oxidation, 5 μmoles of glyoxylate, 170 μmoles of Tris–HCl buffer (pH 7.0), and glyoxylate reductase; for NAD^+ reduction, 220 μmoles of ethanol, 170 μmoles of Tris–HCl buffer (pH 8.0), 15 μmoles of semicarbazide and alcohol dehydrogeanse; and for NADH oxidation, 1.2 μmoles of acetaldehyde, 170 μmoles of Tris–HCl buffer (pH 7.0) and alcohol dehydrogenase. After these reactions were completed (within 40 s), amounts of the substrate cleaved were calculated from the absorbances at 340 nm corrected by those of the corresponding control samples (see Materials and Methods). The reaction mixture B contained 4 μmoles of a substrate, 340 μmoles of Tris–HCl buffer (pH 7.0), 1.4 μmoles of $CoCl_2$ and 2.2 μg of the EDTA-treated enzyme in a final volume of 4.0 ml. The reaction condition and sampling method were as described above, except that 1 ml of the reaction mixture was subjected directly for determination of inorganic orthophosphate.

Reaction system	*Substrate*	*Specific activity**	*Relative activity (%)*
A	$NADP^+$	590	100
	NADPH	600	102
	NADH	350	59
	NAD^+	90	15
B	ADP	1170	198
	UDP	930	158
	NMN	0	0
	AMP	1980	335
	2′,5′-ADP	1720	291
	Thiamine pyrophosphate	0	0
	PP_i	0	0

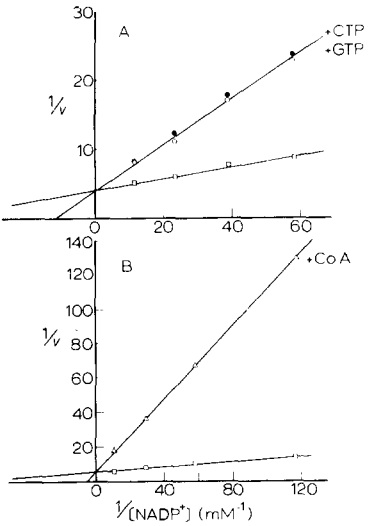
* Specific activity was expressed as μmoles of pyridine coenzyme cleaved per mg protein per h (A) or μmoles of P_i released per mg protein per h (B).

TABLE VII

INHIBITION OF NADP PYROPHOSPHATASE

The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris–HCl buffer (pH 7.0) 0.57 μg of the EDTA-treated enzyme, 1.0 μmole of $CoCl_2$ and the indicated amount of inhibitor in a final volume of 3.0 ml.

Inhibitor	*Concentration (μM)*	*Inhibition (%)*
CoA	133	85
GTP	66	53
CTP	66	55
UTP	66	36
ATP	66	22
2′,5′-ADP	133	44
ADP	133	21
UDP	133	32
FAD	133	35



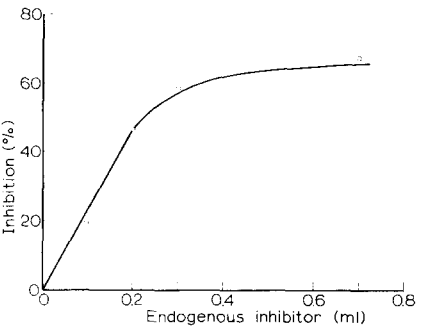


Fig. 4. Effect of CoA and nucleoside triphosphates on NADP pyrophosphatase activity. The reaction conditions were the same as described in Fig. 3 except that CoA (33 μM), CTP (66 μM) or GTP (66 μM) was added.

Fig. 5. Inhibition of NADP pyrophosphatase by endogenous inhibitor. The inhibitor prepared by the method described in the text was diluted with deionized water to a concentration of 0.5 A_{260}/ml, and the indicated volumes of the diluted inhibitor was added to the standard reaction mixture containing 0.57 μg of the EDTA-treated enzyme and 1 μmole of $CoCl_2$.

Tris-HCl buffer (pH 7.4), and dialyzed overnight against the same buffer. The resultant solution has an inhibitory effect on the enzyme reaction (see Fig. 5.)

An attempt was made to evaluate the effects of RNA, DNA and denatured DNA on the enzyme activity. As shown in Fig. 6, RNA and denatured DNA were strong inhibitors of the enzyme, while 5 μg of DNA inhibited only 15%. Despite the production of 80% inhibition by 4 μg of RNA, even when a large amount of RNA (18 μg) was used, complete inhibition did not occur.

Inhibitions by RNA and by denatured DNA were not competitive with $NADP^+$ (Fig. 7). Maximum velocity of the NADP pyrophosphatase was inhibited to 43%

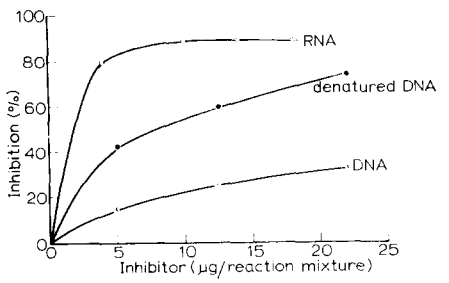


Fig. 6. Inhibition of NADP pyrophosphatase by RNA, DNA and denatured DNA. The reaction mixture contained 0.2 μmole of $NADP^+$, 170 μmoles of Tris-HCl buffer (pH 7.0), 1.0 μmole of $CoCl_2$, 0.57 μg of the EDTA-treated enzyme and the indicated amounts of yeast RNA, wheat DNA or denatured DNA in a final volume of 3.0 ml.

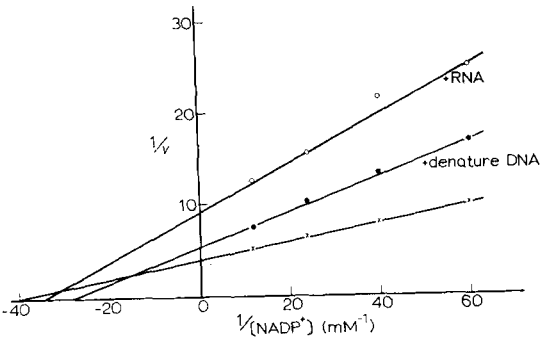


Fig. 7. Lineweaver–Burk plots of inhibition of NADP pyrophosphatase by RNA and denatured DNA. The reaction conditions were the same as described in Fig. 3 except that 0.8 μg of yeast RNA or 3 μg of denatured wheat germ DNA was added or not.

and 74% by 0.8 μg of RNA and by 3 μg of denatured DNA in 3 ml of the reaction mixture, respectively.

DISCUSSION

Several reports on nucleotide pyrophosphatase from a variety of organisms have been published. Most of these reports have been based upon experiments with crude or partially purified enzyme preparations. The nucleotide pyrophosphatases have a broad specificity for substrate and, in general, cleave NAD^+ more rapidly than $NADP^+$ (see refs 2, 4, 11 and 14). Our nucleotide pyrophosphatase purified about 700-fold from *Proteus vulgaris* cleaved $NADP^+$ and NADPH faster than NADH and NAD^+. The rate of NAD^+ cleavage was only 15% of $NADP^+$ or NADPH. Since NAD^+ splitting was much slower than NADH splitting, our enzyme may play the same regulatory role as the pigeon enzyme[5] which was supposed to displace the equilibrium of the reaction which were dominant for NAD^+ formation by removal of NADH. However, because of the rather slow rates of NADH and NAD^+ splitting compared with $NADP^+$ and NADPH splitting, our enzyme may be supposed to regulate an intracellular concentration of NADP and consequently the biosynthetic reactions.

It seems that the nucleotide pyrophosphatases are generally inhibited by EDTA and that the requirement of metal ions for their activities are various in concentration and sort. These enzymes are activated by the addition of bivalent cations such as Co^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} (see refs 4, 9, 11, 16 and 30), with the exception of the enzymes from rat liver microsomes[14], potatoes[2], and rat liver plasma membranes[17]. It is noteworthy that many experiments concerned with the requirement of metal ions were performed by the enzymes which were not treated with chelating agents. It seems that metals firmly bind to the enzyme proteins and are released in various degrees during the preparation of the enzyme. In the present study, the purified enzyme showed a very different requirement for metal ions before and after treatment with EDTA (see Table III). Similar results were obtained in the case of crude extracts, *i.e.* for the activation of the enzyme by Co^{2+}, pretreatment with EDTA was necessary (Table IV). Therefore, it may be concluded that activation by Co^{2+} re-

quires the removal of other metal ions such as Mn^{2+}, Zn^{2+} and Mg^{2+} which seem to be firmly bound to the enzyme protein.

Swartz *et al.*[3] found a heat-labile protein inhibitor of a nucleotide pyrophosphatase in *P. vulgaris* X-19. Although we have not studied any heat-labile inhibitors, we found an inhibitor in boiled extracts of *P. vulgaris* which was heat-stable, precipitable by $(NH_4)_2SO_4$ and undialyzable, and which, therefore, seems to be a large molecular compound. RNA and denatured DNA as well as the endogenous inhibitor were found to be very effective inhibitors of $NADP^+$ splitting, and these inhibitions were not competitive with $NADP^+$ while inhibitions by CoA, FAD, and nucleoside triphosphates were competitive with $NADP^+$. The mechanism of the inhibition by RNA and DNA is still obscure, but some electrostatic interaction may exist between nucleic acids and the enzyme protein since the enzyme protein was adsorbed to a cation exchanger, phosphocellulose. It is therefore proposed that the intracellular activity of the enzyme may be regulated by some nucleic acids. As to the relationship between pyridine coenzymes and nucleic acids, there are reports[31,32] concerning the polynucleotide-joining enzyme in which it was reported that NAD was required as a cofactor of the reaction and that NAD was cleaved to AMP and NMN when the DNA strands were joined through 3',5'-phosphodiester bond. It is interesting to consider the possibility that the NADP pyrophosphatase activity may be regulated by DNA in the living cells of *P. vulgaris*.

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