Study on the Pyridoxal Kinase of Mouse Brain¹

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The properties of the pyridoxal kinase of mouse brain were studied and the following results were obtained.

1. It was concluded that toxopyrimidine seemed to be phosphorylated by the same phosphokinase as pyridoxal based on the following reasons: the mutual competitive inhibition, the coincidence of the metal requirements, the denaturation effects of heat treatment on the enzyme and the behaviors of inhibitors for both substrates.

2. The substrate specificities of the pyridoxal-kinase were investigated and the following results were obtained: (a) The pyridoxal-kinase was able to act on both certain pyridine and pyrimidine derivatives (b) The 2-methyl of the pyrimidine ring was able to be substituted with C_2H_5 or SCH_3 group for CH_3 group (c) The 4 position of the pyridine ring was able to be substituted with CH_2OH , CH_2NH_2 or CH_3 group for CHO group (d) The compounds without a hydroxymethyl group in the 5 position of the pyridine ring were not phosphorylated at all (such as pyrithoxine) or slightly phosphorylated in such a special case as 5-deoxypyridoxine

In the early experiment, the authors (1) studied on the inhibitory action of TXP on the PAL-kinase of mouse brain. It was found that TXP not only inhibited the PAL-kinase, but also was phosphorylated by the enzyme to TXP-P. It is an interesting fact that PAL (one of pyridine compounds) and TXP (one of pyrimidine compounds) are phosphorylated by the same phosphokinase. In this paper, the authors tried to investigate the properties of the purified PAL-kinase of mouse brain, particularly the substrate specificities³.

¹ Abbreviations used: TXP, toxopyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine), Merck Index, 7th edition, Merck and Co. (1960) p. 1055, PAL, pyridoxal; TXP-P, toxopyrimidine phosphate (5); GSH, reduced glutathione; TCA, trichloroacetic acid; PCA, perchloric acid; PIN, pyridoxine; PAM, pyridoxamine.

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³ Concerning the pyridoxal kinase of rat and beef brain there is a brief description of Snell *et al* (7).

EXPERIMENTAL PROCEDURES

1. Preparation of the PAL-kinase

All operations except those specified were conducted at 0 to 4°. 35 g of mouse brain were homogenized with a ten fold excess of cold distilled water. The brain homogenate was centrifuged at $20,000 \times g$ for 30 minutes and the precipitate was discarded. To the supernatant solution were added 10 ml of 0.5 *M* phosphate buffer, pH 7.2, and successively 5 ml of 1 *M* solution of calcium acetate carefully with continuous stirring by a magnetic stirrer. After stirring for five minutes, the Ca-acetate gel formed was removed by centrifugation at $20,000 \times g$ for 20 minutes, and the supernatant was salted out with ammonium sulfate. The protein fraction insoluble between 35 and 60 % saturation was separated and dissolved in a small volume of 0.005 *M* K-phosphate buffer, pH 6.8, containing 0.1 mM GSH and dialyzed against the same buffer overnight. After the precipitate formed was removed by centrifugation, the remaining solution was poured over a column containing a 500 to 600 fold excess of hydroxylapatite gel to protein. Hydroxylapatite gel was prepared by the method of Tiselius (2). After the column was washed with 50 ml of 0.02 *M* K-phosphate buffer, pH 6.8, the PAL-kinase was eluted with 200 ml of 0.075 *M* K-phosphate buffer, pH 6.8. Fractions containing kinase activity were pooled and the protein fraction insoluble between 35 and 55 % saturation with ammonium sulfate was dialyzed overnight as described above. After the precipitated material was removed by centrifugation, the clarified solution, after adjusted to 0.005 *M* as to phosphate content, was poured over a column containing a 100-fold excess (cellulose to protein) of DEAE-cellulose. DEAE-cellulose (Brown Company) was recycled through acid and base before use.

The protein adsorbed on the column and washed with 0.005 *M* phosphate buffer, was eluted fractionally in a linear gradient established between 0.025 and 0.5 *M* K-phosphate buffers, pH 6.8. When the mixing chamber was filled with 100 ml of 0.025 *M* K-phosphate buffer, pH 6.8, the kinase activity appeared in the 30 to 95-ml fractions of the effluent. The kinase activity was determined on the above-mentioned whole fractions which were fractionated 4 ml apiece. The 42 to 72-ml fractions showing equal specific activity were pooled, and ammonium sulfate was added to 60 % saturation. The active protein was collected by centrifugation and dialyzed as mentioned above.

The purified PAL-kinase preparation thus obtained was 60 to 70 % pure ultra-centrifugically and did not contain ATPase activity. The specific activity of this enzyme was increased 3550 times the original homogenate as indicated in Table 1.

TABLE 1
Purification of Pyridoxal Kinase of Mouse Brain

Fraction	Specific activity	Relative purity
1. Homogenate	0.474	1
2. Centrifuged extract	2.92	6.2
3. Ca-phosphate gel fractionation	10.1	21.4
4. $(\text{NH}_4)_2\text{SO}_4$ 35 to 60 % saturation	124.5	265
5. Hydroxylapatite gel	610	1,285
6. $(\text{NH}_4)_2\text{SO}_4$ 34 to 55 % saturation	1,150	2,420
7. DEAE-cellulose	1,680	3,550

2. Determination of the PAL-kinase Activity

The assay conditions were given in the respective figures and tables. When PAL was used as the substrate of the kinase, the reaction was stopped by the addition of 0.2 ml of 50 % TCA and was refrigerated to 0°, and 0.2 ml of 50 % H₂SO₄ and 0.2 ml of the phenylhydrazine reagent (200 mg of phenylhydrazine·HCl dissolved in 10 ml of 10 N H₂SO₄) were added. After 30 minutes the optical density at 410 mμ was measured (Wada and Snell (3)).

When TXP was used as the substrate of PAL-kinase, the reaction was stopped by the addition of 0.5 ml of 4.5 N PCA, and 4.5 N KOH was added for neutralization. The potassium salt precipitated was removed by centrifugation and the supernatant solution was poured on a column (0.8×4.0 cm) of Dowex 1×4 (formate form). After 25 ml of distilled water containing TXP ran out, TXP-P was eluted by 0.1 N HCOOH. As in this fraction a small amount of AMP, contaminated in the sample of ATP, was eluted together with TXP-P, the control without TXP and PAL-kinase was also made. The effluent was pooled 5 ml apiece and the optical density at 245 mμ was read with spectrophotometer.

When other compounds were used as the substrate of PAL-kinase, ADP formed was determined by the method of Potter *et al.* (4). The reaction was stopped by the addition of 0.5 ml of 4.5 N PCA, and 4.5 N KOH was added for neutralization as described above. The salt formed was discarded, 0.9 ml of 1 M NH₄OH was added to the remaining solution, and the solution was poured over a column containing Dowex 1×4 (Cl-form) (0.8×4.0 cm). After 25 ml of distilled water and 170 ml of 0.003 N HCl ran out, ADP was eluted by 45 ml of 0.02 M NaCl-0.01 N HCl solution. The effluent was fractionated in four parts (a) 15, (b) 10, (c) 10 and (d) 10 ml, respectively. ADP content was calculated from the optical density of fractions (b) to (d) at 260 mμ.

Specific activity of the kinase was expressed as units per mg of protein, where 1 unit was the amount of protein that formed 1 mμmole of PAL-P in 1 hour at 38°.

3. Other Methods

On paper chromatography, B₆ derivatives were detected by their ultraviolet absorption, their fluorescence or the coloration by Gibbs' reagent. TXP derivatives were detected by their ultraviolet absorption on paper chromatography. The quantitative analysis of phosphoric acid was carried out by the method of Allen (5). Protein content was determined by the optical density at 280 mμ.

RESULTS AND DISCUSSION

1. Phosphorylation of TXP and PAL and the Mutual Competition

TXP was phosphorylated by the purified PAL-kinase to TXP-P as well as PAL (Figs. 1 and 2). The Michaelis constants were 0.86×10^{-4} M for PAL and 2.67×10^{-4} M for TXP. When the two substrates were incubated in the respective best condition with the kinase in the presence of ATP, TXP was phosphorylated with the rate no less than 2-fold for PAL. Therefore, PAL and TXP served as competitive substrates for this enzyme (Figs. 3 and 4). As an inhibitor for the PAL-kinase PAL was much more potent than TXP, that is, for the phosphorylation of PAL by the kinase, TXP inhibited, at an equal concentration to PAL, the phosphorylation of PAL

FIG. 1 Phosphorylation of Pyridoxal by the Pyridoxal Kinase of Mouse Brain

v , PAL-P formed per minute (μg). Incubation mixture contained 0.15 mM ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer, pH 5.52, 75 μg purified kinase and PAL. Total volume 2.5 ml. Incubation was performed at 38° for 1 hour.

FIG. 2 Phosphorylation of Toxopyrimidine by the Pyridoxal Kinase of Mouse Brain

v , TXP-P formed per minute (μg). Incubation mixture contained 0.25 mM ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer, pH 6.42, 225 μg purified kinase and TXP. Total volume 7.5 ml. Incubation was carried out at 38° for 1 hour.

FIG. 3 Competitive Inhibition of Toxopyrimidine for the Phosphorylation of Pyridoxal

v , PAL-P formed per minute (μg). Incubation mixture contained 0.15 mM ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer, pH 5.52, 75 μg kinase and PAL. Total volume 2.5 ml. Incubation was carried out at 38° for 1 hour.

FIG. 4 Competitive Inhibition of Pyridoxal for the Phosphorylation of Toxopyrimidine

v , TXP-P formed per minute (μg). Incubation mixture contained 0.25 mM ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer, pH 6.42, 225 μg kinase and TXP. Total volume 7.5 ml. Incubation was carried out at 38° for 1 hour.

FIG. 5 *pH Optima of Pyridoxal Kinase of Mouse Brain for the Phosphorylation of Pyridoxal and Toxopyrimidine*

Incubation mixture in (I) contained 0.4 mM TXP and ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer (pH 4.9 to 7.8) and 225 μg kinase. Total volume 7.5 ml. In (II), incubation mixtures contained 0.3 mM PAL and ATP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffer (pH 4.9 to 7.8) and 75 μg kinase. Total volume 2.5 ml. Incubation was carried out at 38° for 1 hour.

about 8 % and 50 % at $10^{-3}M$ 20-fold concentration to PAL. For the phosphorylation of TXP (at 0.25 mM) by the kinase, PAL inhibited about 50 % at 0.05 mM and 80 % at an equal concentration to TXP. The inhibitory constants (K_i) were $3.2 \times 10^{-4}M$ for TXP and $0.29 \times 10^{-4}M$ for PAL.

2. pH Optimum

The optimum pH of the kinase were approximately 5.5 for PAL and 6.4 for TXP (Fig. 5). At pH 5.0 PAL showed high affinity but TXP had rather low affinity for the kinase. On the other hand, TXP showed, at pH 6.4, higher affinity for the enzyme than PAL. As the affinity of PAL and TXP for the kinase varied thus with pH, the inhibition of TXP for phosphorylation of PAL by the kinase was expected to change with pH. Therefore, the reaction mixtures containing 0.15 mM PAL and 1.0 mM TXP were incubated in the presence of the kinase and ATP at several pH levels. It was confirmed, as shown in Table 2, that the inhibition of TXP markedly varied with pH as expected.

TABLE 2
pH-Dependence of the Inhibition of Pyridoxal Kinase of Mouse Brain by Toxopyrimidine

pH	Inhibition ratio
	per cent
4.96	0
5.52	25.6
6.60	57.4
7.25	37.3

Incubation mixture contained 0.15 mM PAL and ATP, 1.0 mM TXP, 0.01 mM ZnSO_4 , 50 mM K-phosphate buffers of various pH levels and 75 μg kinase. Total volume, 2.5 ml. Incubation was carried out at 38° for 1 hour.

3. Identity of PAL-kinase and TXP-kinase

It was concluded from the following experimental data that PAL and TXP were phosphorylated by the same enzyme.

Effects of metal ions — Zn^{2+} or Co^{2+} was essential either to the phosphorylation of TXP and to that of PAL (Fig. 6). The activation effect of Co^{2+} was approximately 80 % of Zn^{2+} . The optimal concentrations of Co^{2+} were approximately 1×10^{-4} and $2 \times 10^{-4}M$ for PAL and TXP, respectively. Mg^{2+} was about 40 % effective compared with Zn^{2+} , and Ca^{2+} showed only a little effect.

FIG. 6 Activation of Pyridoxal Kinase of Mouse Brain by Zn^{2+}

Incubation mixture in (I) contained 0.5 mM TXP, 0.3 mM ATP, 50 mM K-phosphate buffer, pH 6.42, 225 μ g kinase and $ZnSO_4$ of various concentrations. Total volume 7.5 ml. In (II), incubation mixture contained 0.3 mM PAL and ATP, 50 mM K-phosphate buffer, 75 μ g kinase and $ZnSO_4$ of various concentrations. Total volume 2.5 ml. Incubation was performed at 38° for 1 hour.

4. Substrate Specificities

For pyridoxine and pyridoxamine—It has been reported by Snell *et al.* (7) that PAL-kinase from several tissues of animals or bacteria phosphorylated not only PAL but also PIN and PAM. The PAL-kinase from mouse brain also phosphorylated PIN and PAM like PAL. The Michaelis constants were approximately 0.5×10^{-4} and 2×10^{-4} M for PIN and PAM, respectively (Figs. 7 and 8). The optimum

Effects of heat treatment on the enzyme activity—The PAL-kinase was fairly heat labile. When the kinase was incubated at 45, 50 or 55° for 5 minutes at pH 4.96, the decrease in the enzyme activity for PAL was parallel to that for TXP (Table 3).

The inhibition by PAL derivatives—It has been shown by Snell *et al.* (6) that carbonyl derivatives of PAL were potent inhibitors for the PAL-kinase of rat brain. We investigated the effects on the PAL-kinase of mouse brain of six carbonyl derivatives of PAL synthesized by the method of Snell *et al.* (6) (Table 4). It was found that the inhibition ratio was generally parallel for both PAL and TXP though the degree of inhibition was somewhat different in each inhibitor. PAL-azine was the most potent inhibitor, and PAL-isonicotinoylhydrazide and PAL-semicarbazone also inhibited strongly the phosphorylation of PAL or TXP. PAL-thiosemicarbazone and PAL-oxime did not inhibit the kinase activity on TXP at 2×10^{-6} M.

TABLE 3
Effects of Heat Treatment of the Pyridoxal Kinase on the Phosphorylation of Pyridoxal and Toxopyrimidine

Substrates	PAL-P or TXP-P formed (μ g)			
	control	45° ^a	50° ^a	55° ^a
PAL	14.1	7.6	2.2	0
Ratio, activity to control (%)	100	53.8	15.5	0
TXP	119	70	15.6	0
Ratio, activity to control (%)	100	59.0	13.1	0

^a Heat treatment was carried out for 5 minutes at pH 4.96.

Incubation mixture in (I) contained 0.3 mM PAL and ATP, 0.05 mM $ZnSO_4$, 0.05 mM K-phosphate buffer, pH 5.52, and 75 μ g kinase. Total volume 2.5 ml. In (2), incubation mixture contained 0.5 mM TXP, 0.3 mM ATP, 0.1 mM $ZnSO_4$, 50 mM K-phosphate buffer, pH 6.42, and 225 μ g kinase. Total volume 7.5 ml. Incubation was performed at 38° for 1 hour.

TABLE 4
Inhibitory Effect of Certain Carbonyl Reagents and Their Condensation
Products with Pyridoxal on Pyridoxal Kinase of Mouse Brain

Inhibitors ^a	Inhibition ratio (%)	
	(1) PAL	(2) TXP
PAL-thiosemicarbazone	7.5	0
Thiosemicarbazide	0	0
PAL-oxime	12.0	0
Hydroxylamine	0	0
PAL-phenylhydrazine	31.0	18.5
Phenylhydrazine	10	9.5
PAL-isonicotinoylhydrazine	66.3	33.5
Isoniazide	10	3.0
PAL-azine	67.6	51.6
Hydrazine	10	5.0
PAL-semicarbazone	36.5	28.0
Semicarbazide	11.0	3.0

^a The final concentration of inhibitors was $2 \times 10^{-4} M$.

Incubation mixture in (1) contained 0.15 mM PAL and ATP, 0.05 mM $ZnSO_4$, 50 mM K-phosphate buffer, pH 5.52, 75 μg kinase and inhibitors. Total volume 2.5 ml. In (2), incubation mixture contained 0.3 mM TXP and ATP, 0.05 mM $ZnSO_4$, 50 mM K-phosphate buffer, pH 6.42, 225 μg kinase and inhibitors. Total volume, 7.5 ml. Incubation was performed at 38° for 1 hour.

FIG. 7 Phosphorylation of Pyridoxine by the Pyridoxal Kinase of Mouse Brain

v , ADP formed per minute (μg). Incubation mixture contained 0.4 mM ATP, 0.05 mM $ZnSO_4$, 50 mM phosphate buffer, pH 4.90, 330 μg kinase and PIN of various concentrations. Total volume 7.5 ml. Incubation was carried out at 38° for 90 minutes.

FIG. 8 Phosphorylation of Pyridoxamine by the Pyridoxal Kinase of Mouse Brain

v , ADP formed per minute (μg). Incubation mixture contained 0.4 mM ATP, 0.05 mM $ZnSO_4$, 50 mM phosphate buffer, pH 5.52, 330 μg kinase and PAM of various concentrations. Total volume 7.5 ml. Incubation was at 38° for 90 minutes.

pH in phosphate buffer was approximately 5.5 and 4.9 for PAM and PIN, respectively (Fig. 9).

Confirmation of PIN-P and PAM-P by PAL-kinase—As reported in the previous paper (1), the incubation mixture was poured on a column containing Dowex 1 $\times$ 4 (Cl-form), and the fraction eluted by 0.003 N HCl was pooled, concentrated and developed by ascending paper chromatography together with synthetic

FIG. 9 *pH Optima of Pyridoxal Kinase of Mouse Brain for the Phosphorylation of Pyridoxine and Pyridoxamine*

Incubation mixture contained 1 mM PIN or PAM, 0.4 mM ATP, 0.05 mM ZnSO_4 , 50 mM phosphate buffer and 330 μg kinase. Total volume 7.5 ml. Incubation was carried out at 38° for 1 hour. Curve (1), PAM; Curve (2), PIN.

PIN-P and PAM-P, and the formation of PIN-P or PAM-P was confirmed by the coincidence of the R_f values with synthetic PIN-P or PAM-P. The developing solvents used were (a) water-saturated butanol, (b) *n*-butanol-acetic acid- H_2O (4:1:5) and (c) *n*-propanol-water-1 M acetate buffer, pH 5.0 (70:20:10). The R_f values were in solvent (a) PIN-P 0.054; in (b) PIN-P 0.29, PAM-P 0.17 and in (c) PIN-P 0.29, PAM-P 0.19.

The competitive relation between PAL and PIN or PAM—PIN or PAM competitively inhibited the phosphorylation of PAL by PAL-kinase and under a similar condition PIN was more potent than PAM (Figs. 10, 11). In the presence of ATP $4 \times 10^{-4} M$ and PAL $1.5 \times 10^{-4} M$, PAM did not inhibit the kinase at $1.5 \times 10^{-4} M$, inhibited 12 % at $3 \times 10^{-4} M$ and 31 % at $1 \times 10^{-3} M$. In contrast to PAM, PIN showed the inhibition of 34 % at $0.5 \times 10^{-4} M$, 62 % at $1.5 \times 10^{-4} M$ and 95 % at $1 \times 10^{-3} M$. This is presumably due to the difference between the K_m values for PIN and PAM.

Fig. 10 *Competitive Inhibition of Pyridoxine for the Phosphorylation of Pyridoxal*

v , PAL-P formed per minute (μg). Incubation mixture contained 0.4 mM ATP, 0.05 mM ZnSO_4 , 50 mM phosphate buffer, pH 5.52, 75 μg kinase and PAL of various concentrations. Total volume 2.5 ml. Incubation was performed at 38° for 1 hour.

FIG. 11 *Competitive Inhibition of Pyridoxamine for the Phosphorylation of Pyridoxal*

v , PAL-P formed per minute (μg). Incubation mixture contained 0.4 mM ATP, 0.05 mM ZnSO_4 , 50 mM phosphate buffer, pH 5.52, 75 μg kinase and PAL of various concentrations. Total volume 2.5 ml. Incubation was performed at 38° for 1 hour.

The phosphorylation of PAL and TXP derivatives — As PAL derivatives except PIN and PAM, 4-deoxy-PIN (I), 5-deoxy-PIN (II) and pyriothioxine (III), and as TXP derivatives 2-ethyl-4-amino-5-hydroxymethylpyrimidine (IV) and 2-methylmercapto-4-amino-5-hydroxymethylpyrimidine (V) were tested whether they were able to be the substrates for the PAL-kinase of mouse brain (Figs. 13, 14, 15, and 16) or not. 5-Deoxy-PIN was slightly phosphorylated (about 20 % of 4-deoxy-PIN), while pyriothioxine was not phosphorylated at all. Since these compounds seemed to be phosphorylated by the same kinase, the results concerning the substrate specificities of the kinase would be as follows: (a) the PAL-kinase has the ability to act on both certain pyridine and pyrimidine derivatives, (b) the methyl attached to 2-position of pyrimidine ring was able to be substituted with C_2H_5 or SCH_3 group.

FIG. 13 Phosphorylation of 4-Deoxypyridoxine by the Pyridoxal Kinase of Mouse Brain

The reaction condition was the same as that in Fig. 2.

FIG. 14 Phosphorylation of 5-Deoxypyridoxine by the Pyridoxal Kinase of Mouse Brain

The reaction condition was the same as that in Fig. 2.

2-Ethyl-4-amino-5-hydroxymethylpyrimidine, $\times 10^4 M$

FIG. 15 Phosphorylation of 2-Ethyl-4-amino-5-hydroxymethylpyrimidine by the Pyridoxal Kinase of Mouse Brain

The reaction condition was the same as that in Fig. 2.

2-Methylmercapto-4-amino-5-hydroxymethylpyrimidine, $\times 10^4 M$

FIG. 16 Phosphorylation of 2-Methylmercapto-4-amino-5-hydroxymethylpyrimidine by the Pyridoxal Kinase of Mouse Brain

The reaction condition was the same as that in Fig. 2.

(c) the 4-position of the pyridine ring was able to be substituted with CH_2OH , CH_2NH or CH_3 for CHO group and (d) the compounds without a hydroxymethyl group in the 5 position of the pyridine ring were not phosphorylated at all (such as pyrithioxine) or hardly phosphorylated (such as 5-deoxy-PIN).

Confirmation of the phosphorylation of PAL and TXP derivatives — The phosphorylation of these compounds was confirmed as follows: As described in case of identification of PIN-P and PAM-P, the incubation mixture was poured on a column containing Dowex 1×4 (Cl-form) and the fraction eluted by 0.003 N HCl was pooled, concentrated and developed by ascending paper chromatography. The developing solvent used was the mixture consisting of *n*-propanol-water-1 M acetate buffer, pH 5.0 (70 : 20 : 10). The formation of 4-deoxy-PIN-P was confirmed by the coincidence of the *R_f* value with the synthetic 4-deoxy-PIN-P. In case of 5-deoxy-PIN-P, 2-ethyl-4-amino-5-hydroxymethylpyrimidine and 2-methylmercapto-4-amino-5-hydroxymethylpyrimidine whose phosphorylated compounds were not provided synthetically, their unphosphorylated compounds and AMP were developed as controls. The spots different from the unphosphorylated compounds, AMP or PIN-P, were cut out, and extracted for 3 hours at 38°. The extract was again poured on the column containing Dowex 1×4 (Cl-form) and 0.003 N HCl fraction was pooled for measurement of ultraviolet absorption spectrum. As the result, the spectra of the three compounds except 5-deoxy-PIN-P coincided well with those of the unphosphorylated compounds in acidic and basic solutions. (The phosphorylation at 5-hydroxymethyl-position does not affect the spectra of the original compounds. The compound which appears to be 5-deoxy-PIN-P was not sufficient in quantity to measure the ultraviolet absorption spectrum.) Moreover, phosphorylation was confirmed with color test on the paper chromatogram. It was confirmed further by the quantitative analysis of phosphoric acid of the compounds in question that the PAL-kinase in the presence of ATP phosphorylated 4-deoxy-PIN, 2-ethyl-4-amino-5-hydroxymethyl-pyri-

Fig. 12

midine and 2-methylmercapto-4-amino-5-hydroxymethylpyrimidine to their respective monophosphate ester.

It is an interesting fact that a small amount of ADP was formed when 5-deoxy-PIN, which has no hydroxymethyl group in the 5 position, was incubated with ATP and the kinase. The formation of 5-deoxy-PIN-P remains to be investigated.

Requirement of Zn^{2+} ion—Also for the phosphorylation of PIN, PAM, (I), (II), (IV), and (V) Zn^{2+} was found to be essential (Fig. 12).

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