

**NEW REACTIONS ON
MICROBIAL METABOLISM OF VITAMIN B₆**

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ABBREVIATIONS

PIN-P	Pyridoxine 5'-phosphate
PAL-P	Pyridoxal 5'-phosphate
PAM-P	Pyridoxamine 5'-phosphate
Total vitamin B ₆ -P	Total vitamin B ₆ phosphates
PIN-G	Pyridoxine glucoside
<i>p</i> -NPP	<i>p</i> -Nitrophenyl phosphate
<i>p</i> -NP	<i>p</i> -Nitrophenol
Phenyl-P	Phenyl phosphate
<i>p</i> -AAP	<i>p</i> -Aminoacetophenone

INTRODUCTION

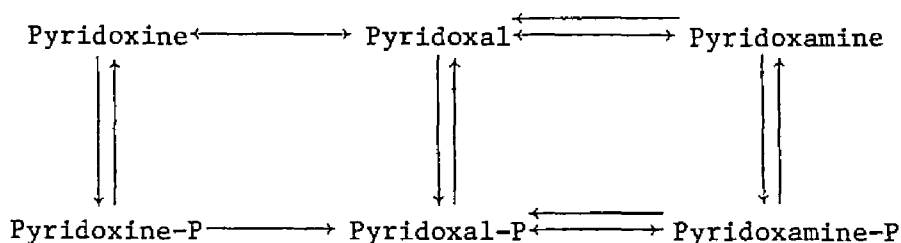
Vitamin B₆ was first defined and delineated to be a distinct entity in the vitamin B₂ complex as the rat pellagra preventive factor!¹⁾ Subsequently, the isolation of pure crystalline vitamin B₆ was reported by Lepkovsky²⁾ and several other groups³⁻⁸⁾ Within a year, the structure and the synthesis were demonstrated by Harris and Folkers⁹⁾ and others!¹⁰⁻¹²⁾ Then, the term "pyridoxine" was proposed by György and Eckhardt¹³⁾ for the vitamin with general acceptance. As for its requirement by the human organism, it suffices to refer to the pioneering studies of Snyderman *et al.*¹⁴⁾ who observed convulsions in one mentally retarded young infant and anemia in an older child both had been restricted to a vitamin B₆-deficient diet!¹⁵⁾

At these stages of the historical development, the microbiological research had entered the scene in the connection of the assay of the vitamin. The discovery of other forms of pyridoxine, *i.e.*, pyridoxal and pyridoxamine, was a consequence of the finding^{16,17)} that although pyridoxine appeared to serve as an essential growth factor for certain lactic acid bacteria, it was essentially inactive in this capacity unless it was heated with the medium. Following, the additional members of pyridoxine were searched in a wide range of natural product¹⁸⁻²³⁾ and defined the structure along with the synthesis²⁴⁻³⁰⁾ Then, it was established that the biological

activity of the vitamin B₆ group was displayed by pyridoxine (pyridoxol), pyridoxal, pyridoxamine, and their 5'-phosphate esters³¹⁾

In various investigations on the function of the vitamin B₆ group, it has been made evident that pyridoxal-P was the main biocatalytically active form of the vitamin, and that the other compounds owed their vitamin activity to the conversion to pyridoxal-P. It serves as a coenzyme for a multiplicity of biosynthetic and catabolic enzyme systems, especially of the amino acid metabolism^{32,33} A number of pyridoxal-dependent enzymes are found under each of the following general categories; oxidoreductases^{34,35)} transferases^{36-lyases⁴¹⁻⁴⁶⁾ isomerases^{47,48)} and ligases⁴⁹⁾ And these enzymes have been available on the elucidation of the coenzymatic action of vitamin B₆ on a molecular level from the first nonenzymatic duplication by Snell *et al.*⁵⁰⁻⁵²⁾ and Braunstein.⁵³⁾}

On the other hand, the knowledges of the enzymatic interconversion of various forms of free vitamin B₆ and of their conversion to pyridoxal-P have been expanded considerably in recent years.⁵⁴⁾ The number of known reactions that vitamin B₆ may undergo in tissues of one or another organism is already large as summarized below.



The enzyme, pyridoxal kinase, which catalyzed the phosphorylation of vitamin B₆ was first purified partially from brewer's yeast by Hurwitz⁵⁵) He established the stoichiometry of the reaction between ATP and pyridoxal, together with the facts that the kinase phosphorylated pyridoxine, pyridoxamine and several other pyridine derivatives in addition to pyridoxal⁵⁶) Subsequently, McCormick *et al.* employed simplified methodology to study the distribution to study the distribution of this enzyme, and to purify and compare properties of kinases from several different sources⁵⁷⁻⁵⁹)

The dephosphorylation of phosphorylated forms of vitamin B₆ has been clarified to be catalyzed by various phosphatase preparations⁶⁰⁻⁶⁶) In these cases, non-specific acid or alkaline phosphatase may act on the hydrolyzed reaction.

The oxidative conversion of pyridoxine into pyridoxal was first found with rabbit liver by the addition of an excess of aldehyde oxidase, which catalyzed the oxidation of pyridoxal to 4-pyridoxic acid, to the reaction mixture^{67,68}) Snell *et al.* purified pyridoxine oxidase from pseudomonad which had FAD as the prosthetic group⁶⁹) While, the existence of a NADP-specific pyridoxine dehydrogenase in baker's and brewer's yeasts was reported^{70,71}) Recently, it has been found that the dehydrogenation of pyridoxine which favored the reduction of pyridoxal could be remarkably catalyzed by the cells of baker's yeast with an addition of suitable carbonyl reagent,⁷²) accompanied with the nonenzymatic Schiffization.^{73,74)}

On the formation of pyridoxal from pyridoxamine, the irreversible oxidation and reversible transamination have been known. Former is weakly catalyzed by an enzyme which oxidizes pyridoxamine-P.⁷⁵⁾ Latter was briefly pointed out the existence in *Escherichia coli* by Gunsalus *et al.*⁷⁶⁾ Although, Wada and Snell have recently shown the occurrence of weakly active transaminase between pyridoxamine and oxalacetate and purified from *Escherichia coli* and rabbit liver, it appeared possible that this enzyme is related to some unidentified amino acid transaminase.⁷⁷⁾ Subsequently, it was found that a related transamination between pyridoxamine and pyruvate was catalyzed by a highly active transaminase present in a pseudomonad grown with pyridoxamine as a sole source of carbon and nitrogen.⁷⁸⁾ The mechanism of the enzyme action which does not require pyridoxal-P as coenzyme attracts the attention from the points of the establishment of the general transamination mechanism.⁷⁹⁻⁸³⁾

The conversion of pyridoxine-P and pyridoxamine-P to pyridoxal-P has an important role on the pyridoxal-P formation from pyridoxine and pyridoxamine. Beechey and Happold⁸⁴⁾ and Pogell⁸⁵⁾ demonstrated the occurrence of an enzyme catalyzing the deamination of pyridoxamine-P. Separately, Morisue *et al.* reported a pyridoxine-P oxidizing enzyme.⁸⁶⁾ These enzymes were purified to a great extent and concluded to be a single enzyme which required FMN as a prosthetic group for both activities.^{75,87)} The study on the enzyme with microorganisms suggested the significance in vitamin B₆ metabolism^{88,89)}

and a large amount production of pyridoxal-P⁹⁰⁾ Furthermore, a transamination between pyridoxamine-P and α -ketoglutarate has recently been recognized in strict anaerobe, clostridia, which lacked the system of pyridoxamine-P oxidation⁹¹⁾

Since the discovery of vitamin B₆, relatively little has been published on the elucidation of its biosynthetic pathway. The lack of a system to produce workable quantities of the vitamin undoubtedly has been the major obstacle to progress in this field. It has been previously known that various sorts of microorganisms could produce the vitamin in or out the cells⁹²⁻⁹⁵⁾ and that the stimulating effect of carbon and nitrogen compounds on the biosynthesis could be observed⁹⁶⁻⁹⁹⁾ The incorporation study of ¹⁴C-labeled substrates resulted too low to permit a definitive interpretation of the biosynthetic pathway^{100,101)} However, it was interest that these studies almost suggested the positive effect of glycerol on the synthesis of the vitamin. Evidence has recently been presented that the biosynthesis may be controlled through a feedback mechanism¹⁰²⁾ The isolation and characterization of pyridoxine auxotrophs of *Escherichia coli* have been also reported^{103,104)} It has been demonstrated that the resistant mutant of yeast to vitamin B₆ antagonist excreted an increased amount of the vitamin.^{105,106)} The introduction of these improved studies with genetical aspect may develop the elucidation of the biosynthetic pathway.

In several derivatives of vitamin B₆ which have been known as

the degradative ones, 4-pyridoxic acid had been first isolated from urine^{107,108}) and shown to be oxidized product by aldehyde oxidase.¹⁰⁹) Snell *et al.* have described the isolation and characterization of several degradation products formed from vitamin B₆ when certain soil pseudomonads are grown with pyridoxine or pyridoxamine as a sole source of carbon and nitrogen.^{110~113}) The sequence in which these products appeared has been studied at the enzymatic level and two distinct enzymatic pathways for the stepwise degradation of vitamin B₆ have been revealed.⁶⁹)

As described above, great many investigations have been available on the metabolism of vitamin B₆. However, our knowledge for the metabolism in the microbial area is still fragmentary. These include, for instance, the biosynthetic study and that relating with the industrial application, and the discovery of the new metabolic pathway.

Present study is concerned to a few new reactions which have been found during the investigation on microbial metabolism of vitamin B₆. Firstly, a new phosphotransferring reaction on the phosphorylation of free forms of vitamin B₆ was found and characterized for the distribution of the activity in microorganisms, and the property and the mechanism of the reaction. Secondly, pyridoxamine-P- α -ketoglutarate transaminase, which had been recognized the existence in strict anaerobe, was purified for the elucidation of the enzymatical mechanism. Moreover, a new derivative was found

in culture filtrate of some bacteria during the course of investigation of microbial metabolism of pyridoxine. The isolation and characterization of the compound, pyridoxine-G, were studied.

Chapter I. Microbial Phosphorylation of Vitamin B₆ through a New Phosphotransferring Reaction

Section 1. Distribution in Microorganisms and Isolation of Phosphorylated Product

INTRODUCTION

On the enzymatic phosphorylation of vitamin B₆, a number of studies have been reported by several groups of workers with mammalian tissues^{58,114,115}) and microorganisms^{19,41,58,114,116}) However, it is only made clear that pyridoxal phosphokinase catalyzes the phosphorylation of vitamin B₆ in the presence of ATP. On the other hand, several phosphotransferring reactions which catalyzed the transfer of the phosphoryl group of organic phosphate have been demonstrated on the phosphorylation of various alcoholic compounds¹¹⁷) Recently, Kumar and Vaidyanathan¹¹⁸) have briefly reported on the enzymatic phosphorylation of pyridoxal and pyridoxamine by the transfer of phosphoryl moiety of FMN during the study of FMN hydrolase from green-gram seeds.

The author found the presence of a new phosphotransferring activity in several microorganisms, which catalyzed the phosphoryl group transfer from *p*-NPP to pyridoxine.

This section deals with the distribution of the new phosphotransferring activity in various kinds of microorganisms, and the

isolation and identification of phosphorylated product from the reaction mixture.

MATERIALS AND METHODS

Microorganisms and Cultures. All microorganisms used were strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University.

Bacteria were grown in a medium containing 1.5 g peptone, 1.0 g glucose, 0.2 g yeast extract, 0.5 g K_2HPO_4 , 0.1 g KH_2PO_4 , 0.2 g NaCl and 0.02 g $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 7.0. Cultures were carried out with 50 ml medium placed in 300-ml shaking flasks, and kept on a reciprocal shaker for 24 hr at 28°C. The cells were harvested by centrifugation, washed twice with deionized water, and suspended in deionized water.

Yeasts were grown in a medium containing 5 g glucose, 0.5 g peptone, 0.5 g yeast extract, 0.4 g K_2HPO_4 , 0.2 g KH_2PO_4 and 0.02 g $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 6.5. Cultures were carried out with 50 ml medium placed in 300-ml shaking flasks, and kept on a reciprocal shaker for 24-48 hr at 28°C. The cells were harvested by centrifugation, washed twice with deionized water, and suspended in deionized water.

Molds were grown in a medium containing 6 g glucose, 0.2 g $NaNO_3$ (0.5 g asparagine instead of $NaNO_3$ for *Rhizopus*), 0.1 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g KCl and 0.001 g $FeSO_4 \cdot 7H_2O$ in 100 ml of

deionized water, pH 6.0. Cultures were carried out with 50 ml medium placed in 300-ml shaking flasks, and kept on a reciprocal shaker for 72 hr at 28°C. The cells were harvested by filtration and washed with deionized water.

Actinomycetes were grown in a medium containing 2.0 g glucose, 0.4 g peptone, 0.4 g liver extract, 0.1 g yeast extract, 0.25 g NaCl and 0.3 g K₂HPO₄ in 100 ml of tap water, pH 7.0. Cultures were carried out with 50 ml medium placed in 300-ml shaking flasks, and kept on a reciprocal shaker for 24-40 hr at 28°C. The cells were harvested by centrifugation or filtration, and washed with deionized water.

Chemicals. *p*-NPP was kindly provided by Dr. K. Mitsugi, Ajinomoto Co., Ltd. Pyridoxine was a gift from Dainippon Pharm. Co., Ltd. Other chemicals were obtained from commercial sources.

Preparation of Dried Cells of *Saccharomyces cerevisiae* and *Aspergillus flavus* Link IFO 5839. The cells of *Saccharomyces cerevisiae* (Baker's yeast) and *Aspergillus flavus* Link IFO 5839 were dried up for 10-15 hr by an electric fan at room temperature.

Activity Measurement. For the screening of the phosphotransferring activity in various microorganisms, the following standard condition was used: The assay mixture containing 10 μmoles of pyridoxine, 50 μmoles of *p*-NPP, 20 μmoles of MgSO₄·7H₂O, 200 μmoles of potassium phosphate buffer, pH 7.0 and approximately 20-30 mg of intact cells as dry matter, in a total volume of 4 ml, was incubated

at 28°C for 14 hr on a reciprocal shaker. In each case, the reaction was stopped by heating the mixture in a boiling water bath for 3 min, and the reaction products were determined after the removal of cells by the centrifugation or filtration.

Analytical Method. Pyridoxal-P was determined by both phenylhydrazine method and apotryptophanase method as noted by Yamamoto *et al.*⁸⁸⁾ Pyridoxine-P was determined as pyridoxal-P after the conversion to pyridoxal-P by pyridoxine-P oxidase obtained from *Alcaligenes faecalis* (30-50% ammonium sulfate-saturated fraction)⁸⁷⁾ Inorganic and organic phosphorus was determined by the method of Fiske and Subbarow.¹⁹⁾

RESULTS AND DISCUSSION

Distribution of the Phosphotransferring Activity in Microorganisms.

The phosphotransferring activity which catalyzed the phosphorylation of pyridoxine with *p*-NPP was searched for various kinds of microorganisms using intact cells. Results of the distribution of the activity are shown in Table I for molds and Table II for yeasts, respectively. The phosphotransferring activity was found in the strains belonging to genera such as *Aspergillus*, *Penicillium*, *Neurospora*, *Endomyces*, *Pichia* and *Saccharomyces*. Especially, the mold strains belonging to *Aspergillus* had the higher activity. In these mold strains, the phosphorylation product of pyridoxine was almost

Table I. Vitamin B₆ Phosphates Formation by Molds

Strains	Pyridoxine-P*		Pyridoxal-P*		
	p-NPP**	+	-	+	-
<i>Mucor javanicus</i> Wehmer IFO 4570		1.6	1.2	0.9	1.2
<i>Mucor javanicus</i> Wehmer IFO 4572		2.7	0.4	1.1	1.3
<i>Mucor fragilis</i> Bainier IFO 6449		1.5	1.2	1.1	1.4
<i>Rhizopus oryzae</i> M-21		1.1	0.9	0.9	1.1
<i>Rhizopus batatas</i> M-24		0.0	0.4	0.9	0.4
<i>Rhizopus javanicus</i> Takeda IFO 5442		0.0	1.4	0.8	1.1
<i>Aspergillus oryzae</i> M-61		14.0	0.4	3.2	1.2
<i>Aspergillus niger</i> No. 4416		5.2	0.0	1.2	1.2
<i>Aspergillus awamori</i> M-66		1.6	0.0	0.8	1.2
<i>Aspergillus candidus</i> M-70		29.2	1.2	4.4	1.4
<i>Aspergillus oryzae</i> var. <i>globosus</i> M-71		28.4	0.0	3.6	1.2
<i>Aspergillus usami</i> IFO 4388		0.8	0.3	1.6	0.8
<i>Aspergillus flavus</i> Link IFO 5839		37.8	0.9	5.4	1.3
<i>Aspergillus nidulans</i> IFO 5719		2.8	0.0	1.4	1.4
<i>Aspergillus oryzae</i> Cohn IFO 4117		8.8	1.0	0.0	0.0
<i>Aspergillus terreus</i> Thom IFO 6123		2.6	1.1	1.4	1.2
<i>Penicillium chrysogenum</i> IFO 4626		1.8	0.7	0.6	0.9
<i>Penicillium oxalicum</i> IFO 5750		4.1	0.0	0.2	0.0
<i>Penicillium notatum</i> IFO 4640		2.0	0.2	1.0	0.8
<i>Monascus purpureus</i> IAM 8010		0.4	0.2	0.2	0.4
<i>Monascus anka</i> IAM 8001		0.5	0.2	0.0	0.0
<i>Neurospora crassa</i> IFO 6068		2.7	0.3	1.7	0.8
<i>Pullularia pullulans</i> IFO 4464		0.5	0.0	0.0	0.0
<i>Fusarium oxysporum</i> IFO 5942		0.2	0.2	0.8	0.8
<i>Fusarium culmorum</i> IFO 5902		0.5	0.2	0.1	0.6
<i>Gibberella fujikuroi</i> IFO 5268		0.0	0.1	0.0	0.0

*Pyridoxine-P or pyridoxal-P formed (μg) per 4 ml of reaction mixture

**p-NPP added (+) or not added (-).

Table II. Vitamin B₆ Phosphates Formation by Yeasts

Strains	Pyridoxine-P*		Pyridoxal-P*	
	p-NPP**	+ -	+ -	
<i>Endomyces hordei</i> IFO 0104		1.6 0.2	1.4 2.0	
<i>Endomyces lindneri</i> IFO 0106		1.0 0.9	1.0 1.1	
<i>Endomyces fibuliger</i> IFO 0103		1.1 0.3	1.3 0.8	
<i>Schizosaccharomyces pombe</i> IFO 0346		0.4 0.6	0.8 1.2	
<i>Saccharomyces logos</i> Y-22		0.4 0.0	1.0 1.8	
<i>Saccharomyces marxianus</i> IFO 0277		0.2 0.2	0.8 1.8	
<i>Saccharomyces sake</i> Kyokai No. 2		0.4 0.2	0.4 1.2	
<i>Saccharomyces carlsbergensis</i> Hansen		0.0 0.0	0.3 0.6	
<i>Saccharomyces rouxii</i> IAM 4011		0.0 0.0	0.6 1.3	
<i>Zygosaccharomyces japonicus</i> IFO 0595		0.2 0.1	0.3 0.4	
<i>Pichia polymorpha</i> Kloecker IFO 0195		2.1 0.0	0.8 0.8	
<i>Pichia farinosa</i> var. <i>japonica</i>		0.0 0.0	0.2 0.5	
<i>Hansenula anomala</i> Y-56		0.5 0.1	0.8 1.1	
<i>Hansenula saturnus</i> Y-57		1.5 1.2	1.7 2.0	
<i>Debaryomyces japonicus</i> IFO 0039		0.1 0.2	0.4 0.8	
<i>Saccharomycopsis capsularis</i> IFO 0672		0.9 0.0	1.0 1.0	
<i>Saccharomycodes ludwigii</i> IFO 0339		0.8 0.3	0.6 0.9	
<i>Nematospora coryli</i> IFO 0658		0.6 0.1	0.2 0.4	
<i>Sporobolomyces salmonicolor</i> Y.U.		0.0 0.0	1.0 3.3	
<i>Cryptococcus neoformans</i> IFO 0410		0.0 0.0	1.0 1.4	
<i>Candida utilis</i> IFO 0619		0.0 0.0	1.8 2.4	
<i>Trichosporon beigeli</i> IFO 0598		0.5 0.0	0.4 0.5	
Awamori yeast (Sakumoto)		1.2 0.4	0.6 0.7	
Brennereihefe Rasse 12		0.2 0.0	0.4 0.5	
<i>Saccharomyces sake</i> Hozan		0.1 0.0	0.3 0.3	
<i>Saccharomyces sake</i> H-31		0.0 0.0	0.0 0.0	
<i>Saccharomyces cerevisiae</i> (Oriental)		0.8 0.4	0.0 0.0	

*Pyridoxine-P or pyridoxal-P formed (μg) per 4 ml of reaction mixture.

** p-NPP added (+) or not added (-).

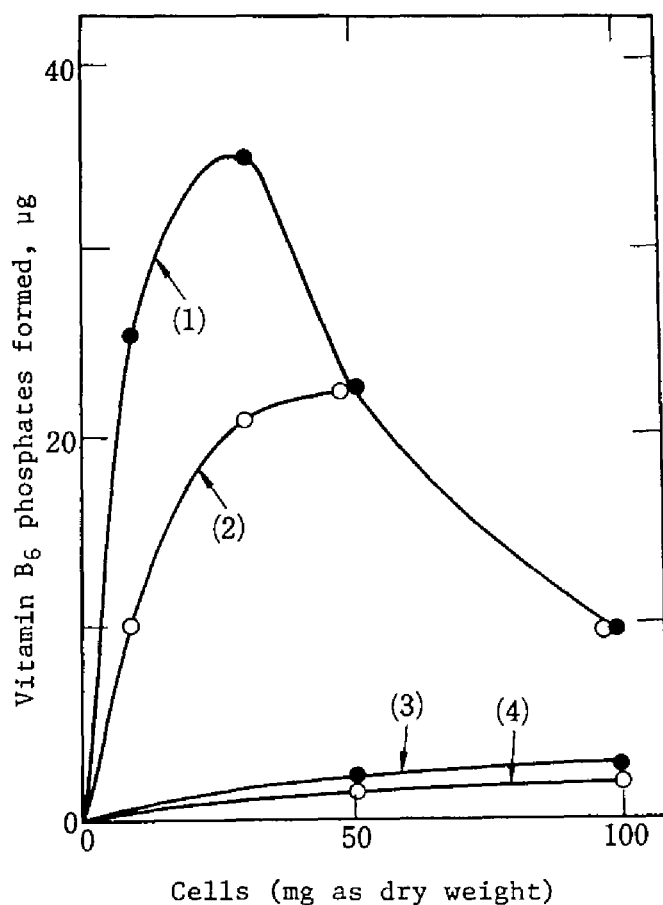


Fig. 1. Effect of Cell Concentration on the Reaction

The reaction was carried out under the standard conditions except dried cell concentration of *Saccharomyces cerevisiae* cells. (1); Total vitamin B₆-P formed with dried cells. (2); Pyridoxal-P formed with dried cells. (3); Total vitamin B₆-P formed with intact cells. (4); Pyridoxal-P formed with intact cells.

exclusively pyridoxine-P, while pyridoxal-P, which was the oxidized product of pyridoxine-P, was scarcely found in the reaction mixture. This suggests that the activity of pyridoxine-P oxidase may be repressed under these assay conditions of the phosphotransferring ac-

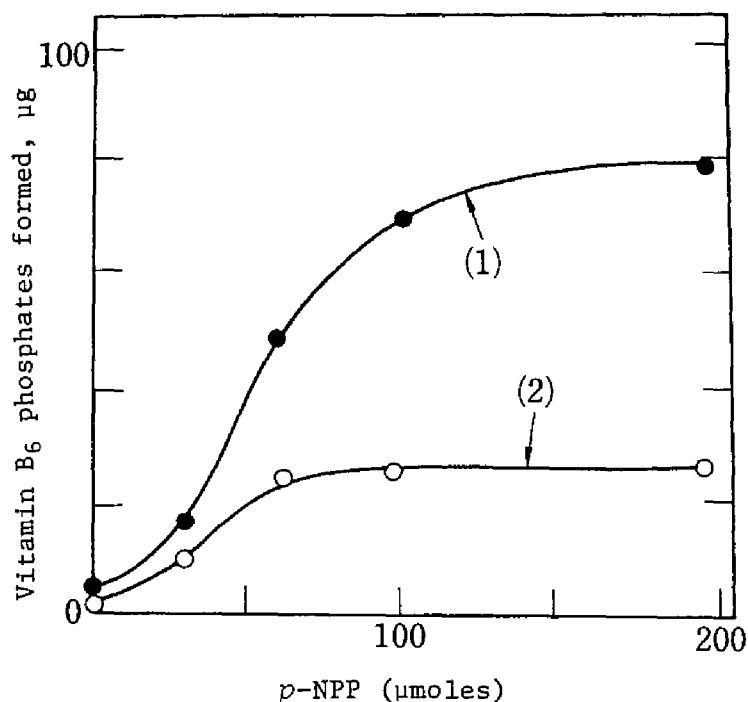


Fig. 2. Effect of *p*-NPP Concentration on the Reaction

The reaction was carried out under the standard conditions with 20 mg of dried cells of *Saccharomyces cerevisiae*. (1); Total vitamin B₆-P formed. (2); Pyridoxal-P formed.

activity. The yeasts such as *Pichia* and *Endomyces* which were ecologically near to mold had also the appreciable activity.

In *Saccharomyces cerevisiae*, the phosphotransferring activity was hardly observed with intact cells, but the activity was observed with dried cells (Fig. 1). The increase of the permeability of substrates through the cells membrane may be suggested. It was also recognized that the amount of vitamin B₆ phosphate formed decreased with the higher concentration of cells. As shown in Fig. 2, the phosphotransferring reaction depends on the concentration of *p*-NPP.

Table III. Formation of Vitamin B₆ Phosphates
with *Saccharomyces cerevisiae* Dried Cells

	Total vitamin B ₆ -P	Pyridoxal-P (μ g)
Complete system	200.0	32.5
Omitted pyridoxine	0.0	0.0
Omitted <i>p</i> -NPP	2.5	2.5
Omitted cells	0.0	0.0
Boiled cells*	0.0	0.0

* Boiling time was 10 min.

The reaction was carried out under the standard conditions with 20 mg of dried cells of *Saccharomyces cerevisiae*. Reaction time was 6 hr.

It was further proved in Table III that the reaction involved *p*-NPP and pyridoxine as phosphoryl donor and acceptor substrates. The formation of an appreciable amount of pyridoxal-P in the reaction mixture was observed in this case. The result suggests that pyridoxine-P formed by the phosphotransferring reaction may be converted to pyridoxal-P by the pyridoxine-P oxidase or that the substrate pyridoxine may be initially converted to pyridoxal by the pyridoxine dehydrogenase,⁷⁰⁾ then phosphorylated to pyridoxal-P.

Isolation and Identification of Pyridoxine-P.

The phosphotransferring reaction between pyridoxine and *p*-NPP was carried out under the optimal conditions with dried cells of *Aspergillus flavus* Link 5839, which had the highest activity as shown in Table I. Then the phosphorylated product, pyridoxine-P,

was isolated and identified as follows.

Isolation. First, the reaction mixture containing 100 mmoles of pyridoxine, 60 mmoles of *p*-NPP, 50 mmoles of Ringer's phosphate buffer, pH 11.0, 0.5 mmoles of nickel sulfate and 6 g of dried cells of *Aspergillus flavus* Link IFO 5839 in a total volume of 500 ml was incubated for 24 hr at 28°C on a reciprocal shaker. The reaction mixture was immersed for 10 min in a boiling water bath and filtered. The filtrate was adjusted to pH 8.0 with 10% ammonium hydroxide and added onto a column of Dowex 1x8 (Cl⁻ form) (200-400 mesh, 3x28cm). The column was washed with 2000 ml of water and 500 ml of 0.001 N hydrochloric acid. The pyridoxine-P adsorbed on the column were eluted with 0.05 N hydrochloric acid. The fractions containing pyridoxine-P (apoptryptophanase reactivation fractions, which were tested after the conversion to pyridoxal-P with pyridoxine-P oxidase) was combined and adjusted to pH 2.0 with sodium hydroxide. To the solution was added 5 g of active carbon. After standing overnight, active carbon was collected and washed with 0.01 N hydrochloric acid, and pyridoxine-P adsorbed on the carbon was eluted with 50% ethanol containing 2% ammonium hydroxide. The eluate was concentrated under reduced pressure at 35°C to about 20 ml and added onto a column of Amberlite IRC-50 (H⁺ form) (200-400 mesh, 2x55cm). Pyridoxine-P on the column was eluted with water. The eluates (160 ml) were evaporated to a small volume under reduced pressure at 35°C and kept in the refrigerator. Crystalline pyridoxine-P obtained after 12 hr

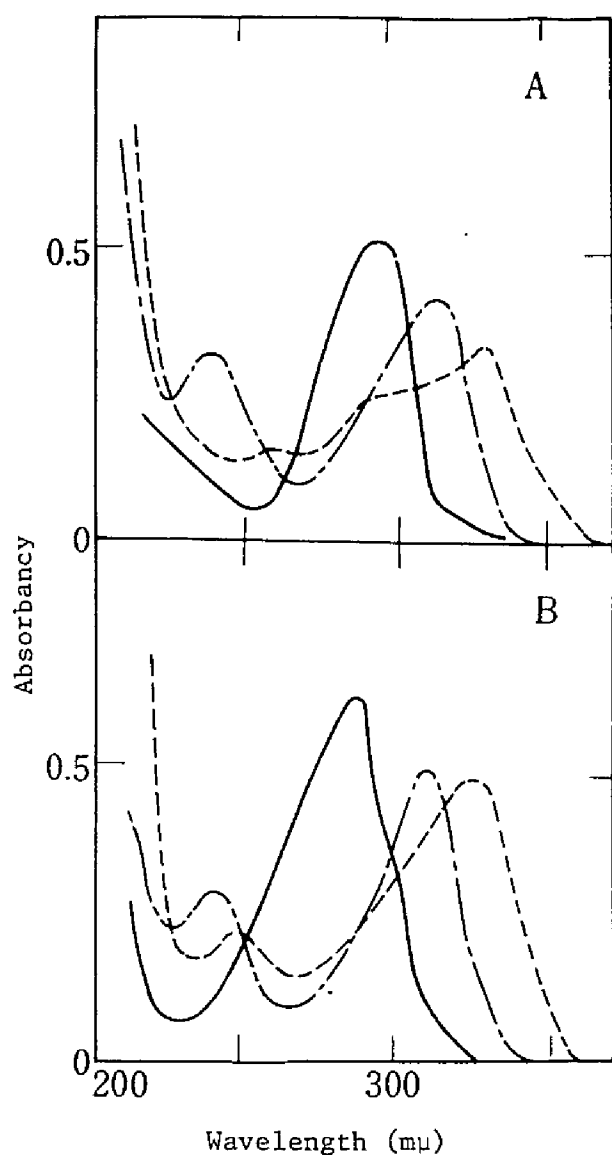


Fig. 3. Ultraviolet Spectra of Isolated Pyridoxine-P
(A) and Authentic Specimen (B)

—; in 0.1 N hydrochloric acid, ----; in 0.1 N sodium hydroxide, - · - · -; in 0.1 M potassium phosphate buffer, pH 7.0.

was recrystallized from hot water containing ethanol and ether.

Above procedure gave a yield of 130 mg of white crystal of pyridoxine-P.

Identification. The ultraviolet absorption spectra of the isolated pyridoxine-P were presented in Fig. 3, and were identical with those of authentic pyridoxine-P, which had λ_{max} at 290 m μ in 0.1 N hydrochloric acid, at 310 m μ in 0.1 N sodium hydroxide and at 325 m μ in 0.1 M potassium phosphate buffer of pH 7.0.

The infrared spectrum of the isolated pyridoxine-P was the same with that of the authentic specimen.

Rf values of the isolated pyridoxine-P on paper chromatogram were identical with those of authentic pyridoxine-P (Table IV). On paper chromatography, the color development on paper with diazotized p-AAP,¹²⁰⁾ Gibbs reagent¹²¹⁾ and ferric chloride⁴⁾ provided further confirmation of its identity as pyridoxine-P (Table IV).

The molar proportions of pyridoxine and inorganic phosphorus after acid hydrolysis of the isolated pyridoxine-P were found to be in the ratio of one to one.

SUMMARY

A new phosphotransferring reaction which phosphorylated pyridoxine through the phosphoryl group transfer from p-NPP was found, and the distribution of the reaction in several microorganisms was investigated. The transferring activity was widely distributed in various kinds of microorganisms, especially in fungi belonging to genus such as *Aspergillus*. The phosphorylated product was isolated from the reaction mixture with the dried cells of *Aspergillus flavus* and identified as pyridoxine-P.

Table IV. Paper Chromatography and Color Development

Solvent Systems	Isolated pyridoxine-P	Authentic pyridoxine-P
<i>n</i> -Propanol 70, NH ₄ OH 10, H ₂ O 10	0.14	0.14
2,4-Lutidine 55, Ethanol 20, H ₂ O 15, Diethylamine 1	0.21	0.21
<i>tert</i> -Butanol 70, H ₂ O 15, 90% Formate	0.44	0.44
<u>Color Reagents</u>		
Diazotized <i>p</i> -AAP	Orange	Orange
Gibbs reagent	Blue	Blue
Ferric chloride	Red-purple	Red-purple

Section 2. Some Properties of the Transphosphorylation

INTRODUCTION

In a previous section, the phosphorylated product of pyridoxine was isolated from the reaction mixture which contained *p*-NPP and pyridoxine as the phosphoryl donor and acceptor substrates, and the cells of *Aspergillus flavus*. It was also demonstrated that the phosphotransferring activity was distributed in various kinds of microorganisms, especially in fungi belonging to genus such as *Aspergillus*. The enzyme system which catalyzed the phosphotransferring reaction seemed to be different from pyridoxal kinase which phosphorylated pyridoxal, pyridoxamine and pyridoxine at the same rate in the phosphoryl group transfer from ATP⁵⁸⁾

It has been known that such enzymatic transphosphorylation, which occurs without the participation of adenine nucleotidic compounds, might be catalyzed by the enzyme functions of the two different types.^{11,122)} One type is that of acid and alkaline phosphatases which have been studied on their phosphotransferring functions from the first work of Axelrod.¹²³⁾ Another type is that of phosphotransferase, such as nucleoside phosphotransferase,^{124,125)} the separation of which from phosphatase has been reported.^{126,127)}

This section describes firstly the isolation of the bacteria which have a higher activity of the transphosphorylation of pyrido-

xine accompanying with that of the hydrolyzation of the phosphoryl donor substrate. With the partially purified enzyme preparation from one of isolated bacteria, *Escherichia freundii* K-1, several properties of the reaction were subsequently characterized.

EXPERIMENTAL

Microorganisms. Seven hundred and thirty bacterial strains were isolated from natural materials by plate culture method after static incubation at 37°C for 24 hr. Other microorganisms were the strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University. These strains were cultured aerobically as described in a preceding section. The cells were harvested by centrifugation or filtration and washed twice with deionized water. The washed cells were used for the following preparation of the enzyme. For the screening of the enzyme activity of the isolated bacteria, the culture fluid and dried cells were used as the enzyme preparations.

Enzyme Preparation. The following operations for the preparation of the phosphotransferring enzyme were performed at 0-5°C.

1) Cell-free extracts preparation: The washed bacterial cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, and treated with a Kaijo-Denki 19 Hz ultrasonic oscillator for 10 min. The intact cells and debris were removed by centrifugation at 12,000xg for 30 min. The resulting supernatant solution was dialy-

zed against the same buffer overnight and then used as the bacterial cell-free extract. While, the washed fungal cells were suspended in 0.1 M potassium phosphate buffer, pH 7.0, and ground with sea sand for about 30 min. After centrifugation, the supernatant solution was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, and then used as the fungal cell-free extract.

2) Partially purified enzyme preparation: The cell-free extract of *Escherichia freundii* K-1 was used as the starting material for the preparation of the partially purified enzyme preparation (Step I). The cell-free extract was heated at 60°C for 10 min in a water bath. After the removal of the precipitate formed, the supernatant solution was dialyzed against deionized water (Step II). To the heat treated enzyme preparation the solid ammonium sulfate was added to 35% saturation with a gentle mixing. The precipitate formed was removed by centrifugation and discarded. An additional ammonium sulfate was added to the supernatant fluid, bringing the ammonium sulfate concentration to 65% saturation. The precipitate formed was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and then dialyzed against the same buffer overnight (Step III). The results of a preparation, in which the enzyme was purified about 10 fold with a recovery of 70% of the enzyme activity, are summarized in Table I.

Chemicals. *p*-NPP was kindly provided by Dr. K. Mitsugi, Ajinomoto Co., Ltd. 5'-Nucleotides was the kind gifts of Takeda Pharm.

Table I. Partial Purification of the Enzyme

Step	Fraction	Total	PTase*		Pase**	
		protein (mg)	S.A.	T.A.	S.A.	T.A.
I	Cell-free extract	15,770	0.03	473	0.9	29,000
II	Heat treatment	2,190	0.18	394	5.0	21,900
III	Ammonium sulfate	882	0.40	353	10.4	18,300

*PTase (phosphotransferase activity) was presented by the amount of pyridoxine-P formation in the standard enzyme assay conditions.

**Pase (phosphatase activity) was presented by the amount of *p*-NP formation in the standard enzyme assay conditions.

S. A. (specific activity) was μ moles of *p*-NP or pyridoxine-P formed per mg of protein per 30 min.

Co., Ltd. Other chemicals were obtained from commercial sources.

Screening Method. On the first screening for the isolation of the bacterial strains having a higher activity of the transphosphorylation, the following standard assay condition was used: The reaction mixture which contained 10 μ moles of pyridoxine, 50 μ moles of *p*-NPP, 200 μ moles of potassium phosphate buffer, pH 7.0, and the bacterial broth, which was cultured for 24 hr and contained about 20 mg of the cells as dry weight, was incubated in a total volume of 4 ml at 28°C for 20 hr on a reciprocal shaker. On the second screening, the following standard assay condition was used: The reaction mixture which contained 10 μ moles of pyridoxine, 50 μ moles of *p*-NPP, 200 μ moles of potassium phosphate buffer, pH 6.6,

or sodium-potassium phosphate buffer, pH 11.0, and approximately 20 mg of the bacterial dried cells, was incubated in a total volume of 4 ml for 20 hr at 28°C on a reciprocal shaker. These reactions were stopped by heating the reaction mixture for 10 min in a boiling water bath. The reaction products were assayed after the removal of the cells with centrifugation at 5000xg for 15 min.

Identification of Bacteria. Standard methods of identification were employed to determine the taxonomic position of the isolated bacteria and referred to the "Bergey's Manual of Determinative Bacteriology", 7th Ed., 1957¹²⁸⁾

Enzyme Assay. The assay of the phosphorylation of pyridoxine accompanying with the hydrolyzation of phosphoryl donor substrate was conducted as follows. (1) For the assay with the cell-free extract, the following standard condition was used: The reaction mixture which obtained 10 μ moles of pyridoxine, 50 μ moles of *p*-NPP, 250 μ moles of potassium phosphate buffer, pH 6.6, or glycine-NaCl-NaOH buffer, pH 10.0, and 1 ml of dialyzed cell-free extract was incubated in a final volume of 3 ml at 37°C for 2 hr. (2) For the assay with partially purified enzyme preparation from *Escherichia freundii* K-1, the following standard condition was used: The reaction mixture which contained 10 μ moles of pyridoxine, 50 μ moles of *p*-NPP, 200 μ moles of Tris-maleate buffer, pH 6.0, and appropriate amount of enzyme was incubated in a final volume of 3 ml at 37°C for 30 min. Pyridoxine-P and *p*-NP measurements were made after

stopping the enzyme reactions by heating for 10 min in a boiling water bath.

Analytical Method. Pyridoxine-P, pyridoxal-P and pyridoxamine-P were determined as described in previous section, using mainly phenylhydrazine method⁷⁵⁾ Para-nitrophenol was measured with the optical density at 430 mμ with addition of saturated sodium carbonate solution¹²⁹⁾ Protein was determined colorimetrically by the method of Lowry *et al*¹³⁰⁾ using egg albumin as a reference standard.

RESULTS

The Transphosphorylation Activities of Microorganisms.

In the first screening about thirty bacterial strains appeared to have the activity of the transphosphorylation between pyridoxine and *p*-NPP, and then several bacteria were selected by the second screening under the acidic and alkaline reaction conditions. The activities with cell-free extract systems of the representative bacterial strains are studied together with the activities of the other microorganisms which were found to give the considerable activity when these dried cell systems were employed. As shown in Table II, it was observed that the cell free extracts of these bacteria, *Aspergillus*, *Penicillium* and *Neurospora* had the ability to form pyridoxine-P. The activities in *Endomyces* and *Pichia*, however, were weakly observed in the dried cell system but were not detected in cell free extracts. The enzyme activity of bacteria at alkaline

Table II. Phosphorylation of Pyridoxine

Strain	Protein added (mg)	Acidic site		Alkaline site	
		PIN-P*	p-NP (μ moles)	PIN-P*	p-NP
Isolated bacterium K-1	8.5	0.42	21.8	0.03	4.5
810	10.5	0.16	15.8	0.03	5.3
68303	5.1	0.57	28.5	0.05	3.0
<i>Asp. tamarit</i> M-68	3.7	0.27	18.6	0.28	41.2
<i>Asp. candidus</i> M-70	0.8	0.16	15.0	0.06	8.3
<i>Asp. oryzae</i> var. <i>globosus</i>	1.5	0.08	6.0	0.02	3.8
<i>Asp. flavus</i> IFO 5839	2.5	0.13	9.0	0.23	30.9
<i>Pen. chrysogenum</i> IFO 4626	2.3	0.02	10.5	0.03	3.8
<i>Pen. oxalicum</i> IFO 5750	2.8	0.04	15.8	0.03	3.8
<i>Neu. crassa</i> IFO 6068	3.5	0.03	14.3	0.02	3.8
<i>End. hordei</i> IFO 0104	1.2	0.00	3.2	0.00	1.2
<i>End. lindneri</i> IFO 0106	1.7	0.00	2.4	0.00	0.9
<i>Pi. polymorpha</i> IFO 0195	1.0	0.00	1.8	0.00	0.6

The reaction was carried out under the standard assay conditions with cell-free extract described in text.

*PIN-P; Pyridoxine-P.

pH range, which appeared to be stronger than at acidic pH range with the experiment employing the bacterial dried cell system, almost disappeared with the treatment for preparing the enzyme extraction. While the cell-free extract of aspergilli had two or three optimal pHs for both of the reactions.

Identification of the Isolated Bacterial Strains.

The taxonomic positions of three bacterial strains contained in Table II were identified as follows:

K-1 strain; 1) Morphological: Short rod, 0.4 to 0.6 by 0.8 to 1.8 microns, occurring singly. Motile with flagella. Gram-negative.

2) Cultural:

Bouillon-agar slant: Filiform, translucent growth.

Gelatin stab: Liquefaction.

Potato: Abundant, grayish white, sebaceous growth.

3) Physiological:

Litmus milk: Acid and coagulation.

Litmus reduced.

Indole is produced.

Lactic acid is produced.

Hydrogen sulfide is produced.

Methyl red test positive. Voges-Proskauer test positive.

Acid and gas from glucose, fructose, arabinose, xylose, raffinose, lactose, maltose, glycerol, mannitol, sucrose. Dulcitol, starch, dextrin, inulin are scarcely or not at all fermented.

Citric acid utilized as a sole source of carbon.

Nitrites are produced from nitrates.

Catalase-positive.

Growth requirements: Good growth on ordinary laboratory media.

Optimum growth temperature, between 30°C and 37°C.

Killed at 70°C.

According to Bergey's Manual, the present strain, K-1, seems to be similar to *Escherichia freundii*. The type culture of *Esche-*

richia freundii S-96 did not showed the appreciable transphosphorylation activity.

The strains, 68303 and 810, were identified by the same manner to be similar to *Pseudomonas pseudomallei* and *Aerobacter aerogenes*, respectively, according to Bergey's Manual.

Properties of the Transphosphorylation.

Several properties of the transphosphorylation, using mainly *p*-NPP and pyridoxine as phosphoryl donor and acceptor substrates, were investigated with the partially purified enzyme preparation from *Escherichia freundii* K-1. In the following results, the enzyme activity of phosphotransferase was estimated by the formation of pyridoxine-P, and that of phosphatase by the liberation of *p*-NP.

Effect of pH. The results of experiments on the effect of the variation of pH on the activities are presented in Fig. 1. The maximum values for the phosphotransferase activity was observed near pH 6.0. The phosphatase also showed the same pH optimum for the activity. The phosphatase in the preparation should be classified to acid phosphatase.

Effect of enzyme concentration. The influence of enzyme concentration on the pyridoxine-P formation was examined. As shown in Fig. 2, the amount of pyridoxine-P was rapidly decreased with the increase of enzyme concentration, when the increase of the amount of *p*-NP was depressed. The amount of pyridoxine-P formed also diminished with further incubation time with relatively low concentra-

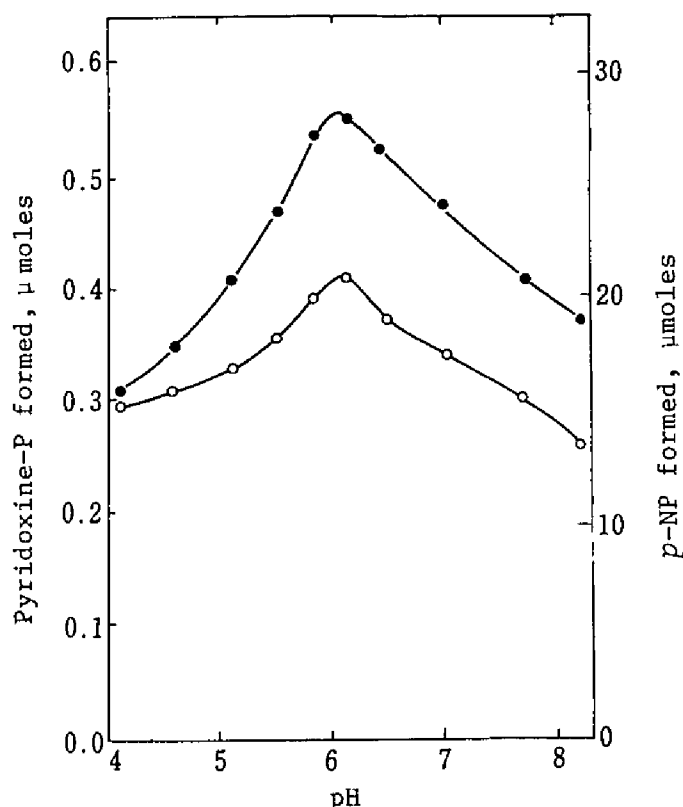


Fig. 1. pH-Activity Curves of Phosphatase and
Phosphotransferase Activities

(●) Pyridoxine-P formed, (○) *p*-NP formed.

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (1 mg of protein) and Tris-maleate buffer at various pH values.

tion of enzyme, and then the hydrolysis of *p*-NPP was rather slow. These might be caused probably because inorganic phosphorus and *p*-NP, which were the effective inhibitors, presented in large amounts in the reaction mixture.

Optimum temperature. The optimum temperature for both the phosphotransferase and phosphatase activities of the enzyme was near

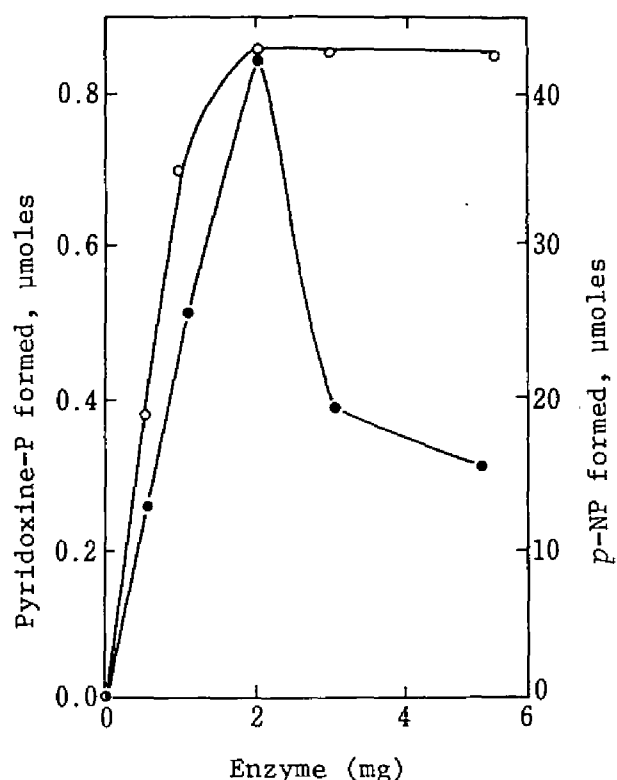


Fig. 2. Effect of Enzyme Concentration on Phosphatase and Phosphotransferase Activities

(●) Pyridoxine-P formed, (○) *p*-NP formed.

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation.

35°C (Fig. 3).

Metal ion effect. The effects of various metal ions listed in Table III were examined at the final concentration of $10^{-3}M$. None of metal ion accelerated either of the enzyme activities. Zn^{2+} , Cu^{2+} , Hg^{2+} and MoO_4^{2-} inhibited the reaction. The inhibitory effect of Co^{2+} on the phosphotransferase activity might be confirmed to be the inhibition against the activity of the enzyme used for the assay,

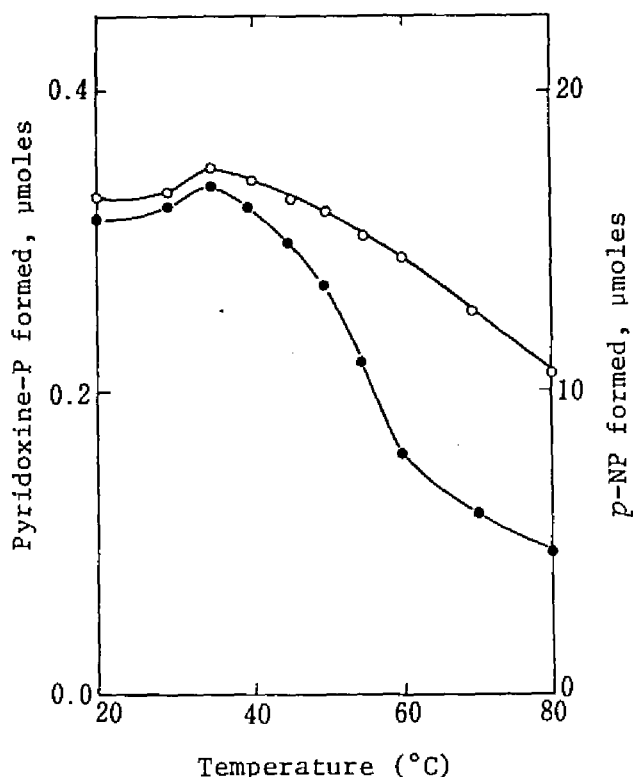


Fig. 3. Effect of Temperature on Phosphatase and Phosphotransferase Activities

(●) Pyridoxine-P formed, (O) *p*-NP formed.

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (0.8 mg of protein) except for the reaction temperature.

pyridoxine-P oxidase. The stimulating effect of several metal ions on nucleoside phosphotransferase has been demonstrated in detail!³¹⁾ The inhibitory or accelerating effect of metal ions on phosphatase has also been reported!^{32,133)} As mentioned above, the metal ions did not affect the enzyme activities in the present preparation.

Inhibitor effect. A number of compounds were tested as inhi-

Table III. Effect of Metal Ion on Phosphatase
and Phosphotransferase Activities

Metal ion (10^{-3}M)	Pyridoxine-P formed (Relative activity)	<i>p</i> -NP formed
None	(100)	(100)
Mg ²⁺	100	102
Mn ²⁺	100	98
Fe ²⁺	85	90
Zn ²⁺	74	83
Ni ²⁺	89	90
Cu ²⁺	85	87
Co ²⁺	56	98
Ca ²⁺	96	90
Hg ²⁺	63	76
Cr ³⁺	85	94
Li ⁺	96	94
MoO ₄ ²⁻	81	81
(EDTA)	96	96

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (0.3 mg of protein), adding 3 μmoles of each metal ions.

bitors of the phosphotransferase and phosphatase activities at different concentrations indicated in Table IV. Sodium fluoride and inorganic phosphorus, which were the typical competitive inhibitors of acid phosphatase, inhibited both of these activities over at 10^{-3} or 10^{-2}M . Hg²⁺ was also inhibitory with lower concentration.

(+)Tartarate and oxalate, which have been reported to inhibit acid

Table IV. Effect of Inhibitor on Phosphatase
and Phosphotransferase Activities

Inhibitor	Pyridoxine-P formed	p-NP formed
(Relative activity)		
None	(100)	(100)
NaF 10^{-4} M	100	100
10^{-3} M	67	73
10^{-2} M	16	45
KCN	95	100
NaAsO ₂	95	100
Na ₂ HAsO ₄	100	100
Pyrophosphate	95	106
KH ₂ PO ₄ 10^{-3} M	100	96
10^{-2} M	84	86
(+)-Tartarate	100	100
Oxalate	98	100
PCMB	100	103
o-Phenanthroline	100	103
α,α' -Dipyridyl	100	100
EDTA	96	97
HgCl ₂ 10^{-4} M	82	94
10^{-3} M	64	83
10^{-2} M	23	60
Deoxypyridoxine	100	103
4-Pyridoxic acid	100	103

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (0.3 mg of protein), adding inhibitor, 3 μ moles except for PCMB (0.3 μ moles), and NaF, KH₂PO₄ and HgCl₂ (indicated) in a total volume of 3 ml.

phosphatase on the hydrolysis of *p*-NPP,¹³⁴) did not inhibit the activity of this enzyme preparation.

Substrate specificity. Various phosphates were tested as phosphoryl donor substrate for the pyridoxine-P formation (Table V). It was shown that the enzyme was capable of transferring the phosphoryl moiety of a variety of the compounds to pyridoxine, and that *p*-NPP and fructose 1,6-diphosphate were the most excellent donor substrates.

Table VI shows the acceptor specificity of the free form of vitamin B₆. Pyridoxine was phosphorylated in rather high rate. The phosphorylation of pyridoxal was lower with the transphosphorylation under the condition employed. It has been shown that all of pyridoxal phosphokinase phosphorylated pyridoxal, pyridoxamine and that pyridoxal is generally the preferred substrate for the kinase.⁵⁸)

DISCUSSION

As for the enzymatical phosphorylation, phosphokinase system, capable of transferring the phosphoryl moiety of ATP to acceptor, is known to be the most common system.

On the phosphorylation of vitamin B₆ it has been demonstrated to be catalyzed by the action of pyridoxal phosphokinase in the presence of ATP as phosphoryl donor.¹¹⁶) It was also found that a few other nucleoside triphosphates were also available as phosphoryl donor substrate in the enzyme system, though ATP was the preferred subst-

Table V. Phosphoryl Donor Specificity

Donor substrate	Pyridoxine-P formed (μ moles)
<i>p</i> -NPP	0.36
Phenyl-P	0.18
Phenolphthalein 2-phosphate	0.08
Fructose 1,6-diphosphate	0.47
α -Glycerol phosphate	0.12
5'-Adenylic acid	0.03
5'-Cytidylic acid	0.03
5'-Guanylic acid	0.05
5'-Inosinic acid	0.07
5'-Uridylic acid	0.08
Acetyl phosphate	0.25
Creatine phosphate	0.00
ATP	0.02
Pyrophosphate	0.00
None	0.00

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (0.45 mg of protein) without *p*-NPP. The phosphoryl donor substrate added was 50 μ moles.

ate⁵⁸)

On the other hand, the transphosphorylation between a variety of organic phosphates not involved ATP and other phosphoryl acceptor as been investigated with several enzyme systems. These contain the transphosphorylation activities of phosphatases and that of phosphotransferases. The mechanism of the reaction, however, has not

Table VI. Phosphoryl Acceptor Specificity
of Vitamin B₆

Acceptor substrate	Vitamin B ₆ -P formed	<i>p</i> -NP formed
	μmoles	μmoles
Pyridoxine	0.46 (0.41*)	16.8
Pyridoxamine	0.12 (0.03*)	16.5
Pyridoxal	- (0.01*)	16.5

The enzyme assay was carried out under the standard conditions with partially purified enzyme preparation (0.45 mg of protein) without pyridoxine. Each vitamin B₆ added were 10 μmoles.

* The values were obtained with the assay of apotryptophanase method.

still been elucidated satisfactorily especially the identity of both the enzymes. Since the works of Axelrod¹²³⁾ and Appleyard¹³⁵⁾ with acid phosphatase, many works on the phosphotransferase activity of certain phosphatase preparations have shown that phosphoryl transfer occurs in the absence of high-energy donors to acceptors like simple alcohols and sugars. While, Brawerman and Chargaff¹²⁴⁾ were the first to discover a group of enzymes which they designated as "nucleoside phosphotransferase" which were capable of transferring esterified phosphoric acid to nucleosides. The enzyme has been also demonstrated in the various bacteria¹³⁶⁾ A few phosphotransferases, which were considered to be different from phosphatase, such as glucose-1-phosphate phosphotransferase have been also discovered¹³⁷⁾

The phosphorylation of pyridoxal by enzymes other than pyridoxal kinase was attempted by Axelrod and resulted in no success using plant phosphatase.¹²³⁾ Recently, Kumar and Vaidyanathan have been shown that the preparation of the enzyme hydrolyzing FMN from green gram extracts possessed the phosphotransferase activity.¹¹⁸⁾ They have been briefly reported that the enzyme could transfer the phosphoryl group cleaved from FMN to pyridoxal and pyridoxamine resulted in the formation of their corresponding phosphate esters.

The phosphorylation of vitamin B₆ presented here provides the first clear-cut evidences for the formation of phosphorylated coenzymes by a phosphotransferase reaction. The enzyme preparation used in these experiments also appeared to gave a strong acid phosphatase activity. Both the enzyme activities were compared using mainly *p*-NPP and pyridoxine as phosphoryl donor and acceptor substrates in this section.

The optimum pH for the phosphotransferase activity was observed to be 6.0, the same as that of phosphatase. The optimum temperature for the transphosphorylation was found to be 35°C, and the enzyme was not inactivated by the heat treatment for 10 min below 60°C. These effects of temperature on the transphosphorylation were also the same with that of the hydrolyzation. The inhibition of inorganic phosphorus and sodium fluoride on phosphatase activity has been investigated by several groups of workers. The phosphatase described

here was moderately inhibited by the addition of these compounds to the reaction mixture, accompanying with the same extent inhibition on the phosphotransferase activity. The behavior of the phosphotransferase to the action of metal ions and other inhibitors was also identical with that of phosphatase. All the available data thus made to suggest that the phosphoryl group transfer from *p*-NPP to pyridoxine might be an intrinsic and inherent function of acid phosphatase. The crystallization of the phosphatase in next section also contributed to the conclusion.

Experiments on phosphoryl donor specificity for the enzyme system indicated that the phosphatase was nonspecific and the phosphotransferase reaction might occur under the wide variety of conditions in microorganisms. On phosphoryl acceptor specificity of vitamin B₆, pyridoxine was the most excellent substrate followed by pyridoxamine. Pyridoxal phosphokinases from various enzyme sources could phosphorylate the three forms of vitamin B₆, though individual kinases differ greatly in their affinities for these compounds.⁵⁸⁾ Pyridoxal-P is known as active coenzyme form of vitamin B₆ for various metabolism. In spite of these facts and the relative inactivity of pyridoxal for the transphosphorylation described here, the reaction may give the physiological significance because an enzyme, pyridoxine-P oxidase, which is capable of conversion of pyridoxine-P and pyridoxamine-P to pyridoxal-P, exists widely in various aerobic bacteria⁸⁸⁾ and also in the strains used here.

SUMMARY

A large number of bacteria were searched for the activity of the synthesis of pyridoxine-P by the transphosphorylation between pyridoxine and *p*-NPP. Several properties of the transphosphorylation by the partially purified enzyme prepared from one of the isolated bacteria, *Escherichia freundii* K-1, were investigated accompanying with phosphatase activity. The behavior of the phosphotransferase and phosphatase activities in various reaction conditions were almost parallel. It was pointed out that the transphosphorylation might be catalyzed by the function of acid phosphatase. The phosphoryl donor specificity for the enzyme system was found to be broad.

Section 3. Crystallization and Characterization of Acid Phosphatase Having Pyridoxine-Phosphorylating Activity

INTRODUCTION

The transphosphorylation reactions catalyzed by phosphatases have been demonstrated as their intrinsic functions.¹³⁸⁾ Though it is considered to be not necessary to assume the existence of a separate class of phosphotransferase in the phosphatase preparation,¹³⁹⁾ the detailed mechanism on the function still remains to be elucidated.

In the previous section, several properties of the enzyme, which catalyzed the phosphoryl group transfer from various organic phosphates to vitamin B₆, did not depend on ATP, were investigated with the partially purified enzyme from *Escherichia freundii* K-1. The enzyme preparation also showed the high phosphatase activity at the same acidic pH. During the course of further investigation on the transphosphorylation, the crystalline enzyme having both the phosphotransferase and phosphatase activities was obtained from the bacterial cell extract.

The present section will describe the studies on the purification, crystallization, and some of the properties of the enzyme. The results indicate that the phosphotransferase activity is functioned by an acid phosphatase.

MATERIALS AND METHODS

Microorganism and Cultures. *Escherichia freundii* K-1, which was isolated from soil and identified in the previous section, was used throughout this work. The culture medium for the organism consisted of 1.5 g peptone, 1.0 g glucose, 0.2 g yeast extract, 0.5 g K_2HPO_4 , 0.1 g KH_2PO_4 , 0.2 g NaCl and 0.02 g $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 7.0. The bacterial cultivation was made aerobically for 20-25 hr at 28°C with 2-liter shaking flask contained 500 ml of the medium.

Activity Measurement. Enzymatic activity was routinely measured in a system with a final volume of 3 ml containing 10 μ moles of pyridoxine, 50 μ moles of *p*-NPP, 200 μ moles of Tris-maleate buffer, pH 6.0, and enzyme. Incubation was carried out for 30 min at 37°C and the reaction was stopped by heating for 10 min in a boiling water bath. Any precipitate formed was removed by centrifugation and the supernatant fluid was used for the determination of pyridoxine-P, *p*-NP and inorganic phosphorus. When the heat-labile substrates were used in the assay, the control tube contained substrate and the buffer. After the 30 min incubation period, the assay was performed in the same manner.

Specific activity of the enzyme is expressed as units per mg of protein where one unit is defined as the amount of protein that forms one μ moles of *p*-NP in 30 min at 37°C.

Analytical Method. *p*-NP, pyridoxine-P and protein were deter-

mined as noted in previous section. Inorganic phosphorus was determined according to the method of Takahashi¹⁴⁰⁾ Sedimentation velocities were measured with a Spinco model E ultracentrifuge operating at 59,780 rev./min. The ultracentrifuge runs for the diffusion constant were made in the Spinco model E ultracentrifuge operating at 6995 rev./min. The temperature of the ultracentrifugation was maintained at 21.5 and 12.8°C, respectively.

Chemicals. Hydroxylapatite was prepared according to the method of Tiselius *et al.*¹⁴¹⁾ Other chemicals were used the same as in the previous section.

RESULTS

Purification of Acid Phosphatase Having Pyridoxine-Phosphorylating Activity.

All operations were performed at 0-5°C throughout the purification procedures.

Step I. Preparation of crude extract. The crude extract of *Escherichia freundii* K-1 was prepared from cells cultivated on 300 liters of the culture medium. The cells were harvested by centrifugation and washed twice with deionized water. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, and disrupted with Kaijo-Denki 19 Hz ultrasonic oscillator. The cell debris was removed by centrifugation at 12,000xg for 30 min.

Step II. Heat treatment. The cell-free extract was immediately

heated for 10 min at 60°C in a water bath by a gently stirring. The resulted precipitates were centrifuged off at 10,000 $\times$ g for 30 min. The supernatant solution was then dialyzed against deionized water overnight.

Step III. First ammonium sulfate fractionation. To the dialyzed enzyme solution was added solid ammonium sulfate to 35% saturation, adjusting pH to 7.0 with 10% ammonium hydroxide solution. After stirring for 30 min, the precipitated protein was removed by centrifugation at 10,000 $\times$ g for 20 min and discarded. The ammonium sulfate concentration was then increased to 65% saturation by further addition of solid ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation at 10,000 $\times$ g for 20 min. This process was repeated separately until 340 g of the cells as dry weight were treated and the active precipitates were combined for the further purification. The combined precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, the final volume was 1000 ml. The solution was then dialyzed overnight against the same buffer. The inactive precipitate appeared was removed by centrifugation at 12,000 $\times$ g for 20 min.

Step IV. Protamine sulfate treatment. Hundred ml of freshly prepared 2.0% protamine sulfate solution neutralized with sodium hydroxide, was slowly added under stirring to 1100 ml of the dialyzed enzyme solution from step III and allowed to stand for 15 min. The precipitate formed was removed by centrifugation at 12,000 $\times$ g

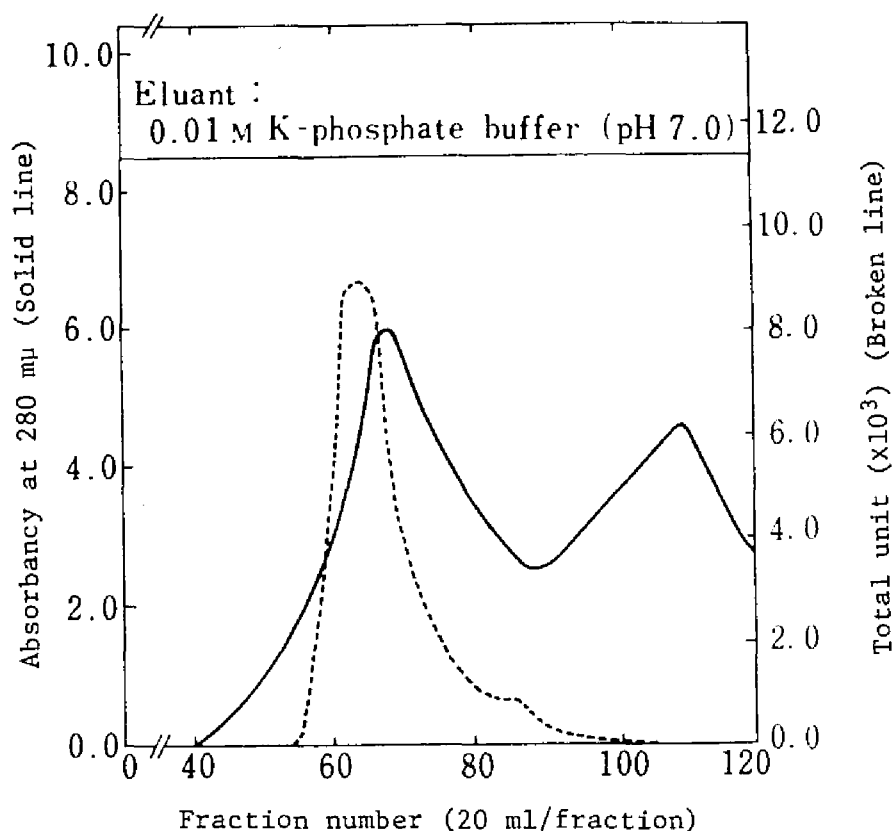


Fig. 1. Chromatography of Phosphatase on DEAE-Cellulose

Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

for 30 min and the resultant supernatant solution was dialyzed against three changes of 0.01 M potassium phosphate buffer, pH 7.0, 12 liters each, overnight under continuous stirring.

Step V. DEAE-Cellulose column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, was packed into a column 3x40 cm. The enzyme solution was placed on the column and eluted with 2.5 liters of 0.01 M potassium phosphate buffer,

pH 7.0 (Fig. 1). The active fractions were combined to give 860 ml of solution and concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate obtained by centrifugation at 12,000xg for 30 min was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and dialyzed overnight against 10 liters of the same buffer.

Step VI. Second ammonium sulfate fractionation. Solid ammonium sulfate to 45% saturation was added to 90 ml of the dialyzed enzyme solution from step V, adjusting pH to 7.0 with 10% ammonium hydroxide solution. After stirring for 30 min, the precipitate was removed by centrifugation at 12,000xg for 20 min. The ammonium sulfate concentration was then increased to 55% saturation by the further addition of solid ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation at 12,000xg for 20 min and dissolved in a small amount of 0.01 M potassium phosphate buffer, pH 7.0. The fractionated solution was dialyzed against three changes of the same buffer, 5 liters each, overnight.

Step VII. Hydroxylapatite column chromatography. The dialyzed enzyme solution (25 ml) was subjected to hydroxylapatite column chromatography. The absorbent equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, was used to pack a column 5x5 cm. The dialyzed enzyme was placed on the column and the column was washed with 650 ml of 0.03 M of the same buffer. The enzyme was subsequently eluted with 0.1 M of the same buffer containing 0.1 M sodium chloride and

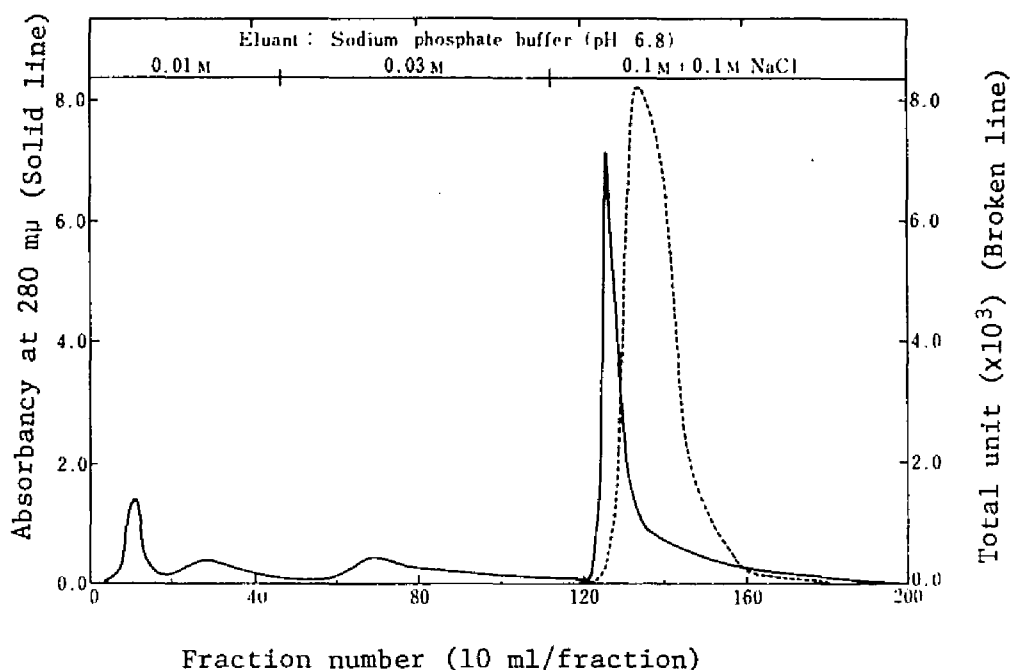


Fig. 2. Chromatography of Phosphatase on Hydroxylapatite

Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

combined to give 150 ml of solution. The elution pattern is shown in Fig. 2. The active fraction was concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate obtained by centrifugation at $12,000\times g$ for 30 min was dissolved in 0.01 M potassium phosphate buffer, pH 6.6, and dialyzed against two changes of the same buffer, each 5 liters, overnight.

Step VIII. CM-Sephadex column chromatography. The dialyzed enzyme solution (9.4 ml) was subjected to CM-sephadex column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 6.6, was used to pack a column 2.2×90 cm. The

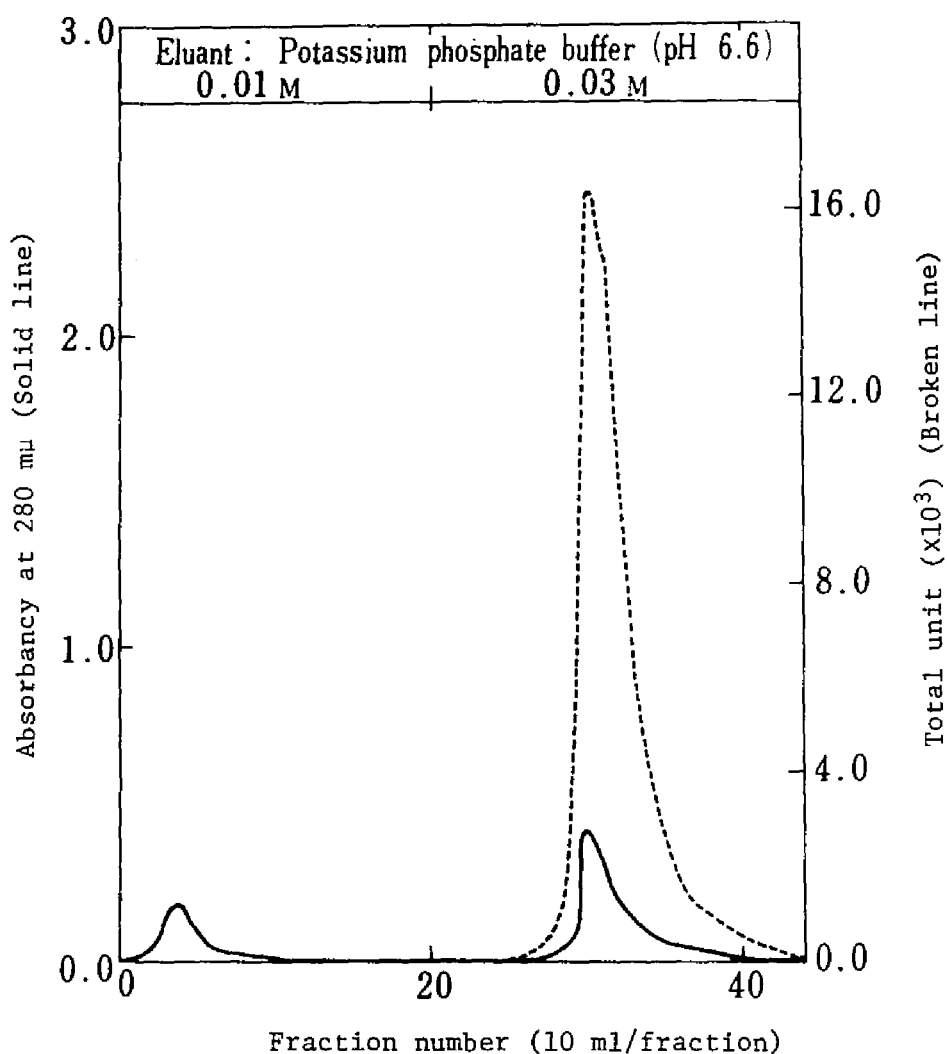


Fig. 3. Chromatography of CM-Sephadex Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

enzyme solution was placed on the column and the column was washed with the same buffer. The enzyme was eluted with 0.03 M of potassium phosphate buffer, pH 6.6. The elution pattern is shown in Fig. 3. The active fractions which had approximately 3000-fold specific activity to

the original extract were combined to give 63 ml of solution and precipitated by the addition of ammonium sulfate to 80% saturation.

Step IX. Crystallization. The purified enzyme preparation obtained from CM-sephadex column chromatography was used for the crystallization of the enzyme. The precipitate was collected by centrifugation at $15,000\times g$ for 30 min and dissolved in a small volume of 0.03 M potassium phosphate buffer, pH 7.0. The insoluble precipitate was removed by centrifugation. Solid ammonium sulfate was added gradually to the supernatant solution until the solution became faintly turbid. The turbid suspension was left in the refrigerator for about one week. Fig. 4 is a photomicrograph of crystalline acid phosphatase having pyridoxine-phosphorylating activity which appeared as highly refractive needles.

The results of the purification procedure are summarized in Table I.

Properties of the Enzyme.

Ultracentrifuge analysis. The analysis of the crystalline enzyme preparation showed a single and symmetric schlieren peak in the ultracentrifuge as shown in Fig. 5. The sedimentation coefficient in water at 20°C ($s_{20,w}$) was found to be 7.5 Svedberg constant, when the protein concentration of 7 mg per ml was used in 0.03 M potassium phosphate buffer, pH 7.0, assuming values found for most proteins of V , the partial specific volume of the enzyme, equal to 0.75. The diffusion coefficient in water at 20°C ($D_{20,w}$) was found

Table I. Purification of Acid Phosphatase

Step	Fraction	Total protein	Total activity	Specific activity	Ratio <i>p</i> -NP/PIN-P
I	Cell-free extract	343,300mg	308,970	0.9	33
II	Heat treatment	58,200	302,640	5.2	35
III	Ammonium sulfate	27,500	299,750	10.9	35
IV	Protamine treatment	18,200	305,760	16.8	35
V	DEAE-cellulose	4,000	228,400	57.1	32
VI	Ammonium sulfate	1,420	116,080	81.7	33
VII	Hydroxylapatite	113	133,815	1184.2	32
VIII	CM-sephadex	18	37,803	2716.1	31
IX	Crystallization	12	33,602	2800.2	32

to be $6.15 \times 10^{-7} \text{ cm}^2/\text{sec}$. From these values, the molecular weight of the enzyme was calculated to be about 120,000 according to the equation of Svedberg and Erikson:¹⁴²⁾

Absorption spectrum. The absorption spectrum of crystalline acid phosphatase was taken with a Shimazu Model MPS-50 L recording spectrophotometer (Fig. 6). Over the wave length investigated, the enzyme exhibited the maximum at 279 m μ . The shoulders at 285 and 293 m μ were also noted in the spectrum. The ratio of 260 m μ /280 m μ was 0.52.

Heat stability. The purified enzyme was heated in a water bath for 10 min with temperature indicated in Fig. 7, and then the residual activity was measured. The enzyme was found to be stable up to 60 to 65°C, but, above 70°C, it was gradually destroyed. It was also observed that both the enzyme activities of phosphatase

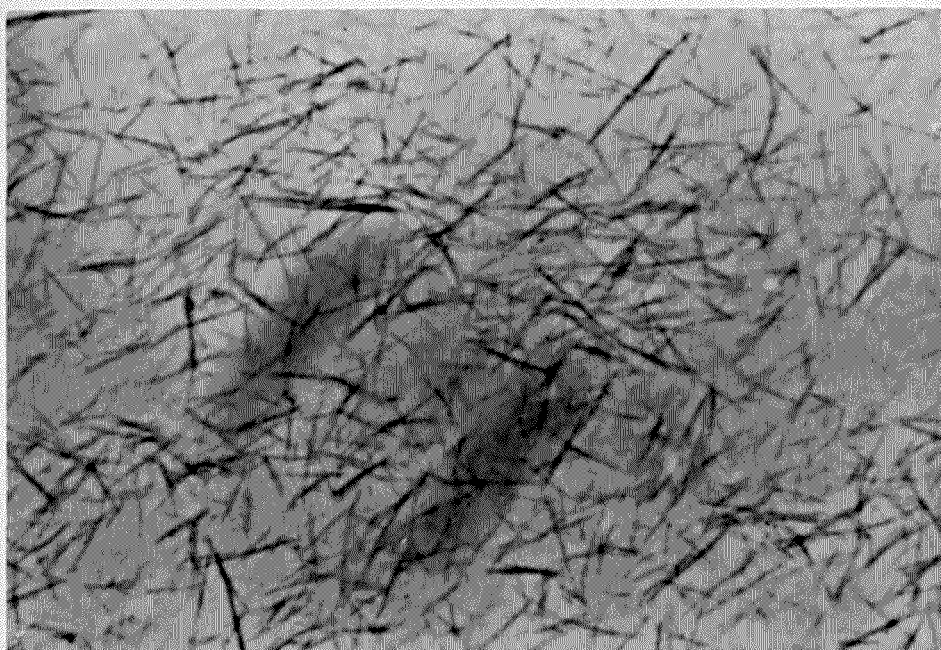


Fig. 4. Photomicrograph of Crystalline Acid Phosphatase
of *Escherichia freundii* K-1

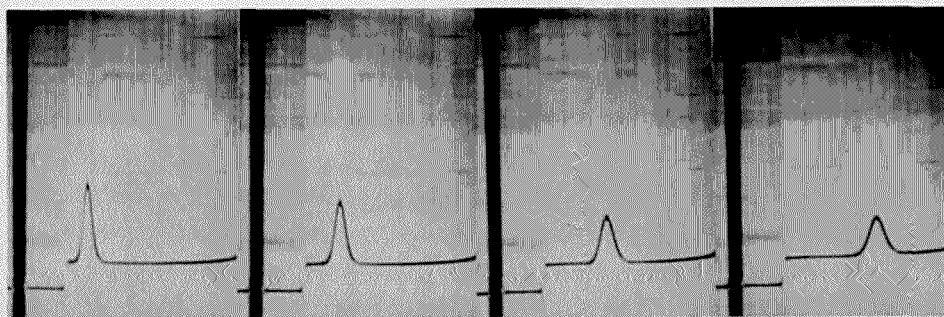


Fig. 5. Ultracentrifugal Analysis of Crystalline Acid
Phosphatase

Schlieren patterns after (left to right) 8, 16, 32, 48 min at 59,780 rpm in the Spinco model E analytical ultracentrifuge. Protein, 0.67% in 0.01 M potassium phosphate buffer of pH 7.0.

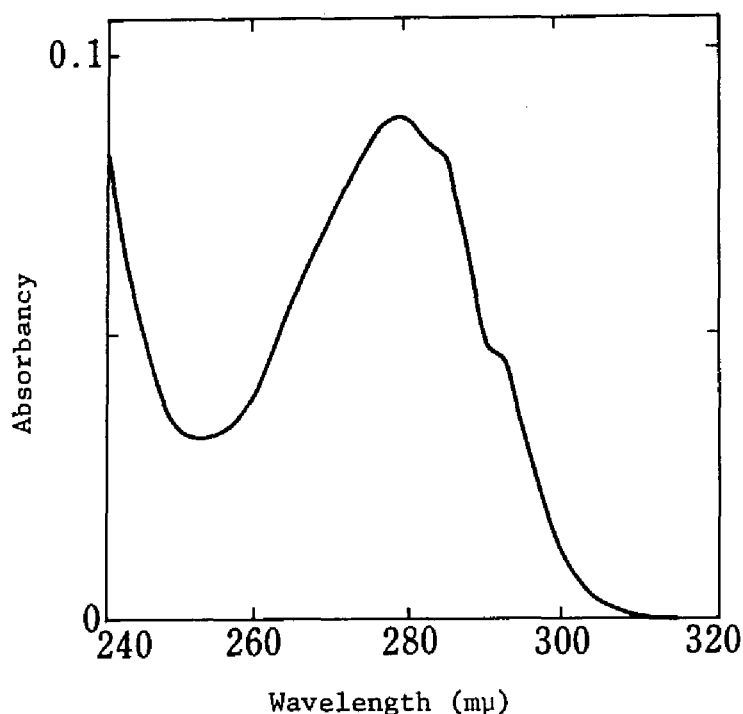


Fig. 6. Ultraviolet Spectrum of Phosphatase

The enzyme solution contained 132 μg per ml of 0.006 M potassium phosphate buffer of pH 7.0.

and phosphotransferase were to be parallel on the heat-resistant property.

Substrate specificity. Seventeen phosphoryl compounds were tested as substrates for the phosphatase and phosphotransferase activities of the purified enzyme under the conditions of the standard assay (Table II). The specificity of the enzyme for both the reactions was found to be fairly broad and to be almost similar. Various sugar phosphates and 5'-mononucleotides other than phenolic compounds were readily served, but 2' (& 3')-mononucleotides were slightly effective as the substrate for the enzyme.

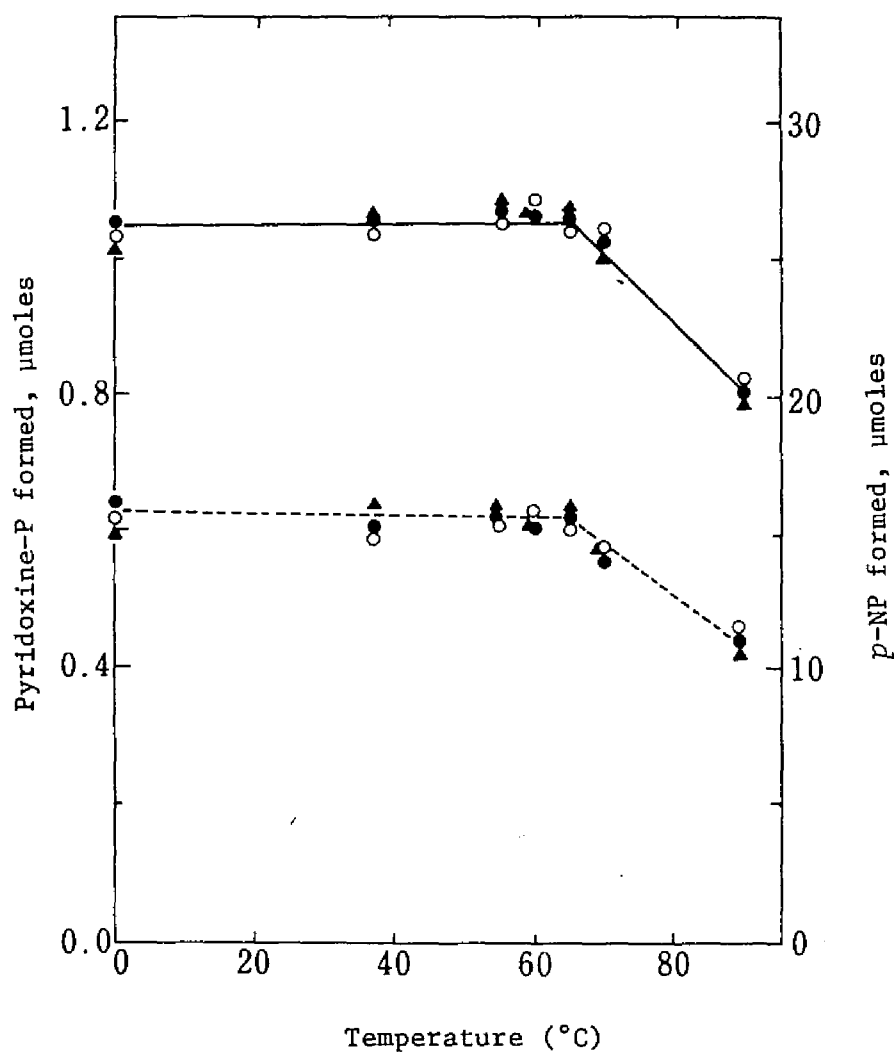


Fig. 7. Heat Stability of the Phosphatase

The enzyme (18 μ g) was heated for 10 min at each temperature in 0.02 M Tris-maleate buffer of pH 5.85 (○), 7.00 (●) and 8.20 (▲), and the residual activity was measured under the standard assay conditions.

—; p-NP formed. - - - - -; pyridoxine-P formed.

Table II. Phosphoryl Donor Specificity

Donor Substrate	Pyridoxine-P	Inorganic-P
	formed (μ moles)	formed
<i>p</i> -NPP	0.43	19.5
Phenyl-P	0.31	16.2
Glucose 1-phosphate	0.03	1.8
Glucose 6-phosphate	0.14	4.5
Fructose 6-phosphate	0.15	6.0
Fructose 1,6-diphosphate	0.15	14.6
β -Glycerol phosphate	0.02	7.8
α -Glycerol phosphate	0.11	4.1
5'-Adenylic acid	0.05	1.3
5'-Cytidylic acid	0.03	5.7
5'-Guanylic acid	0.11	5.4
5'-Inosinic acid	0.13	10.9
5'-Uridylic acid	0.14	10.8
2'(3')-Adenylic acid	0.00	0.0
2'(3')-Cytidylic acid	0.00	0.0
2'(3')-Guanylic acid	trace	0.7
2'(3')-Uridylic acid	0.00	0.0
ATP	0.03	0.9
Acetyl phosphate	0.25	15.0

The enzyme assay was carried out under the standard conditions with purified enzyme preparation (18 μ g of protein) without *p*-NPP. The phosphoryl donor substrate added was 50 μ moles.

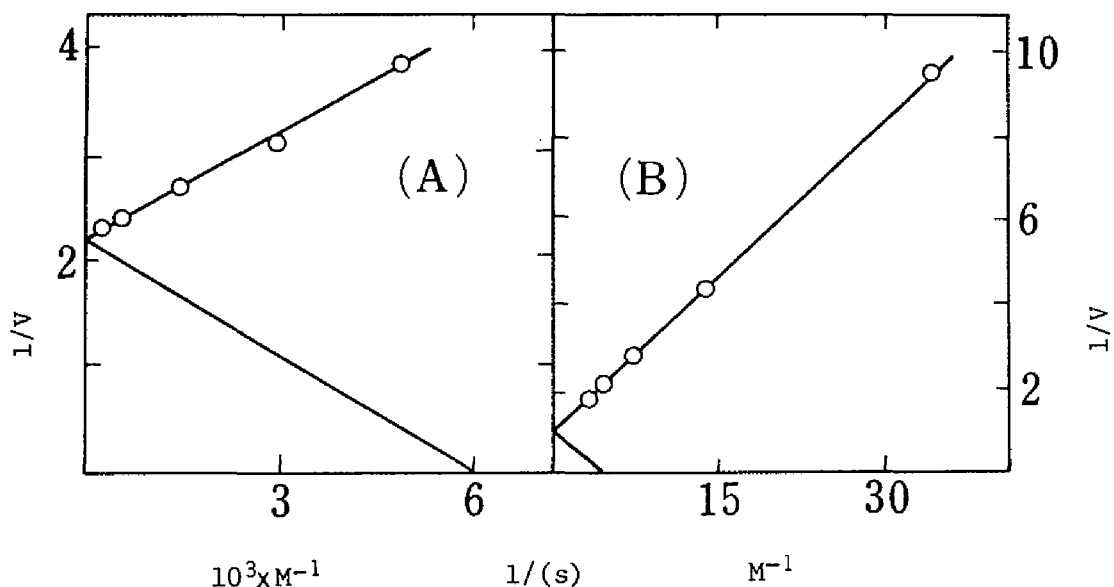


Fig. 8. Effect of Substrate Concentration, (S), on the Reaction Rate, V, by Phosphatase

The enzyme assay was carried out under the standard conditions with purified enzyme (18 μ g of protein) without substrate concentration. The results are plotted by the method of Lineweaver and Burk¹⁴³) (A); *p*-NPP, (B); Pyridoxine.

Michaelis constant. The effect of various concentration of substrates, *p*-NPP on the phosphatase activity and pyridoxine on the phosphotransferase activity, was examined as shown in Fig. 8. The Michaelis constants as calculated by the method of Lineweaver and Burk¹⁴³) were found to be 1.7×10^{-4} M for *p*-NPP and 2.6×10^{-1} M for pyridoxine.

DISCUSSION

Although the purification of phosphatase has been attempted

by many workers, the enzyme has never been crystallized, nor have purified preparations been demonstrated to be completely free of other contaminating proteins, until recent work on the crystallization of alkaline phosphatases of *Escherichia coli*¹⁴⁴⁾ and *Bacillus subtilis*¹⁴⁵⁾ These crystallization procedures contained the release of the enzyme bound in insoluble fractions of the organisms by the conversion of cells to spheroplasts or the extraction with a high concentration of magnesium ion. The transphosphorylation catalyzed by alkaline phosphatase has been reported with purified enzyme preparation^{146,147)} but the investigation with the crystalline enzyme has not been performed. While, several reports on the purification of acid phosphatase have been made,^{148,149)} success in complete purification has not yet been reported.

The present investigation described the first successful crystallization of acid phosphatase of the isolated bacterial strain, *Escherichia freundii* K-1. It has been recognized that the acid phosphatase of *Escherichia coli* was easily solubilized upon disintegration of the cells and that the activity might be separated into several fractions which had different properties.¹⁵⁰⁾ The purification procedures of the acid phosphatase presented herein was performed using cell-free extract (sonicated) as starting material. When the acid phosphatase activities of the intact cells and the extract were compared, no difference was found. From the results of the elution patterns on column chromatographies and substrate

specificities on the purification steps, the separation of the enzyme activity could not be observed. The purified preparation showed the optimum pH of 6.0 with *p*-NPP as phosphoryl substrate, differed from that of *Escherichia coli*!⁵¹⁾

The purified enzyme preparation also hydrolyzed various phosphates other than *p*-NPP as shown in Table II, and accompanied with the transphosphorylation of pyridoxine under similar pH range. Though the *Neurospora* acid phosphatase which has been studied in detail on the substrate specificity had the similar optimum pH!⁵²⁾ the *Escherichia* enzyme utilized the substrates with a somewhat different rate from the phosphatase.

The physiological function of acid phosphatase is still obscure in despite of its widespread occurrence in animal, plant and micro-organism. Hofsten!⁵¹⁾ has reported that the acid phosphatase of *Escherichia coli* was not repressed by inorganic phosphate and the enzyme content of cells grown on carbohydrates was very much lower than that of cells metabolizing organic acids, suggesting the metabolic function. A more anabolic function of the enzyme is, however, not excluded in view of the transferase activity observed for various phosphatases!²²⁾ which might occur with a competition at the enzyme between the acceptor alcohol and water for the phosphoryl moiety from the donor substrate!⁵³⁾ The transphosphorylation of vitamin has been investigated using the partially purified preparations of the phosphatase!^{118,154)} or phosphotransferase!¹³⁷⁾ In these cases,

the identities of the phosphatase and phosphotransferase activities are not elucidated. In the experiment with *Escherichia freundii* K-1 reported here, it was recognized with the obtaining of crystalline enzyme that the transphosphorylation of vitamin B₆ might be catalyzed by the action of phosphatase.

SUMMARY

The enzyme which catalyzed the transphosphorylation of pyridoxine accompanying with the hydrolyzation of phosphoryl donor substrates was purified and crystallized from the cell-free extract of *Escherichia freundii* K-1. The purification procedures involved heat treatment, ammonium sulfate fractionation and DEAE-cellulose, hydroxylapatite, and CM-sephadex column chromatographies. The crystalline enzyme showed the sedimentation coefficient of 7.5 S and the diffusion coefficient of 6.15×10^{-7} cm /sec. The molecular weight was calculated to be 120,000. Several properties of the purified enzyme were also investigated. It was recognized that the transphosphorylation of pyridoxine might be catalyzed by the action of acid phosphatase.

Section 4. Formation of Pyridoxine-P from Pyridoxine

INTRODUCTION

In preceding sections, the author observed a new phosphotransferring reaction, in which phosphoryl group was transferred from various organic phosphates to pyridoxine without the participation of adenine nucleotidic compounds, with various kinds of microorganisms. The enzyme catalyzing the transphosphorylation was purified and crystallized from one of the isolated bacteria, *Escherichia freundii* K-1. Then it was pointed out that the transphosphorylation might be catalyzed by an acid phosphatase.

In spite of many works on the enzymatic interconversion of vitamin B₆, the study on the accumulations of the vitamin and its phosphate esters by microorganism has been scarcely attempted. The microbial transphosphorylation between vitamin B₆ and an organic phosphate may produce a considerable amount of the phosphorylated product of vitamin B₆, although the hydrolysis of the product occurs concurrently.

In this section, several conditions for the phosphorylation of pyridoxine by transphosphorylation from *p*-NPP are investigated with dried cell systems of *Aspergillus flavus* and three isolated bacteria.

MATERIALS AND METHODS

Microorganisms and Cultures. *Aspergillus flavus* Link IFO 5839 was grown in a medium containing 6 g glucose, 1 g peptone, 0.2 g yeast extract, 0.1 g K_2HPO_4 , 0.05 g $MgSO_4 \cdot 7H_2O$ and 0.05 g KCl in 100 ml of tap water, pH 6.0. Cultures were aerobically carried out for 40 hr at 28°C with 50 ml medium placed in 300-ml shaking flasks. The cells were filtrated and washed thoroughly with deionized water.

Escherichia freundii K-1, *Pseudomonas pseudomallei* 68303 and *Aerobacter aerogenes* 810, which were isolated and identified in a previous section, were grown in a medium containing 1.5 g peptone, 1.0 g glucose, 0.2 g yeast extract, 0.5 g K_2HPO_4 , 0.1 g KH_2PO_4 , 0.2 g NaCl and 0.02 g $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 7.0. Cultures were carried out aerobically for 20-25 hr at 28°C. Cells of three bacteria were harvested by centrifugation and washed twice with deionized water.

Preparation of Dried Cells. The washed cells of *Aspergillus flavus* and three bacteria were dried up for 10-15 hr by an electric fan at room temperature. The dried cells were stored at -15°C.

Chemicals. All chemicals were the same as in the previous section and used without further purification.

Standard Reaction Mixture for Transphosphorylation of Pyridoxine. To estimate the formation of pyridoxine-P with the cells of *Aspergillus flavus*, the following standard condition was used: The assay mixture which contained 25 μ moles of pyridoxine, 125 μ moles of *p*-NPP,

300 μ moles of potassium phosphate buffer of pH 6.6 or 250 μ moles of Ringer's phosphate buffer, pH 11.0, and 30 mg of dried cells in a total volume of 4 ml, was incubated at 28°C for 4 hr on a reciprocal shaker.

To estimate the phosphorylation of pyridoxine with the bacterial cells, the following standard condition was used: The assay mixture which contained 10 μ moles of pyridoxine, 300 μ moles of *p*-NPP, 200 μ moles of potassium phosphate buffer, pH 6.6 or sodium-carbonate buffer, pH 9.0, and 30-40 mg of each dried bacterial cells in a total volume of 4 ml, was incubated at 28°C for 24 hr on a reciprocal shaker. The reaction was stopped by heating the mixture in a boiling water bath for 10 min and then cooled in ice water. The reaction products were assayed after the removal of cells by centrifugation.

Analytical Method. Pyridoxine-P, pyridoxal-P and *p*-NP were determined as described in the previous section. Referring to experiments using bacteria, the term "total vitamin B₆-P" is used to stand for the amount of pyridoxal-P plus pyridoxine-P.

RESULTS

Several conditions for transphosphorylation of pyridoxine were investigated with the dried cell systems of *Aspergillus flavus* Link IFO 5839, *Escherichia freundii* K-1, *Pseudomonas pseudomallei* 68303 and *Aerobacter aerogenes* 810, using *p*-NPP as phosphoryl donor subst-

rate. The phosphorylated product by the reaction with *Aspergillus flavus* was almost exclusively pyridoxine-P, while a considerable amount of pyridoxal-P was formed in the reaction mixture with the bacterial cells. The pyridoxal-P formation by the bacterial cell system is described in the next section.

Formation of Pyridoxine-P by the Cells of *Aspergillus flavus*.

Effect of pH. The transphosphorylation between pyridoxine and *p*-NPP occurred at the acid and broad alkaline ranges. The hydrolyzation of phosphoryl donor substrate was found to result in the liberation of *p*-NP at both the pH ranges. The transphosphorylation was performed at pH 6.6 on the acid side, since the strong hydrolyzing activity decomposed the phosphorylated product at lower pH. When the reaction was carried out using various buffers around pH 6.6, phosphate buffer was found to favor the formation of pyridoxine-P and repress the liberation of *p*-NP as shown in Fig. 1-A. The hydrolyzing activity was repressed remarkably with phosphate buffer under alkaline condition, contrasted with no change of pyridoxine-P formation (Fig 1-B).

Effect of temperature. The rates of pyridoxine-P formation measured at different temperatures are shown in Fig. 2. The optimal temperature on the formation of pyridoxine-P under acid and alkaline conditions was found to be around 35°C and 45°C, respectively.

Time course of the reaction. The time course of pyridoxine-P

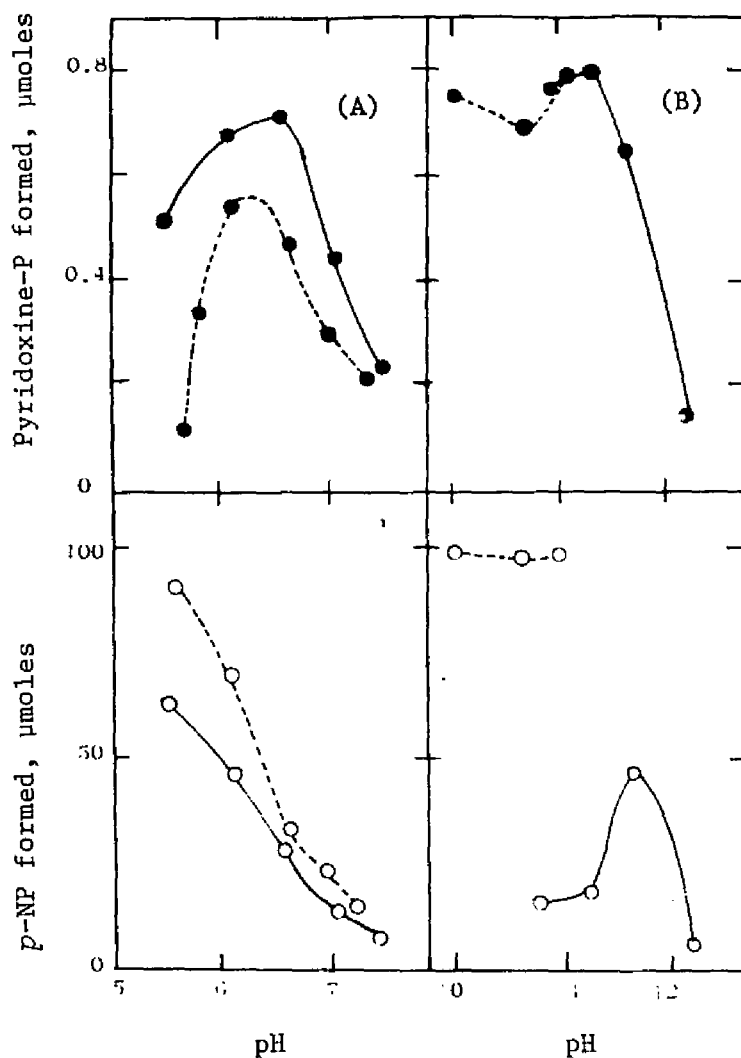


Fig. 1. pH-Activity Curves of the Reaction

The reaction was carried out under the standard conditions with *Aspergillus flavus* cells, and the values obtained were represented in μ moles per 4 ml.

(●) Pyridoxine-P formation, (○) *p*-NP formation.

——; Phosphate buffer, - - - -; Citrate buffer (A) or Sodium-carbonate buffer (B).

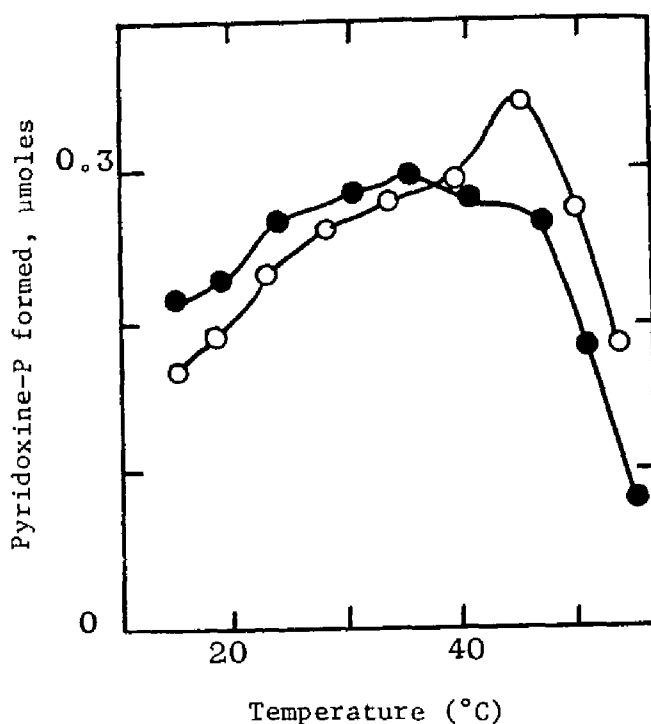


Fig. 2. Effect of Temperature on Pyridoxine-P Formation

The reaction was carried out under the standard conditions with *Aspergillus flavus* cells except reaction time for 1 hr.

(●) at pH 6.6, (○) at pH 11.0.

formation is shown in Fig. 3. When the reaction was carried out in acid media, the formation of pyridoxine-P reached a maximum in 5 hr. The pyridoxine-P formed was gradually decomposed after 10 hr, while the liberation of *p*-NP continued for 20 hr at a constant rate. Under alkaline conditions, both reactions were nearly linear with time for 10 to 15 hr.

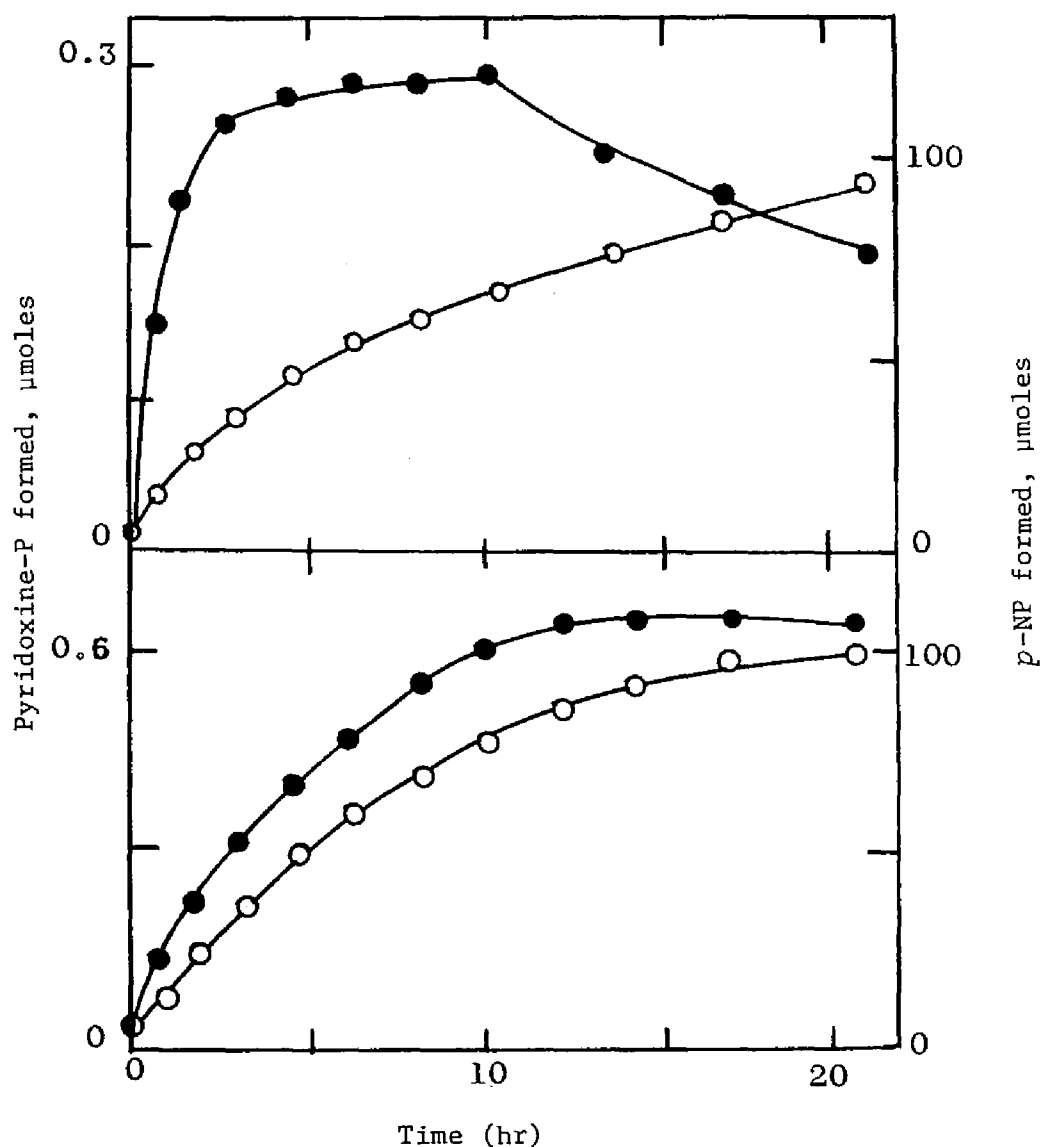


Fig. 3. Time Course of the Reaction

The reaction was carried out under the standard conditions at pH 6.6 (A) or pH 11.0 (B) with *Aspergillus flavus* cells.

(●) Pyridoxine-P formation, (○) p-NP formation.

Table I. Phosphoryl Donor Specificity

Donor substrate	Pyridoxine-P formed, μ moles	
	(A)	(B)
<i>p</i> -NPP	0.23	0.31
Phenyl-P	0.24	0.45
β -Glycerol phosphate	0.22	0.07
α -Glycerol phosphate	0.06	0.05
Glucose 1-phosphate	0.10	0.04
5'-Adenylic acid	0.15	0.24
5'-Inosinic acid	0.16	0.15
5'-Cytidylic acid	0.17	0.12
5'-Uridylic acid	0.06	0.13
ATP	0.02	0.03
None	0.00	0.01

The reaction was carried out under standard conditions at pH 6.6 (A) or pH 11.0 (B) with *Aspergillus flavus* cells. Phosphoryl donors contained were 100 μ moles.

Phosphoryl donor activity. Fifteen compounds were tested as the phosphoryl donor substrate for the formation of pyridoxine-P under acid and alkaline conditions. As shown in Table I, phenyl-P and *p*-NPP were the most effective as donor substrate for the reaction. Other physiological phosphoryl compounds such as β -glycerol phosphate and 5'-mononucleotides were also effective as donor substrate. ATP, which is the most excellent substrate in phosphokinase systems,⁵⁸⁾ was ineffective as phosphoryl donor substrate for the reaction.

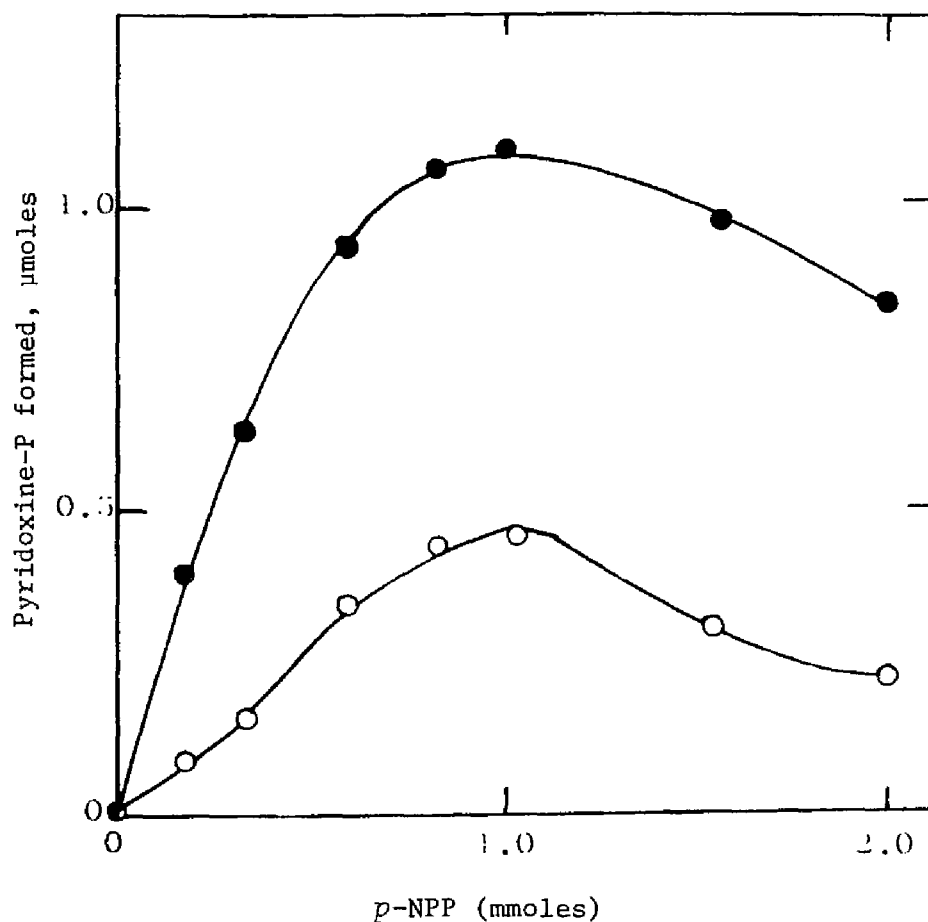


Fig. 4. Effect of *p*-NPP Concentration on Pyridoxine-P Formation

The reaction was carried out under the standard conditions with *Aspergillus flavus* cells except reaction time at pH 11.0 for 24hr.

(●) at pH 6.6, (○) at pH 11.0.

Effect of substrate concentration. The effects of various concentrations of phosphoryl donor and acceptor substrates on the reaction were studied. As shown in Fig. 4, the maximum formation of pyridoxine-P as a function of *p*-NPP concentration was obtained with

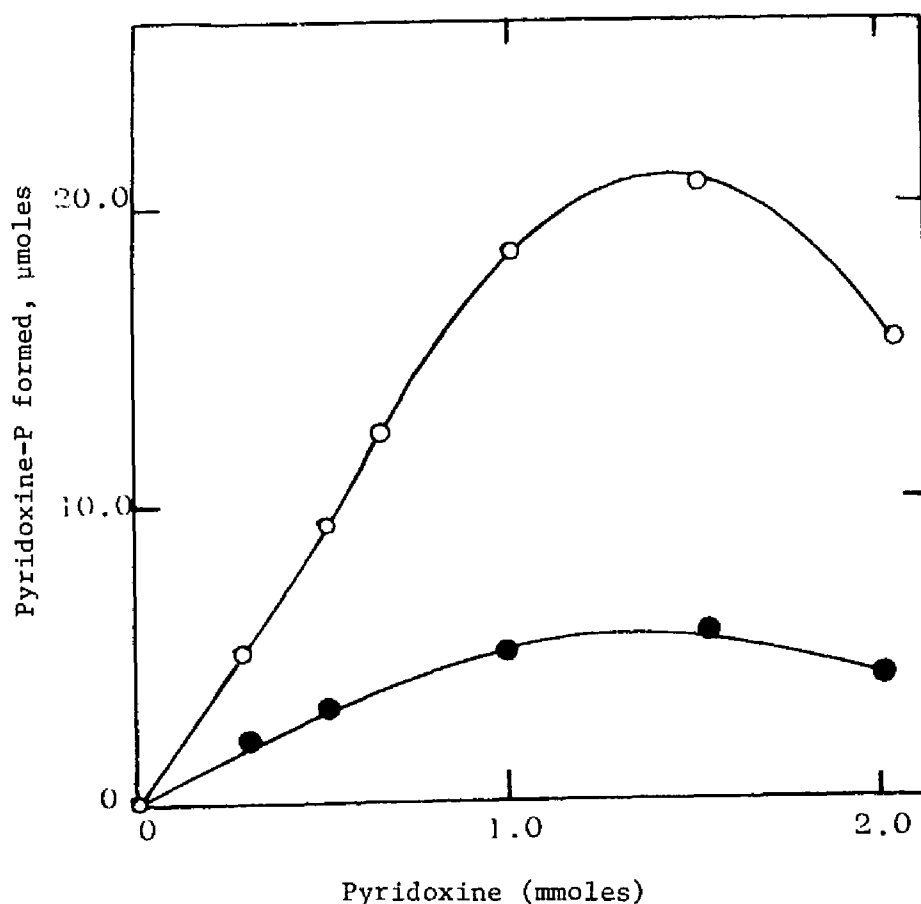


Fig. 5. Effect of Pyridoxine Concentration on Pyridoxine-P Formation

The reaction was carried out under the standard conditions with *Aspergillus flavus* cells except reaction time at pH 11.0 for 24 hr.

(●) at pH 6.6, (○) at pH 11.0.

the addition of 0.5 to 1.0 mmoles of *p*-NPP per 4 ml of the reaction mixture under the standard reaction conditions. The liberation of *p*-NP increased with the addition of an increased amount of *p*-NPP.

Fig. 5 shows the effect of pyridoxine concentration on the reaction.

The amount of pyridoxine-P formed in the reaction mixture increased with the addition of an increased amount of pyridoxine and reached a maximum value of 20 μ moles (ca. 1.3 mg per ml) at alkaline condition. The higher concentration of pyridoxine was inhibitory for pyridoxine-P formation. The liberation of *p*-NP decreased with the higher pyridoxine concentration.

Metal ion effect. The effects of various metal ions on pyridoxine-P formation and *p*-NP liberation were shown in Table II. When the reactions were carried out under the alkaline pH condition, the formation of pyridoxine-P was remarkably accelerated with the addition of Ni^{2+} , Zn^{2+} , Fe^{2+} and MoO_4^{2-} . The addition of Ni^{2+} , for example, made the amount of formed pyridoxine-P 1.5-fold as large. The liberation of *p*-NP was increased by the addition of all metal ions tested. Under acidic conditions, the pyridoxine-P formation was not accelerated by the metal ions tested, but *p*-NP liberation was promoted by the addition of Fe^{3+} , Cr^{3+} and others.

Phosphorylation of Pyridoxine with Bacterial Cells.

Effect of pH. The pH-activity curves for the formation of total vitamin B₆-P by transphosphorylation were investigated with the dried cells of three bacteria (Fig 6). *Escherichia freundii* showed the activity both in acid and alkaline media. *Pseudomonas pseudomallei* and *Aerobacter aerogenes* had their pH optimum in acid or neutral range. The hydrolyzation of phosphoryl donor substrate also occurred in the same pH ranges.

Table II. Effect of Metal Ion on Reaction.

Metal ion	(A)		(B)	
	Pyridoxine-P	p-NP	Pyridoxine-P	p-NP
	(Relative activity)			
None	(100)	(100)	(100)	(100)
Mn ²⁺	88	110	57	119
Mg ²⁺	86	121	83	200
Co ²⁺	29	124	77	190
Ni ²⁺	57	92	153	176
Zn ²⁺	95	110	133	190
Cu ²⁺	67	68	83	143
Fe ²⁺	105	116	120	167
Fe ³⁺	105	137	120	167
Cr ³⁺	105	137	120	167
Al ³⁺	76	110	73	138
MoO ₄ ²⁻	24	47	123	162

The reaction was carried out under the standard conditions at pH 6.6 (A) or pH 11.0 (B) with *Aspergillus flavus* cells. Metal ions were added at the concentration of 10⁻³M.

Effect of cell concentration. The effect of cell concentration on the phosphorylation of pyridoxine was illustrated in Fig. 7. About forty per cent of pyridoxine added as phosphoryl acceptor substrate was phosphorylated at suitable cell concentrations. The decrease in products after attaining the maximal formation was observed with *Escherichia freundii* cells only in alkaline media, in which the phosphorylation of pyridoxine occurs most effectively.

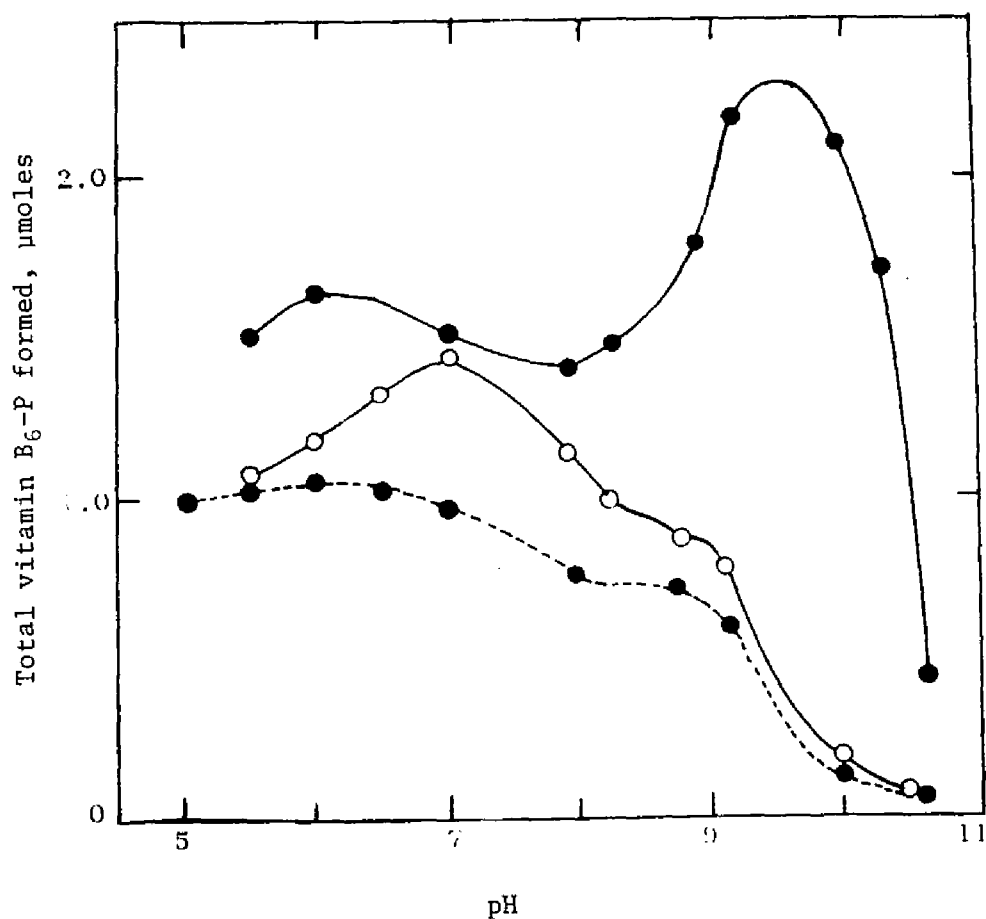


Fig. 6. pH-Activity Curves of Phosphorylation of Pyridoxine

The reaction was carried out under the standard conditions with the cells of *Escherichia freundii* (—●—), *Pseudomonas pseudomallei* (—○—) and *Aerobacter aerogenes* (---●---). The buffers used were: potassium phosphate buffer, pH 5.0-7.8; Tris-HCl buffer, pH 8.2-9.2; sodium-carbonate buffer, pH 9.9-10.5.

Effect of substrate concentration. The formation of total vitamin B₆-P with dried cells of the three bacterial strains was studied in relation to substrate concentration. The amount of phosphorylated products increased with the addition of an increased

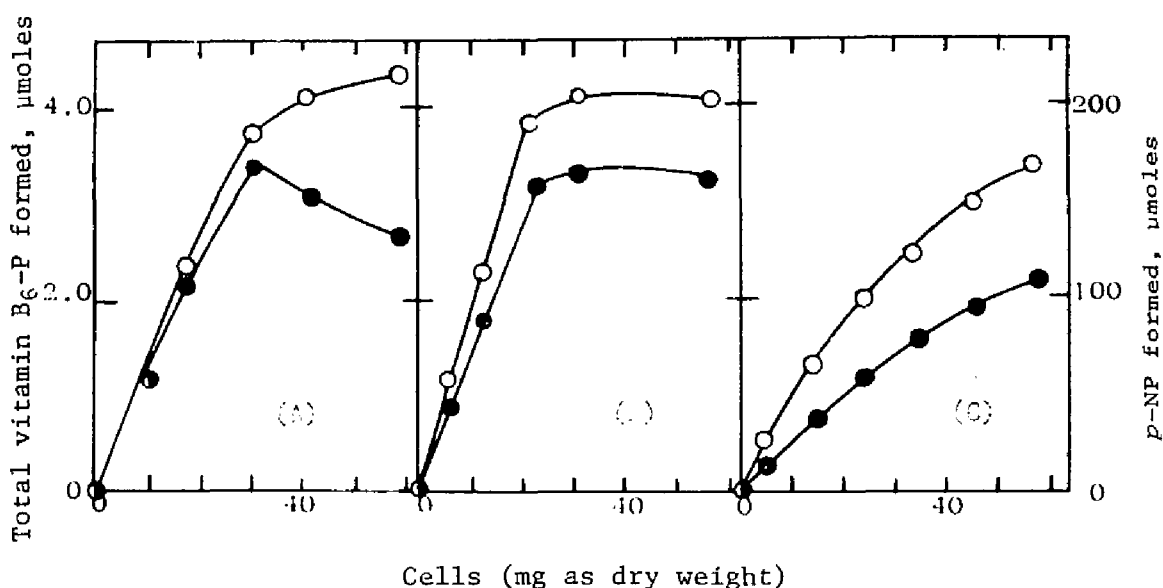


Fig. 7. Effect of Cell Concentration on Phosphorylation of Pyridoxine

The reaction was carried out under the standard conditions at pH 9.0 with *Escherichia freundii* cells (A), at pH 6.6 with *Pseudomonas pseudomallei* cells (B) and at pH 6.6 with *Aerobacter aerogenes* cells (C).

(●) Total vitamin B₆-P formation, (○) p-NP formation.

amount of pyridoxine and reached a maximum value of 120 μmoles (ca. 8 mg per ml) as shown in Fig. 8. Fig. 9 shows that an increase of donor concentration also resulted in a higher yield of the phosphorylated product and the maximal amount of total vitamin B₆-P was obtained with the addition of about 300 μmoles of p-NPP.

DISCUSSION

The primary route of formation of pyridoxal-P appears to be *via* phosphorylation by ATP of pyridoxal, pyridoxamine and pyridoxine

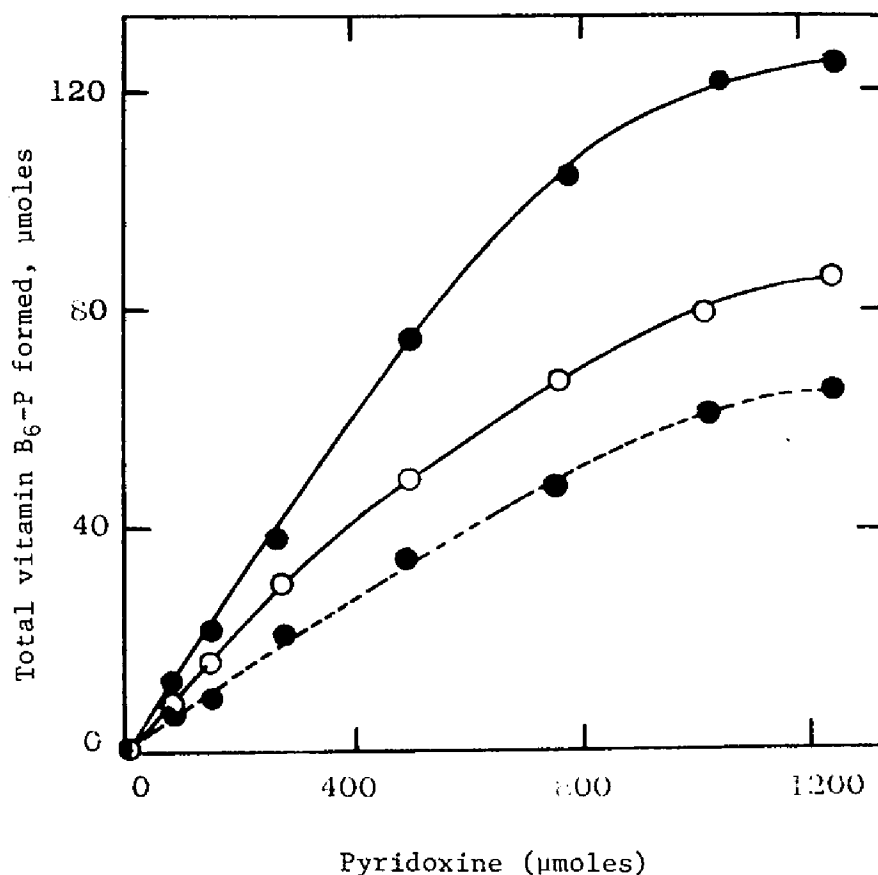


Fig. 8. Effect of Pyridoxine Concentration on
Vitamin B₆-P Formation

The reaction was carried out under the standard conditions at pH 9.0 with *Escherichia freundii* cells (—●—), at pH 6.6 with *Pseudomonas pseudomallei* cells (—○—) and at pH 6.6 with *Aerobacter aerogenes* cells (---●---).

to the corresponding 5'-phosphates, followed by oxidation of the phosphates of pyridoxamine and pyridoxine to pyridoxal-P by pyridoxine (pyridoxamine)-P oxidase¹⁵⁶) Pyridoxine-P is thought to be the main intermediate in the pathway of the conversion of pyridoxine to pyridoxal-P, though the route for the oxidation of pyridoxine

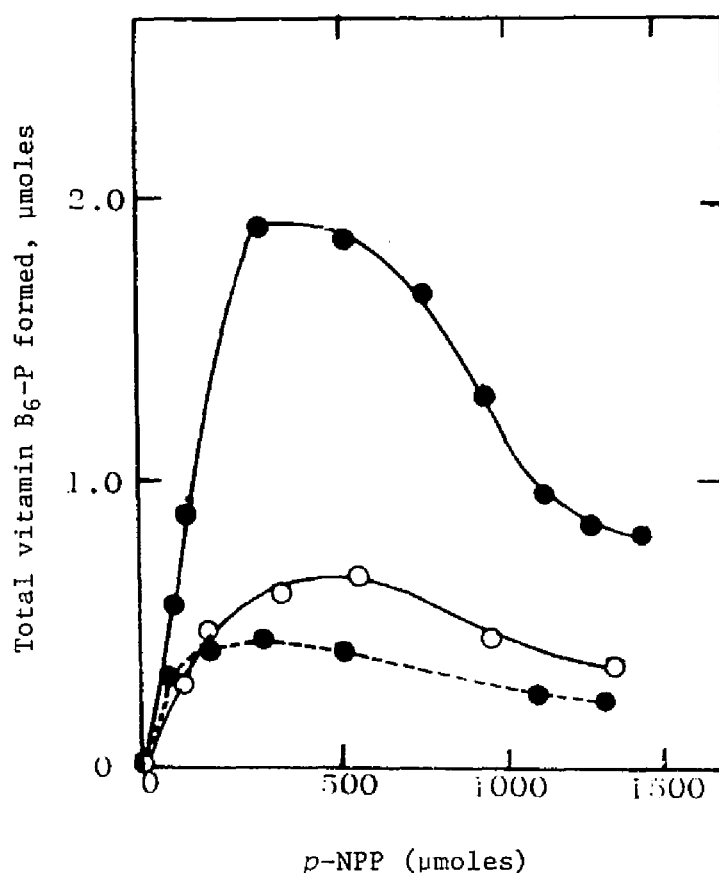


Fig. 9. Effect of *p*-NPP Concentration on Phosphorylation of Pyridoxine

The reaction was carried out under the standard conditions at pH 9.0 with *Escherichia freundii* cells (—●—), at pH 6.6 with *Pseudomonas pseudomallei* cells (—○—) and at pH 6.6 with *Aerobacter aerogenes* cells (---●---).

to pyridoxal also exists^{67,157}). The presence of pyridoxine-P *in vivo* was demonstrated in tissues¹⁵⁸) and microorganisms¹⁰²)

In the preceding section, the author has reported the formation of pyridoxine-P through the transphosphorylation between pyridoxine and various organic phosphates with microorganisms. The reaction

was recognized to be catalyzed by an acid phosphatase, using the enzyme from *Escherichia freundii*. Then the reaction always accompanied the hydrolyzations of phosphorylated product and phosphoryl donor substrate. It was observed, however, that a considerable amount of pyridoxine-P was formed in the reaction mixture. With dried cell systems of *Aspergillus flavus* and three isolated bacterial strains, several conditions for the phosphorylation of pyridoxine were investigated, here, using *p*-NPP as phosphoryl donor substrate.

Aspergillus flavus and *Escherichia freundii* showed the activity of transphosphorylation in acid and alkaline pH ranges, while other bacteria acted in acid or neutral medium. These activities were almost in parallel with the hydrolytic activity under various reaction conditions. It is most likely that the activities other than that of *Escherichia freundii* at acid pH, might be also catalyzed by phosphatase. Then, the increase of cell concentration or reaction time resulted in the decomposition of phosphorylated product which might be caused by the decrease of phosphoryl donor substrate or increase of its hydrolyzed product. It has been demonstrated that alkaline phosphatase was activated by the addition of various metal ions¹⁴⁵⁾ and that Zn^{2+} was the prosthetic group of *Escherichia coli* alkaline phosphatase.¹⁵⁹⁾ Some metal ions showed an accelerating effect on the formation of pyridoxine-P by *Aspergillus flavus* under alkaline pH condition. The hydrolysis of phos-

phoryl donor substrates was also accelerated by the addition of metal ions. Under optimal condition with dried cells of *Escherichia freundii*, the formation of total vitamin B₆-P in the reaction mixture reached *ca.* 8 mg per ml with increasing amount of pyridoxine as phosphoryl acceptor substrate, while the maximum rate of conversion of pyridoxine to vitamin B₆ phosphates was obtained in about 40% yield using a suitable amount of bacterium in the reaction mixture.

The most excellent activity of vitamin B₆ as phosphoryl acceptor substrate was obtained with pyridoxine, followed by pyridoxamine, under the reaction conditions presented in this report. On the phosphoryl group transfer from low-energy phosphates to vitamin B₆, a brief study has been reported by Kumar and Vaidyanathan during their investigation of FMN hydrolase from green-gram seeds.¹¹⁸⁾ They described that FMN hydrolase could transfer the phosphoryl group of FMN to pyridoxal, pyridoxamine and other acceptors, and pyridoxine was inactive as the phosphoryl acceptor substrate. They obtained the values of the phosphorylated products, about 20 µg per ml, under the conditions appeared the higher rate of phosphoryl transfer.

It has been demonstrated by many groups of workers that phosphatases could catalyze the phosphoryl group transfer to various compounds which have alcoholic group.¹²²⁾ Pyridoxine has two hydroxymethyl group which may be phosphorylated by the action of phosphatase. But the formation of pyridoxine 4'-phosphate in the reaction

mixture could not be detected on the paper chromatogram or paper electrophoregram.

SUMMARY

Several conditions for the formation of pyridoxine-P by the transphosphorylation between pyridoxine and *p*-NPP was investigated using *Aspergillus flavus* and three isolated bacteria, *Escherichia freundii*, *Pseudomonas pseudomallei* and *Aerobacter aerogenes*.

Dried cells of *Aspergillus flavus* and *Escherichia freundii* showed the transphosphorylation activity at acid and alkaline pH ranges. The other bacteria showed the activity at acid or neutral pH range, respectively. These bacteria also formed pyridoxal-P beside pyridoxine-P. The transphosphorylation always accompanied the strong hydrolytic activity of phosphoryl donor substrates. Metal ions accelerated the formation of pyridoxine-P by *Aspergillus flavus* under the alkaline condition. The amount of phosphorylated products reached ca. 8 mg per ml of reaction mixture when *Escherichia freundii* was used. The maximum phosphorylation rate of pyridoxine observed with the bacterial cells was 40%. The specificity of phosphoryl donor substrate was observed to be broad. *p*-NPP and phenyl-P showed the highest activity for the phosphorylation of pyridoxine.

Section 5. Formation of Pyridoxal-P from Pyridoxine

INTRODUCTION

It has been demonstrated with microorganisms and mammalian tissues that the enzymatic conversion of pyridoxine to the active form of vitamin B₆, pyridoxal-P, was performed through two pathways.³⁴⁾ One of them is the phosphorylation of pyridoxal after dehydrogenation of pyridoxine and another is the oxidation of pyridoxine-P after the phosphorylation of pyridoxine.

In an earlier section, it has been demonstrated that pyridoxine could be phosphorylated by microbial transphosphorylation to produce a considerable amount of pyridoxine-P and a small amount of pyridoxal-P. Subsequently, the isolation of several bacteria having the higher activity of the transphosphorylation, has been accomplished. During the course of investigation on the transphosphorylation with the intact cell systems of these bacteria, it was found that a considerable amount of pyridoxal-P was produced in the reaction mixture which contained pyridoxine and phenyl-P as phosphoryl acceptor and donor substrates under alkaline pH range, although *p*-NPP was the most excellent phosphoryl donor substrate for the phosphorylation of pyridoxine.

This section deals with the several conditions on the formation of pyridoxal-P from pyridoxine with the transphosphorylation and the

oxidation by *Escherichia freundii* K-1 cells. The reaction product, pyridoxal-P, was also isolated for the further confirmation of the reaction. Moreover, the clarification of the rather accelerating effect of phenyl-P than *p*-NPP on pyridoxal-P formation was attempted.

MATERIALS AND METHODS

Microorganisms and Cultures. *Escherichia freundii* K-1, which was isolated from soil and identified in previous section, was used in this work. The culture of the organism was performed as described in a previous section.

Chemicals. All chemicals were obtained from commercial sources and were used without further purification.

Preparation of Intact Cells and Cell-Free Extract. The cells of *Escherichia freundii* K-1 in cultured broth were harvested by centrifugation and washed twice with deionized water. The washed cells were suspended in deionized water and used as the intact cells in the experiments for pyridoxal-P formation. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.001% 2-mercaptoethanol and treated with a Kaijo-Denki 19 Hz ultrasonic oscillator for 20 to 40 min. The intact cells and debris were removed by centrifugation at 12,000*g* for 30 min. The resulted supernatant solution was dialyzed against the same buffer overnight, and then used as cell-free extract preparation.

Standard Reaction Mixture for Pyridoxal-P Formation. To estimate the formation of pyridoxal-P from pyridoxine, the reactions were aerobically carried out in a total volume of 4.0 ml at 28°C for 24 hr. The standard composition was: 10 μ moles of pyridoxine, 200 μ moles of phenyl-P, 300 μ moles of Tris-HCl buffer, pH 9.0, and the intact cells. The reactions were terminated by heating the mixture in a boiling water bath for 10 min and then cooled in ice water. The reaction products were assayed after the removal of cells by centrifugation.

Analytical Method. Pyridoxal-P and pyridoxine-P were determined as described in a previous section using the methods of phenylhydrazine and apotryptophanase. In the following experiments, the term mentioned as "total vitamin B6-P" involved pyridoxine-P in addition to pyridoxal-P. Pyridoxal was determined by phenylhydrazine method reported by Wada and Snell⁷⁵⁾ Protein and p-NP were estimated by the method as described previously.

RESULTS

Pyridoxal-P Formation from Pyridoxine.

Several conditions of the transphosphorylation which formed pyridoxal-P from pyridoxine were studied with the intact cell systems of *Escherichia freundii* K-1.

It has been briefly described in a previous section that the whole cells of the bacterium showed the activity of the transphos-

Table I. Transphosphorylation of Pyridoxine

Donor Substrate	Enzyme	pH	Pyridoxal-P (μ moles)	Total vitamin B ₆ -P
<i>p</i> -NPP	Intact cells	6.0	0.01	0.83
		8.6	0.17	0.53
	Cell free extract	6.0	0.05	0.66
		8.6	0.25	0.39
	Intact cells	6.0	0.10	0.54
		8.6	0.21	0.25
Phenyl-P	Cell free	6.0	0.17	0.50
	extract	8.6	0.28	0.30

The reaction was carried out under the standard conditions with 36 mg as dry weight of intact cells or cell free extract of 27 mg protein except reaction time of 2 hr and Tris-maleate buffer, pH 6.0, or Tris-HCl buffer, pH 8.6.

phorylation between pyridoxine and *p*-NPP at both acidic and alkaline pH ranges and that the activity at alkaline pH disappeared with the cell-free extract preparation. Table I shows the phosphorylation of pyridoxine in the presence of phosphoryl donor substrate. The remarkable formation of pyridoxal-P was observed at alkaline pH. When phenyl-P was used as phosphoryl donor substrate, it was also recognized that the relatively considerable amount of pyridoxal-P was formed.

The comparative effect of donor concentration on the phosphorylation of pyridoxine was examined with *p*-NPP and phenyl-P as phosphoryl donor substrate (Fig. 1). The result shows that phenyl-P is the preferred substrate for pyridoxal-P formation. The formation

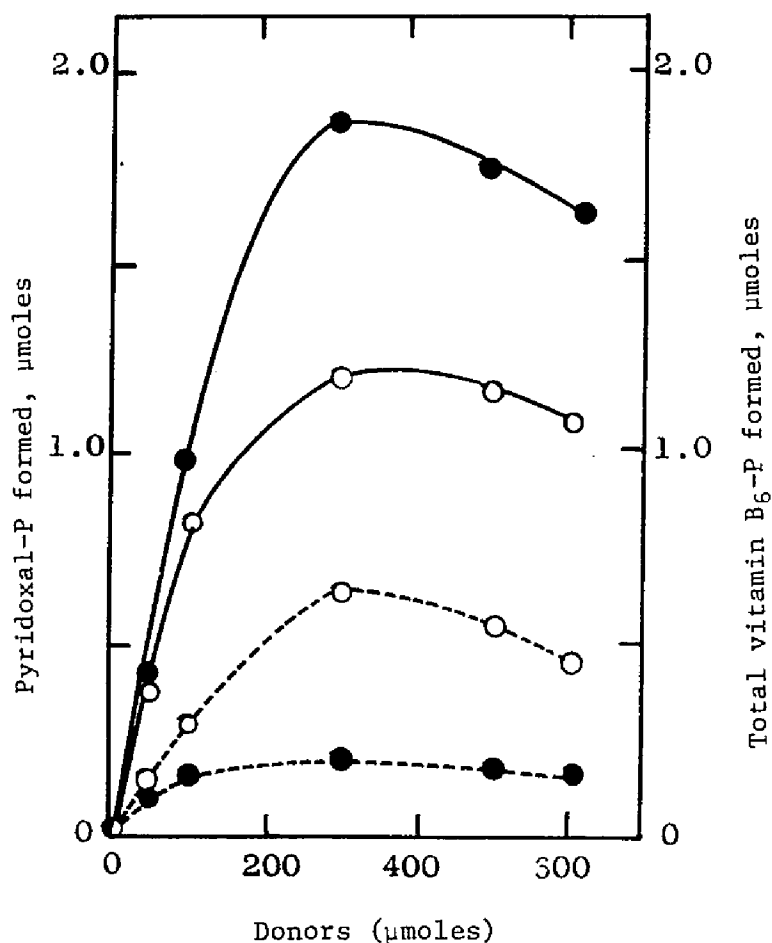


Fig. 1. Comparative Effects of Donor Concentration
on Phosphorylation of Pyridoxine

The reaction was carried out under the standard conditions with 20 mg as dry weight of intact cells.

(●) *p*-NPP added, (O) phenyl-P added as phosphoryl donor substrate.

-----; pyridoxal-P formed. ———; total vitamin B₆-P formed.

of total vitamin B₆-P, which involved the formation of pyridoxine-P, however, was larger in the case of the addition of *p*-NPP than in that of phenyl-P. The higher concentration of donor substrates

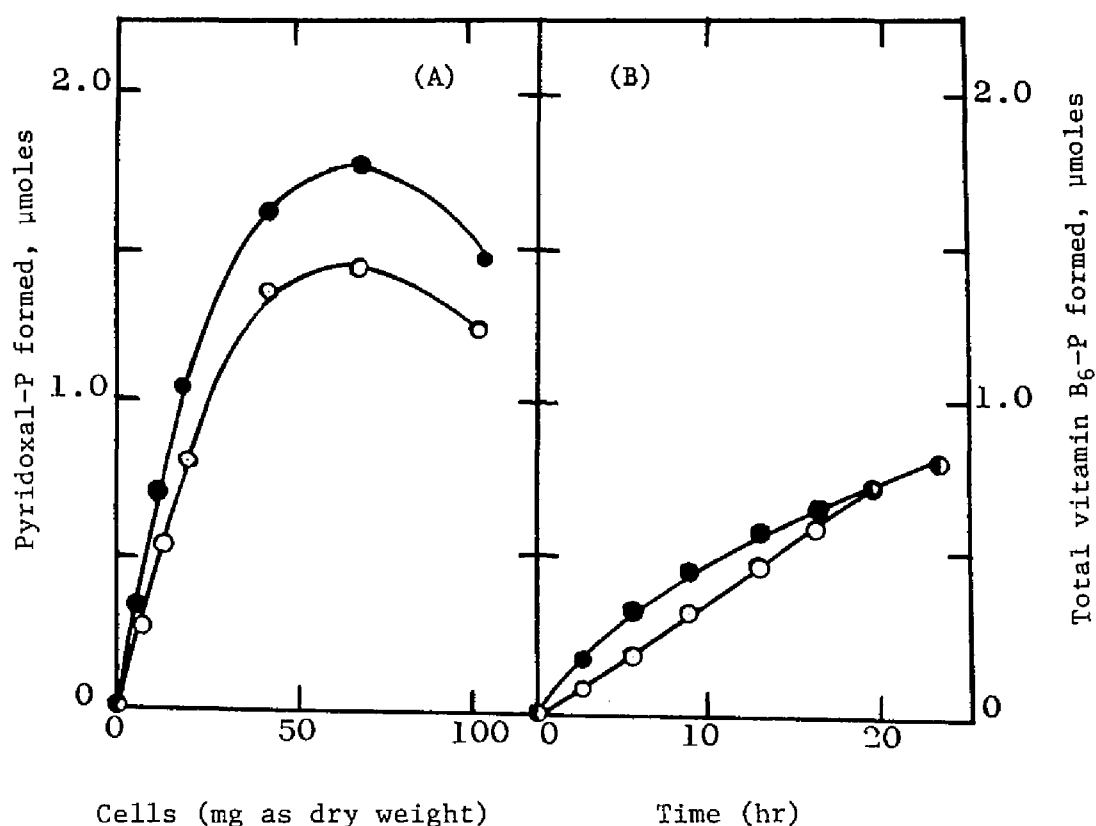


Fig. 2. Effects of Cell Concentration (A) and Reaction Time (B) on Phosphorylation of Pyridoxine

The reactions were carried out under the standard condtions with 30 mg as dry weight of intact cells (B).

(O) pyridoxal-P formed. (●) total vitamin B₆-P formed.

inhibited both the formations of pyridoxine-P and pyridoxal-P.

The effect of cell concentration on the phosphorylation of pyridoxine is shown in Fig. 2-A. The amount of pyridoxal-P formed in the reaction mixture reached a maximum value of *ca.* 1.5 μmoles per 4 ml (0.4 mg). The addition of higher concentration of cells in reaction mixture resulted in the decomposition of both the phos-

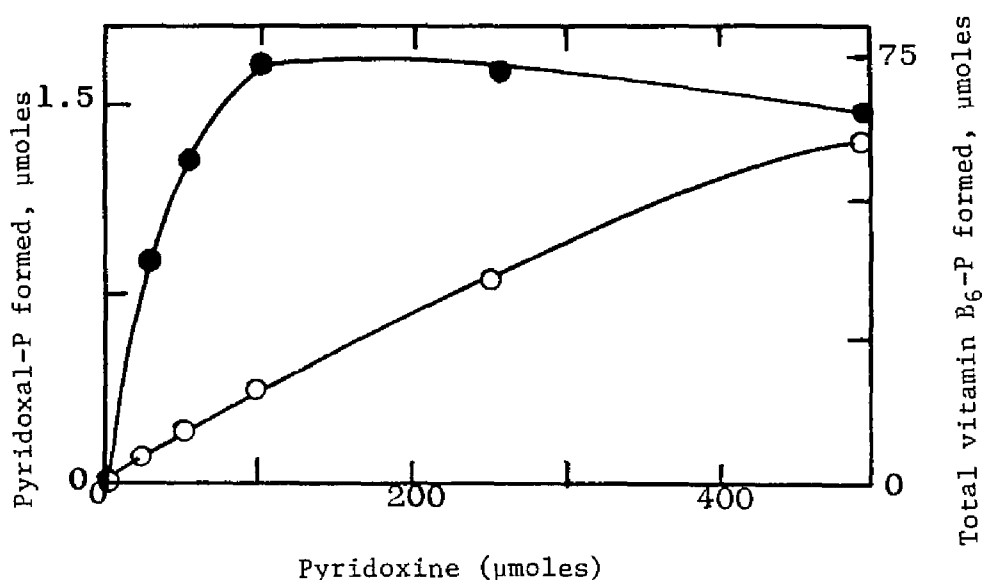


Fig. 3. Effect of Pyridoxine Concentration on Phosphorylation of Pyridoxine

The reaction was carried out under the standard conditions with 30 mg as dry weight of intact cells.

(O) pyridoxal-P formed. (●) total vitamin B₆-P formed.

phorylated products. The formations of pyridoxal-P and total vitamin B₆-P were the nearly linear function of the reaction time (Fig. 2-B). It was observed that the increase of reaction time increased the ratio of pyridoxine-P to pyridoxal-P.

The effect of pyridoxine concentration on the formation of vitamin B₆ phosphates is shown in Fig. 3. The amount of pyridoxal-P formed increased with the increased addition of pyridoxine as phosphoryl acceptor, while pyridoxine-P was no more accumulated in the reaction mixture above the addition of 100 μmoles of pyridoxine per 4 ml.

Table II. Effects of Metal Ion and Inhibitor

on Phosphorylation of Pyridoxine

Metal ion and inhibitor	Pyridoxal-P	Total vitamin B ₆ -P
(Relative activity)		
None	(100)	(100)
Cu ²⁺ 10 ⁻³ M	102	82
3x10 ⁻³ M	115	80
Al ³⁺	105	110
Mg ²⁺	100	100
Zn ²⁺	67	74
Ni ²⁺	85	96
MoO ₄ ²⁻	112	112
Hg ²⁺	55	67
NaF	85	96
PCMB	34	41
EDTA	101	100
Borate	29	33

The reaction was carried out under the standard conditions with 28 mg as dry weight of intact cells and additions of 10⁻³M metal ion and inhibitor.

The effects of metal ions and inhibitor at concentrations of 1 mM on the formations of pyridoxal-P and total vitamin B₆-P were examined (Table II). Cupric ion showed the stimulating effect only on pyridoxal-P formation; Hg²⁺, Zn²⁺ and *p*-chloromercuribenzoate inhibited both of the formations to the extent of 40 to 60%.

Isolation of Pyridoxal-P from the Reaction Mixture.

Pyridoxal-P was isolated from the reaction mixture employing

anion and cation exchangers and then identified for the confirmation of the reaction as follows: The reaction mixture was prepared by addition of 1 mmole of pyridoxine, 30 mmoles of phenyl-P, 50 mmoles of Tris-HCl buffer, pH 9.0, and 4 g as dry weight of the intact cells of *Escherichia freundii* K-1 in a total volume of 1000 ml. The reaction was aerobically carried out at 28°C for 24 hr and then terminated by heating in a boiling water bath for 10 min. The resulting precipitate was centrifuged off. The supernatant solution was adjusted to pH 7.0 with hydrochloric acid, and applied onto a Dowex 1x8 (Cl⁻ form) column. The column was washed thoroughly with deionized water and 0.001 N hydrochloric acid, followed by elution with 0.005 N and 0.01 N hydrochloric acid. The fractions eluted with 0.005 N hydrochloric acid consisted of pyridoxine-P. The fractions containing pyridoxal-P eluted with 0.01 N hydrochloric acid were combined and adjusted to pH 7.0 with sodium hydroxide. The solution was concentrated under reduced pressure at 30°C and then applied onto a Dowex 50X8 (H⁺ form) column. The elution was performed with deionized water. The active fractions were combined and then concentrated to an appropriate volume under reduced pressure at 30°C. To the concentrated fluid adjusted to pH 4.0 with acetic acid, calcium acetate was added, followed by the addition of absolute alcohol. The precipitated calcium salt of pyridoxal-P (20 mg) was obtained by filtration and recrystallized from water. Calcium salt of pyridoxal-P isolated was found to have one spot on paper

chromatogram¹⁷⁴⁾ and paper electrophoregram¹⁶⁰⁾ and to coincide with the Rf value of authentic specimen. Further investigation with infrared analysis⁸⁸⁾ gave additional confirmation. The ultraviolet spectra were also identical with those of authentic pyridoxal-P, which had λ_{max} at 330 m μ and 388 m μ in 0.1 M phosphate buffer, pH 7.0, at 307 m μ and 388 m μ in 0.1 N sodium hydroxide, and at 295 m μ in 0.1 N hydrochloric acid.

Properties of Enzymes Concerning to Conversion of Pyridoxine to Pyridoxal-P.

From the results mentioned above, it was apparent that pyridoxal-P formation from pyridoxine through the transphosphorylation was larger when phenyl-P was added as phosphoryl donor substrate than when *p*-NPP was added. It has been demonstrated that the oxidation of pyridoxine to pyridoxal and that of pyridoxine-P to pyridoxal-P were catalyzed by the two distinct enzymes, pyridoxine dehydrogenase and pyridoxine-P oxidase.⁵⁴⁾ In the transphosphorylation of pyridoxine and pyridoxal by the enzyme of *Escherichia freundii* K-1, *p*-NPP was the preferred phosphoryl donor substrate. Then the effect of phenyl-P on the pyridoxal-P formation might be caused by the different properties of the enzymes concerning to the oxidation of pyridoxine or pyridoxine-P for these phenolic compounds. From this point of view, the effects of both the phosphoryl donor substrates and the hydrolyzed products on the enzyme concerning to the oxidations of pyridoxine and pyridoxine-P were investigated using the cell-free

extract and the partially purified enzyme preparation from *Escherichia freundii* K-1.

a) Pyridoxine-P Oxidase.

Partial purification of enzyme. For the separation of pyridoxine-P oxidase from phosphatase, which catalyzed the hydrolysis and transphosphorylation, the partial purification was carried out by adopting the ammonium sulfate fractionation. All operations were performed at 0-5°C. The buffer used in this preparation was 0.01 M potassium phosphate buffer, pH 7.0, containing 0.001% 2-mercaptoethanol. The cell-free extract was fractionated by an addition of solid ammonium sulfate, adjusting to pH 7.0 with 10% ammonium hydroxide solution. The insoluble fraction between 10 to 50% saturation was collected and dissolved in the buffer. The enzyme solution was dialyzed overnight against the buffer. Subsequently, protamine treatment was carried out by the addition of one tenth amount of neutralized protamine sulfate of protein content and the precipitate formed was centrifuged off. After dialysis overnight against the buffer, the supernatant solution was again fractionated with an addition of ammonium sulfate to yield 0-10, 10-20, 20-30, 30-40, 40-50 and over 50% levels, adjusting to pH 7.0 with 10% ammonium hydroxide solution. The insoluble fraction between 30 to 40% saturation which involved a large part of pyridoxine-P oxidase activity was dissolved in the buffer and used in the following experiments after dialysis overnight against the buffer. A part of phosphatase activity remained

Table III. Effect of Phenolic Compound on
Pyridoxine-P Oxidase

Phenolic Compound	Concentration(M)	Pyridoxal-P formed		
		10 ⁻³	10 ⁻²	10 ⁻¹
(Relative activity)				
Phenyl-P		106	117	128
<i>p</i> -NPP		118	130	119
Phenol		101	96	6
<i>p</i> -NP		97	100	5

The reaction mixture containing 0.5 μ mole of pyridoxine-P, 200 μ moles of Tris-HCl buffer of pH 8.6, 100 μ moles of sodium fluoride, 11.9 mg of partially purified enzyme protein and phenolic compound in a total volume of 3 ml was incubated for 30 min at 37°C. Relative activity was indicated against the value of no addition of the phenolic compound.

in this preparation. A few ion exchange cellulose column chromatographies and acid treatment did not affect the separation of both the enzyme activities.

Properties of enzyme. The effects of phenyl-P, *p*-NPP and their hydrolyzed products on the oxidation of pyridoxine-P were investigated with an addition of sodium fluoride, which was a typical inhibitor for acid phosphatase.¹⁶¹⁾ As shown in Table III, the additions of higher concentrations of both phenol and *p*-NP remarkably inhibited the pyridoxine-P oxidase activity. While, *p*-NPP or phenyl-P showed a rather stimulating effect, which might be owing to the residual

Table IV. Conversion of Pyridoxine to Pyridoxal

Condition	Pyridoxal formed
Complete system	0.14 μ mole
Omitted pyridoxine	0.00
Omitted NADP	0.04
Omitted Enzyme	0.00
Boiled enzyme	0.01

The complete system contained 10 μ moles of pyridoxine, 200 μ moles of Tris-HCl buffer of pH 8.6, 1 μ mole of NADP and 27 mg protein of cell free extract in a total volume of 3 ml. The enzyme assay was carried out for 2 hr at 37°C, and stopped by an addition of 0.5 ml of 50% trichloacetic acid.

activity of phosphatase catalyzing the transphosphorylation. The results suggested that pyridoxine-P oxidase did not relate to a cause of the difference between *p*-NPP and phenyl-P as phosphoryl donor substrate for pyridoxal-P formation.

b) Pyridoxine Dehydrogenase.

The conversion of pyridoxine to pyridoxal in the dialyzed cell-free extract was observed with an addition of NADP under alkaline conditions (Table IV). Then, the effect of phenolic compounds on the pyridoxine dehydrogenase was studied with the extract. It was recognized that only *p*-NP inhibited the dehydrogenation at a low concentration (Fig. 4). The result suggested that the pyridoxal-P formation from pyridoxine and phenyl-P or *p*-NPP through the transphosphorylation with the intact cell systems might be differentiated

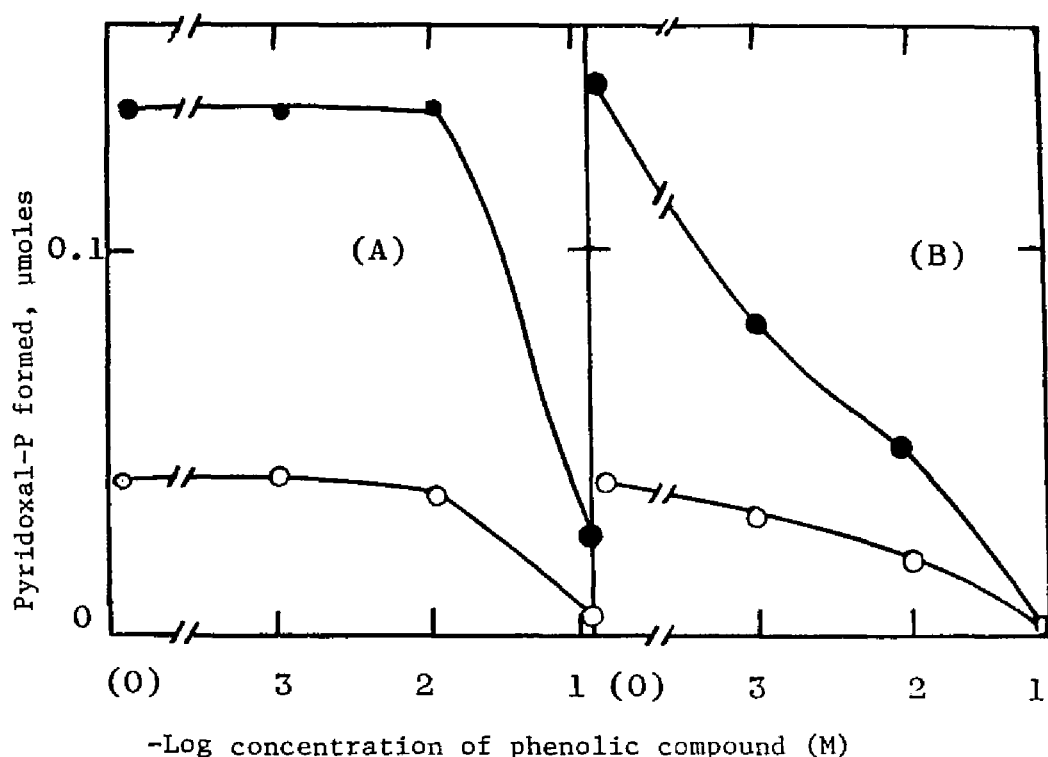


Fig. 4. Effect of Phenolic Compound on Pyridoxine Dehydrogenase

The reaction mixture containing 10 μmoles of pyridoxine, 200 μmoles of Tris-HCl buffer of pH 8.6, 1 μmoles of NADP, 27 mg protein of cell-free extract and phenolic compound was incubated for 2 hr 37°C.

(A) Phenol added, (B) *p*-NP added. (●) NADP added, (○) NADP not added.

by less inhibitory effect of phenol on pyridoxine dehydrogenase.

The effect of metal ion on the enzyme activity was examined, as shown in Table V. Cupric ion remarkably accelerated the dehydrogenation. This phenomenon coincided with the result in Table II, which showed the stimulating effect of cupric ion only on the pyridoxal-P formation.

Table V. Effect of Metal Ion on Pyridoxine

Dehydrogenase	
Metal ion	Pyridoxal formed
	(Relative activity)
None	(100)
Cu ²⁺	421
Al ³⁺	200
Mg ²⁺	100
Zn ²⁺	63
Ni ²⁺	43
MoO ₄ ²⁻	180
Co ²⁺	98
Mn ²⁺	98
(EDTA)	43

The reaction was carried out under the conditions as described in Table IV with an addition of 10⁻³M metal ion and 11.3 mg protein of cell-free extract.

DISCUSSION

The enzymatic conversion of free forms of vitamin B₆ to the active form was demonstrated in the primitive state of the studies on vitamin B₆ function.^{19,114}) Subsequently, a few studies on the formation of pyridoxal-P were reported with the growing cultures of microorganisms without the addition of pyridoxine derivative, though the amount of pyridoxal-P formed was found to be rather small.¹⁶²⁻¹⁶⁴) The author has demonstrated the formation of pyridoxine-P from pyridoxine by the transphosphorylation with fungal and bacterial cell or cell-free extract system in previous sections. Under the

alkaline conditions with the intact cell system of *Escherichia freundii* K-1, it was found that no small amount of pyridoxal-P was formed with the addition of pyridoxine and phosphoryl donor substrates through the transphosphorylation and that phenyl-P was a more suitable phosphoryl donor for pyridoxal-P formation than *p*-NPP which was the most excellent donor in the transphosphorylation of pyridoxine. The amount of pyridoxal-P formed reached a maximum value of 0.4 mg from 2 mg pyridoxine under the appropriate conditions.

Regarding the conversion of pyridoxine to pyridoxal-P, recent studies have shown the presence of two pathways.⁵⁴⁾ One of them is that pyridoxine is phosphorylated after being oxidized to pyridoxal, another is that pyridoxine is oxidized to pyridoxal-P after being phosphorylated to pyridoxine-P. In the cells of *Escherichia freundii* K-1, the presences of the enzyme activities concerning the oxidation of pyridoxine or pyridoxine-P was recognized. It was also observed that these enzymes had pH optima at alkaline range. Pyridoxine-P oxidase was partially purified in order to remove phosphatase which had also the transphosphorylation activity to pyridoxine. But these separations could not be completed. Pyridoxine dehydrogenase activity in dialyzed cell-free extract could be observed with an addition of NADP. It is well expected that the formation of pyridoxal-P was mainly proceeded *via* pyridoxine-P from pyridoxine, since a considerable amount of pyridoxine-P was formed in the reaction mixture. The study on the properties of the enzymes

concerning pyridoxal-P formation from pyridoxine showed the strong inhibiting effect of *p*-NP on pyridoxine dehydrogenase. Then, this finding raised the possibility that the enzyme might contribute to the over-all reaction for pyridoxal-P formation and differentiate the effect of *p*-NPP and phenyl-P on the pyridoxal-P formation from pyridoxine through the transphosphorylation. Though the activity of the dehydrogenation of pyridoxine and the phosphorylation of pyridoxal were rather weak, the long reaction time employed in this experiment might bring about the difference of pyridoxal-P formation between the addition of phenyl-P and *p*-NPP as phosphoryl donor substrate.

Several inhibition studies on pyridoxine dehydrogenase^{70,71)} and pyridoxine-P oxidase^{75,86~89)} have been reported. The phenolic compounds employed here are conceivable as unphysiological ones, but the comparative properties of these enzymes may give the problem for the control mechanism of pyridoxal-P formation from pyridoxine.

SUMMARY

It was found that a considerable amount of pyridoxal-P was formed from pyridoxine through the transphosphorylation by the cells of *Escherichia freundii* K-1 and that phenyl-P was more effective on the formation as phosphoryldonor substrate than *p*-NPP. The effects of these phenolic compounds on the enzymes concerning with the

conversion of pyridoxine to pyridoxal-P were studied. Then it was suggested that the more stimulating effect of phenyl-P on the pyridoxal-P formation than *p*-NPP might be caused by less inhibition of phenol than *p*-NP on pyridoxine dehydrogenase. Calcium salt of the product was isolated and identified.

Chapter II. Purification and Characterization of Pyridoxamine-P-
 α -Ketoglutaric Acid Transaminase from
Clostridium kainantoi

INTRODUCTION

Pyridoxamine-P is the possible intermediate in the biological conversion of pyridoxamine to pyridoxal-P and its natural occurrence has been demonstrated in the course of investigation on the microbiological determination of vitamin B₆.^{21,22,165)} The enzyme, which catalyzed the irreversible oxidation of pyridoxamine-P forming pyridoxal-P in substrate level, has been studied to be the same with pyridoxine-P oxidase.^{75,87)}

While, it has been known that the enzymatic transfer of the amino group from pyridoxamine-P to keto acid occurred with the level of coenzyme bound with various amino acid transaminases¹⁶⁶⁻¹⁶⁸⁾ and other enzymes containing pyridoxal-P as the prosthetic group.¹⁶⁹⁾ The nonenzymatic transamination in the presence of metal ion has been also studied relating with the mechanism of enzymatic transamination.^{52,170,171)}

Recently, an enzyme "pyridoxamine-P transaminase (pyridoxamine-P: 2-oxoglutarate aminotransferase)" which catalyzed the transamination between pyridoxamine-P and α -ketoglutaric acid was found in certain strict anaerobic bacteria such as clostridia, which were

lacking pyridoxamine-P oxidizing system.¹⁷²⁾ The enzyme was partially purified from *Clostridium kainantoi* and some properties were investigated roughly.⁹¹⁾

Further purification of pyridoxamine-P transaminase was attempted from *Clostridium kainantoi*, and, then, the further characterization of the enzyme was investigated using the purified enzyme. These results were described in this chapter.

MATERIALS AND METHODS

Microorganisms and Cultures. *Clostridium kainantoi* IFO 3353, which was provided by the Institute of Fermentation, Osaka, was used throughout this work. The culture medium for the organism, which was modified the Speakman's medium,¹⁷³⁾ consisted of 3 g glucose, 0.5 g peptone, 0.3 g K_2HPO_4 , 0.02 g $MgSO_4 \cdot 7H_2O$, 0.001 g $MnSO_4 \cdot xH_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, 0.001 g NaCl, 0.1 g yeast extract and 1.0 g $CaCO_3$ (precipitated) in 100 ml of tap water, pH 7.0. The bacterial cultivation was made anaerobically at 28°C with 10-liter Ehrenmeyer flask contained 9 liters of the medium. The cells were harvested at or near the end of logarithmic phase, 15-20 hr cultivation time, at which the specific and total activities of the enzyme involved in the cells were found to be the highest.

Chemicals. Pyridoxamine-P was prepared from pyridoxal-P by the nonenzymatic transamination with an excess glutamic acid,²¹⁾ followed by the purification with ion exchange column chromatogra-

phy.¹⁷⁴⁾ Hydroxylapatite was prepared according to the method of Tiselius *et al.*¹⁴¹⁾ Other chemicals were obtained from the commercial sources.

Activity Measurement. Enzymatic activity was routinely measured in a system with a final volume of 1.75 ml containing 5 μ moles of pyridoxamine-P, 5 μ moles of α -ketoglutaric acid, 250 μ moles of Tris-HCl buffer, pH 7.0, 50 μ moles of EDTA and enzyme. Incubation was carried out for 60 min at 37°C and the reaction was stopped by the addition of 0.25 ml of 50% trichloroacetic acid. Any precipitate formed was removed by centrifugation and the supernatant fluid was used for the determination pyridoxal-P.

Specific activity of the enzyme is expressed as units per mg of protein where one unit is defined as the amount of protein that forms one μ moles of pyridoxal-P in 60 min at 37°C.

Analytical Method. Pyridoxal-P was determined by phenylhydrazine method.⁷⁵⁾ Protein was determined by the method of Lowry *et al.*¹³⁰⁾

RESULTS AND DISCUSSION

Purification of Pyridoxamine-P Transaminase.

All operations were performed at 0-5°C throughout the purification procedures.

Step I. Preparation of crude extract. The crude extract of *Clostridium kaimantoi* was prepared from cells cultivated on about

500 liters of the culture medium. The cells were harvested by a continuous centrifugation at 10,000 $\times$ g and washed twice with deionized water. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 7.0, and disrupted with Kaijo-Denki 19 Hz ultrasonic oscillator. The cell and cell debris were removed by centrifugation at 12,000 $\times$ g for 30 min. The supernatant solution was then dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01% 2-mercaptoethanol, overnight.

Step II. First ammonium sulfate fractionation. To the dialyzed enzyme solution was added solid ammonium sulfate to 40% saturation, adjusting pH to 7.0 with 10% ammonium hydroxide solution. After stirring for 30 min, the precipitated protein was removed by centrifugation at 10,000 $\times$ g for 20 min and discarded. The ammonium sulfate concentration was then increased to 75% saturation by further addition of solid ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation at 10,000 $\times$ g for 20 min. This process was repeated separately until 7 liters crude extracts were treated and the active precipitates were combined for the further purification. The combined precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.0. The solution was then dialyzed overnight against the same buffer. The final volume was 530 ml.

Step III. Protamine sulfate treatment. Fifty ml of freshly prepared 2.0% protamine sulfate solution neutralized with sodium

hydroxide, was slowly added under stirring to the dialyzed enzyme solution and allowed to stand for 20 min under stirring. The precipitate formed was removed by centrifugation at 12,000xg for 30 min and the resultant supernatant solution was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, throughly for 40 hr under continuous stirring.

Step IV. DEAE-cellulose column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, was plated on the column. The enzyme was eluted with 0.1 M of the same buffer and combined to give 3.5 liters of solution. The elution pattern is shown in Fig. 1. The active fraction was concentrated by the addition of solid ammonium sulfate to 90% saturation. The precipitate obtained by centrifugation at 12,000xg for 30 min was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight.

Step V. Second ammonium sulfate fractionation. The dialyzed enzyme solution of 305 ml was fractionated by solid ammonium sulfate to yield fraction between 0 to 40, 40 to 45, 45 to 50, 50 to 55, 55 to 60, 60 to 65, 65 to 70 and over 70% saturation. pH was kept to 7.0 during the fractionation with 10% ammonium hydroxide solution. The protein precipitated after stirring for 30 min was collected by centrifugation at 12,000xg for 20 min and dissolved in 0.01 M potassium phosphate buffer, pH 7.0. The fractionated solution was dialyzed against the same buffer overnight. Most of the activity

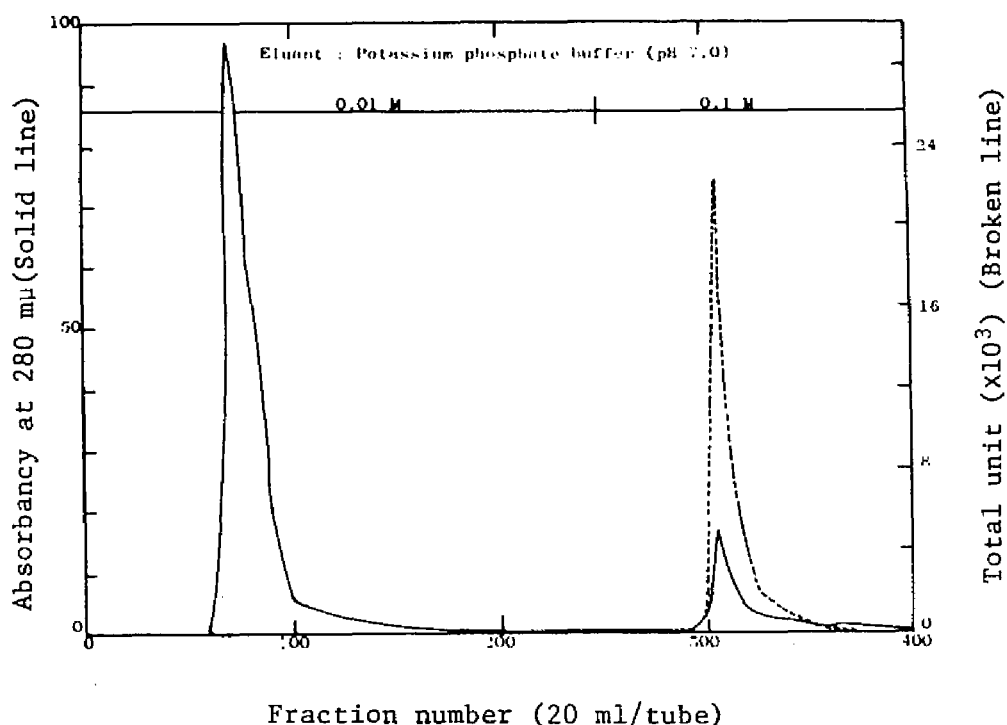


Fig. 1. Chromatography of Pyridoxamine-P Transaminase on DEAE-Cellulose Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

was found to the fraction precipitated between 45-65% saturation. These fractions were combined to yield the solution of 133 ml.

Step VI. First hydroxylapatite column chromatography. The dialyzed enzyme solution was subjected to hydroxylapatite column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, was used to pack a column 6X11cm. The enzyme was placed on the column and eluted with 0.04 M of the same buffer. The active fractions were combined to give 1640 ml of solution. The elution pattern is shown in Fig. 2. The combined

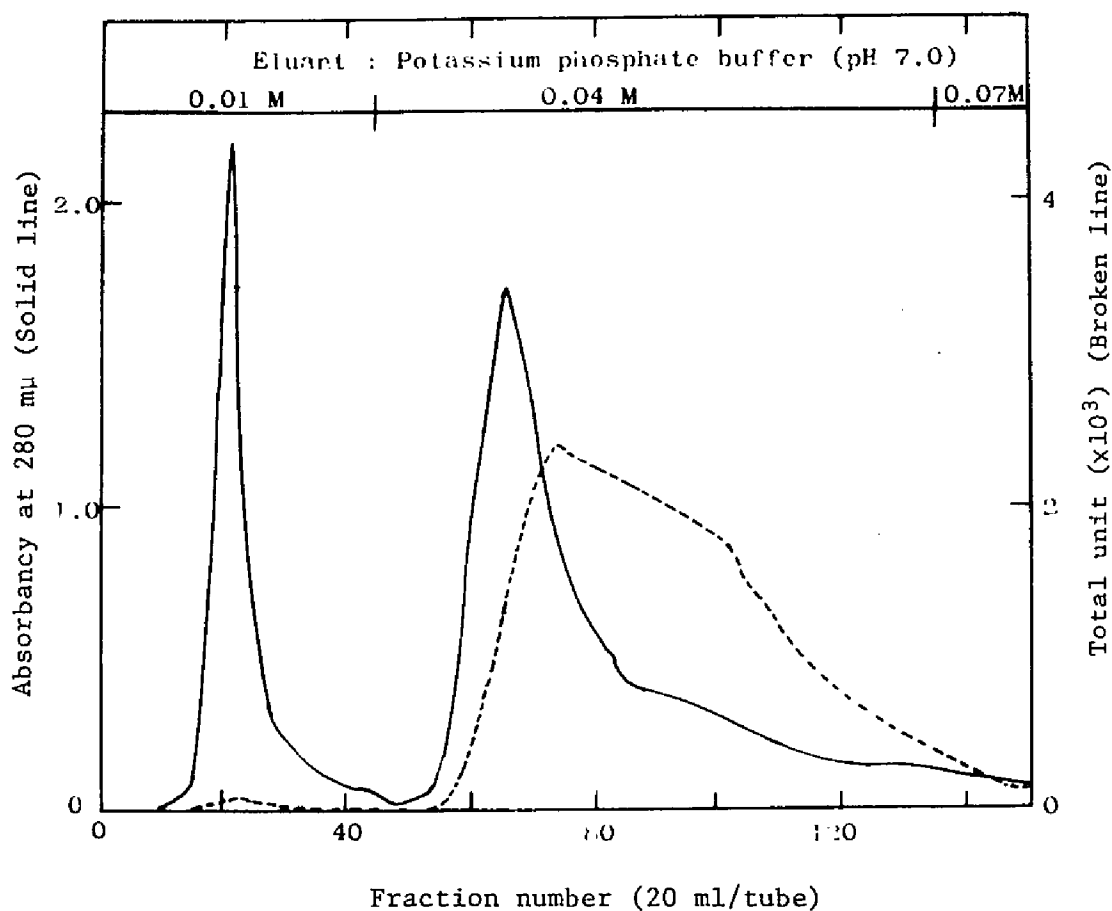


Fig. 2. Chromatography of Pyridoxamine-P Transaminase on Hydroxylapatite Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

solution was concentrated by the addition of solid ammonium sulfate to 80% saturation. The precipitate obtained by centrifugation at 12,000xg for 30 min was dissolved in 0.01 M potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight.

Step VII. DEAE-sephadex column chromatography. The dialyzed enzyme solution (50 ml) was subjected to DEAE-sephadex column

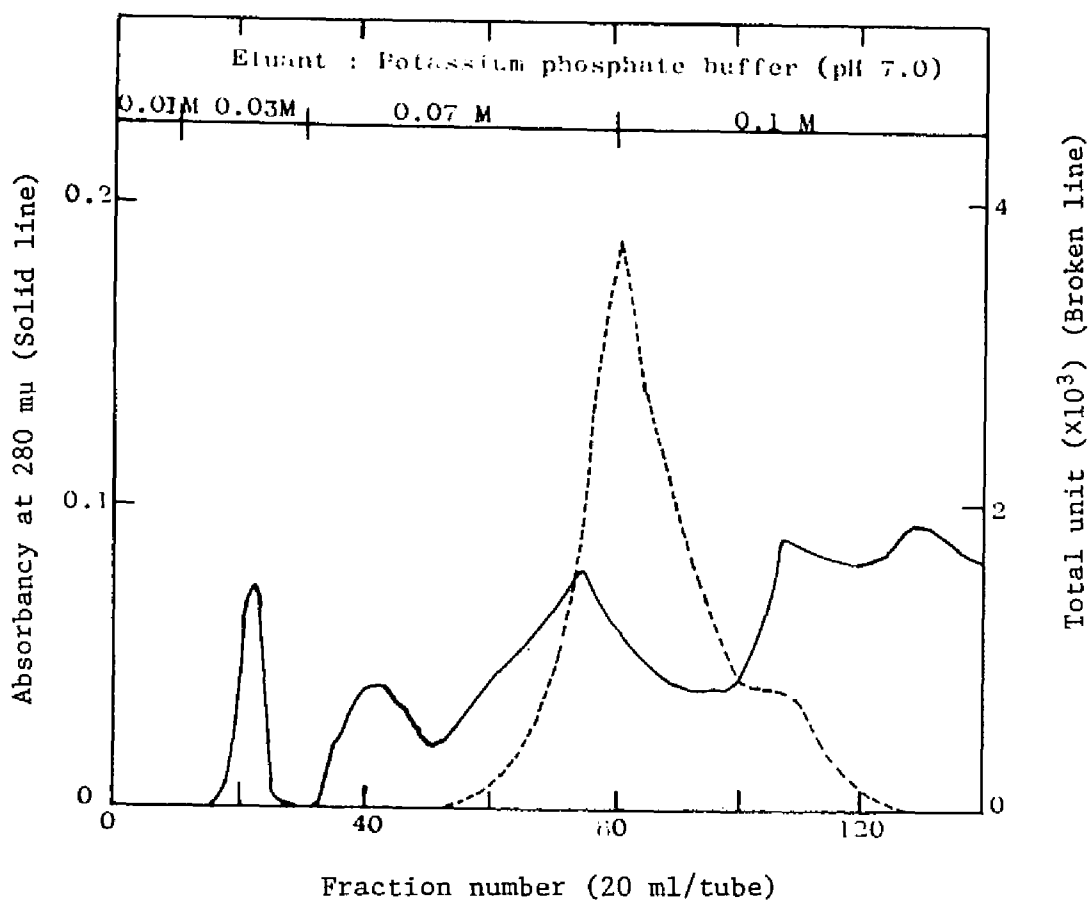


Fig. 3. Chromatography of Pyridoxamine-P Transaminase on
DEAE-Sephadex Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, was used to pack a column 3x75cm. The enzyme solution was placed on the column and the column was washed with 0.03 M of the same buffer. The enzyme was subsequently eluted with 0.07 M to 0.1 M of the same buffer and combined to give 1025 ml of solution. The elution pattern is shown in Fig. 3. The active

fraction was concentrated by the dialysis against the super-saturated ammonium sulfate solution, adjusting pH to 7.0 with 10% ammonium hydroxide solution overnight. The precipitate formed was collected by centrifugation at 12,000Xg for 30 min and dissolved in 0.01 M potassium phosphate buffer, pH 7.0. The solution was dialyzed against the same buffer overnight and slight precipitate appeared was discarded off by centrifugation.

Step VIII. Second hydroxylapatite column chromatography.

The dialyzed enzyme solution (35 ml) was subjected to hydroxylapatite column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, was used to pack a column 5X5cm. The dialyzed enzyme was placed on the column and the column was washed with 0.03 M of the same buffer. The enzyme was then eluted with 0.07 M of the same buffer and combined to give 200 ml of solution. The elution pattern is shown in Fig. 4. The active fraction was concentrated by the dialysis against the super-saturated ammonium sulfate solution, adjusting pH to 7.0 with 10% ammonium hydroxide solution, overnight. The precipitate formed was collected by centrifugation at 12,000Xg for 30 min and dissolved in small volume of 0.01 M potassium phosphate buffer, pH 7.0. The solution was dialyzed against the same buffer overnight.

Step IX. Sephadex G-200 column chromatography. The dialyzed enzyme solution was subjected to sephadex G-200 column chromatography. The absorbent equilibrated with 0.01 M potassium phosphate

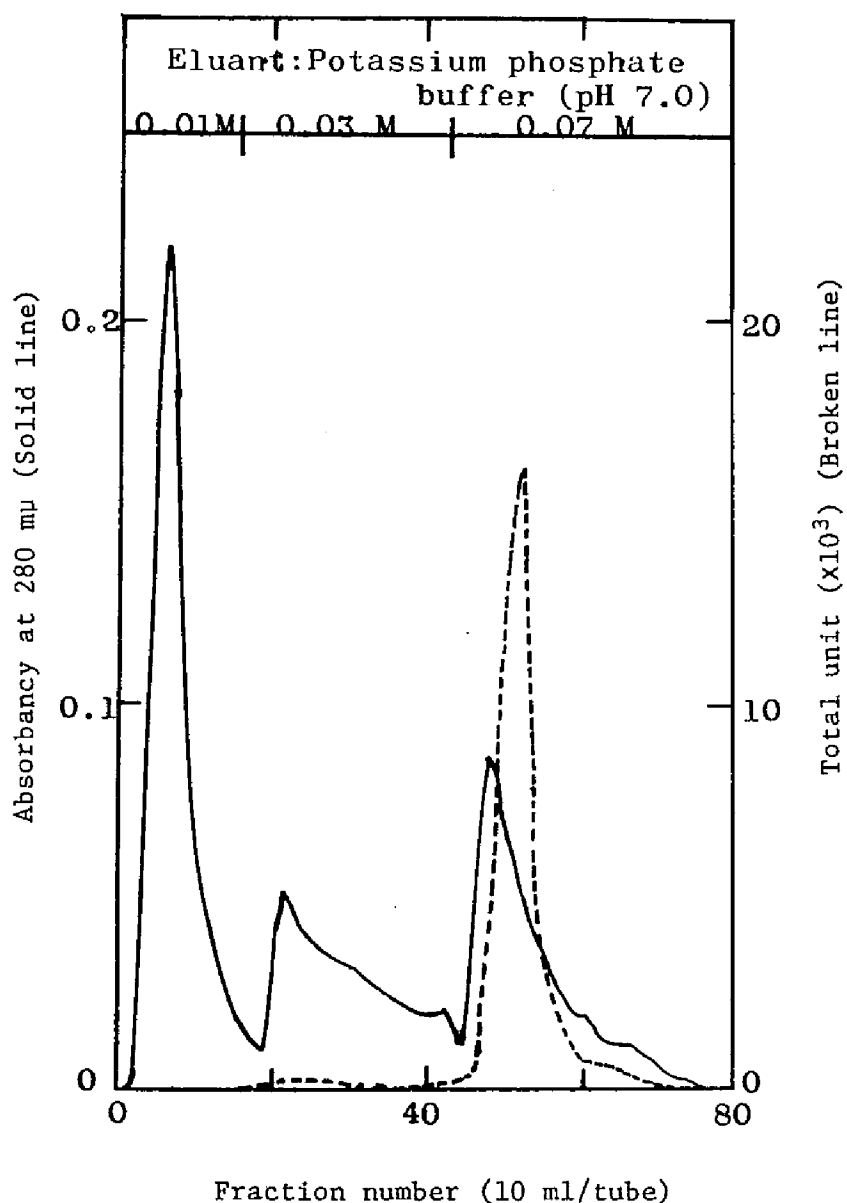


Fig. 4. Chromatography of Pyridoxamine-P Trans-aminase on Hydroxylapatite Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

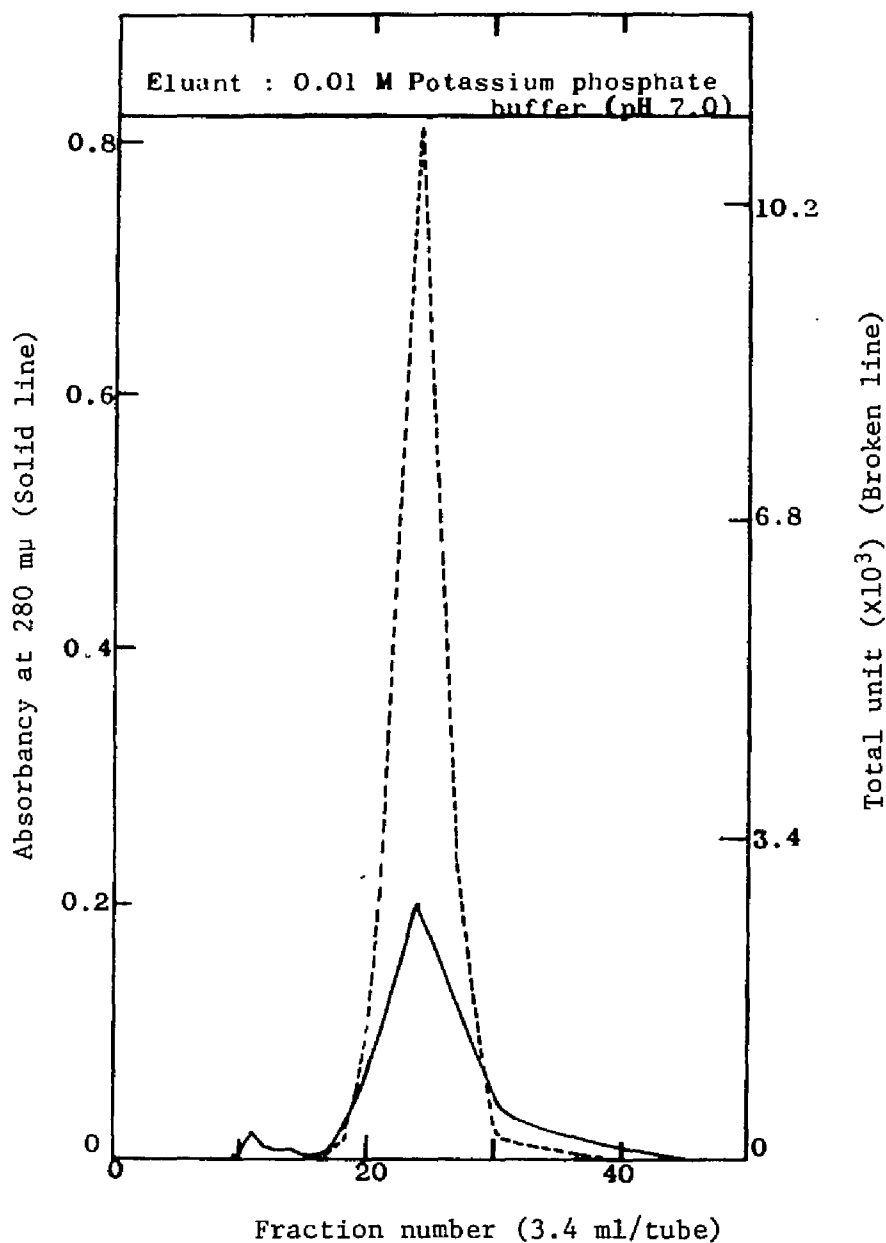


Fig. 5. Chromatography of Pyridoxamine-P Trans-aminase on Sephadex G-200 Column

Chromatography was performed as stated in the text, and the enzyme activity was assayed under the standard assay conditions.

buffer, pH 7.0, was used to pack a column 1.2X110cm. The enzyme solution was placed on the column and eluted with the same buffer. The elution pattern is shown in Fig. 5. The most active fractions were combined to give 26 ml of solution and concentrated by collodion bag for 4 hr to 0.7 ml. The purified enzyme preparation obtained showed approximately 2700-fold specific activity to the original extract and had a slight yellow color. The crystalline enzyme could not be obtained by an addition of solid ammonium sulfate to the preparation.

The results of the purification procedure are summarized in Table I.

Properties of the Purified Enzyme.

Ultracentrifuge analysis. The analysis of the purified enzyme preparation showed a single and symmetric schlieren peak in the ultracentrifuge by Spinco model E ultracentrifuge operating 59,780 rev./min as shown in Fig. 6. But the further ultracentrifugal analysis of the preparation could not be accomplished for the too small content of protein.

Absorption spectrum. The absorption spectrum of purified pyridoxamine-P transaminase was taken with a Shimazu Model MPS-50 L recording spectrophotometer. As shown in Fig. 7, the enzyme in 0.01 M potassium phosphate buffer, pH 7.0, exhibited the absorption maxima near at 235 m μ and 280 m μ over the wave length investigated. The spectrum did not appeared the significant change by an addition

Table I. Purification of Pyridoxamine-P Transaminase

Step	Fraction	Total protein	Total activity	Specific activity
I	Cell-free extract	141,100mg	980,400	6.2
II	Ammonium sulfate	42,900	401,100	9.4
III	Protamine treatment	37,200	398,000	10.7
IV	DEAE-cellulose	9,300	300,000	34.8
V	Ammonium sulfate	3,400	168,000	37.4
VI	Hydroxylapatite	1,280	103,000	80.5
VII	DEAE-sephadex	42.7	70,700	1,650
VIII	Hydroxylapatite	8.8	75,400	8,560
IX	Sephadex G-200	3.0	50,900	16,850

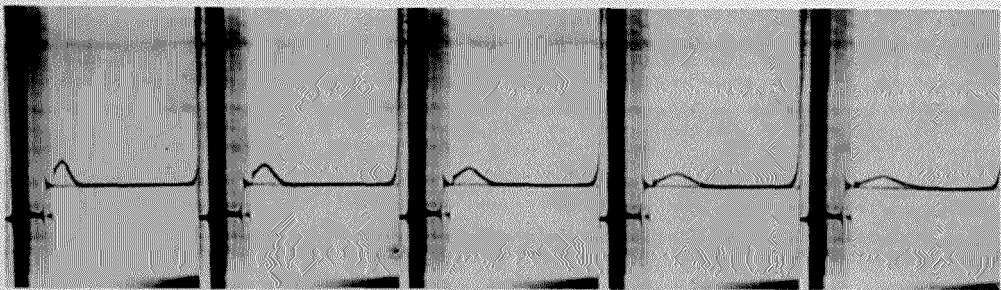


Fig. 6. Ultracentrifugal Analysis of Purified Pyridoxamine-P Transaminase

Schlieren patterns after (left to right) 26, 34, 42, 58, 66 min at 59,780 rev./min in the Spinco model E analytical ultracentrifuge. Protein, 0.43% in 0.01 M potassium phosphate buffer of pH 7.0.

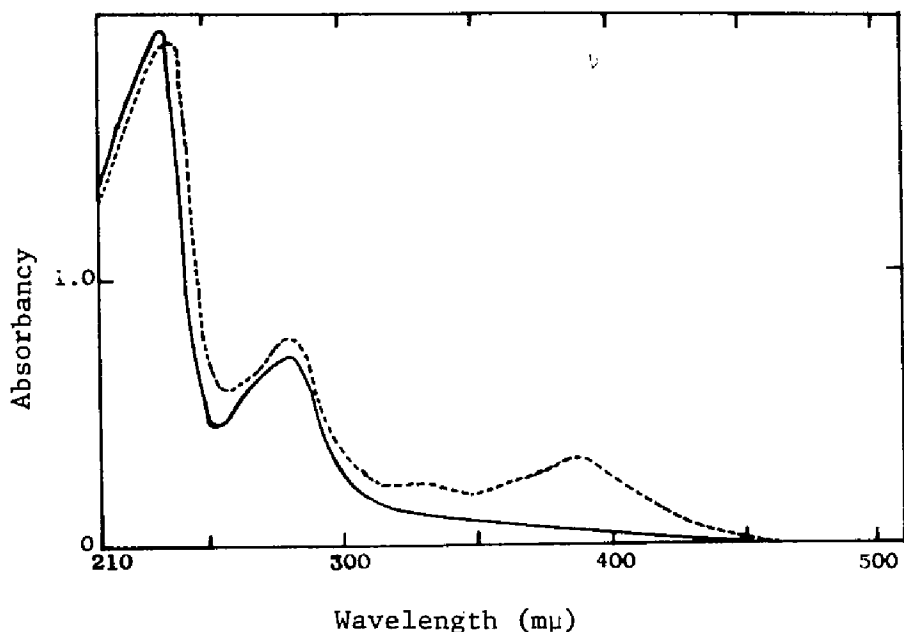


Fig. 7. Absorption Spectrum of Purified, Pyridoxamine-P
Transaminase

The enzyme solution contained 900 μg protein per ml of 0.01 M potassium phosphate buffer, pH 7.0, with addition of 0.04 μmoles pyridoxal-P per ml(-----) and no addition(——).

of pyridoxal-P, except additive absorption peak of pyridoxal-P itself, *i.e.*, 324 $\text{m}\mu$ and 388 $\text{m}\mu$. It has been recently demonstrated that the enzymes contained pyridoxal-P as the prosthetic group showed the typical absorption maximum near at 410 $\text{m}\mu$ by the formation of aldimine bond between pyridoxal-P and lysine in apoenzyme¹⁷⁵⁾ These pyridoxal-enzymes were also classified to three groups¹⁷⁶⁾ which were 1) cystathionase type¹⁷⁷⁾ 2) glutamic acid-oxalacetic acid transaminase type¹⁷⁸⁾ and 3) phosphorylase type¹⁷⁹⁾ Then it appeared that pyridoxamine-P transaminase may not contain pyridoxal-P as a

prosthetic group of the enzyme. While, the extinction of absorption peak at 235 m μ was also observed by an addition of α -ketoglutaric acid to the enzyme solution. The same observation could not be obtained with the absorption peak at 220 m μ of bovine albumin.

Effect of pH. The result of the experiment on the effect of pH on the enzyme activities showed the optimum pH to be 8.0. The value somewhat differed from that obtained by the experiment at 50°C.⁹¹⁾

Effects of metal ion and inhibitor. The effects of metal ion and inhibitor on pyridoxamine-P transaminase were investigated with the exception of EDTA from the standard assay conditions. In the presence of 1 mM of Cu²⁺ and Ni²⁺, a considerable nonenzymatic transamination forming pyridoxal-P was observed. But any metal ion did not accelerate the enzymatic transamination. SH-inhibitor such as PCMB, phenylmercuric acetate and Hg²⁺ showed no inhibition effect. Diisopropylphosphofluoride, which was known as a specific inhibitor against the hydrolase having serine in the active site, inhibited completely at the concentration of 3x10⁻⁴M. This phenomenon has not yet been recognized with other transaminases.

Effect of amino compound on the reaction. The addition of various sort of amino compounds effected pyridoxal-P formation as shown in Table II. These effects may be caused by the exception of the reaction product, pyridoxal-P, forming the corresponding Schiff base. The different effects between D- and L-isomer of amino acids,

Table II. Effect of Amino Compound on

Pyridoxamine-P Transaminase	
Amino compound	Pyridoxal-P formed
	(Relative activity)
None	(100)
Glycine	137
γ -Aminobutyric acid	177
L-Glutamic acid	166
D-Glutamic acid	95
L-Cysteine	64
D-Cysteine	135
L-Methionine	114
D-Methionine	127
L-Asparagine	112
D-Asparagine	112
L-Ornithine	112
DL-Ornithine	112
L-Lysine	105
DL-Lysine	104

The reaction mixture containing 1.25 μ moles of pyridoxamine-P, 5 μ moles of α -ketoglutaric acid, 250 μ moles of Tris-HCl buffer, pH 8.0, 3 μ moles of amino compound and 1 μ g of purified enzyme protein in a total volume of 1.5 ml was incubated for 30 min at 37°C.

such as glutamic acid and cysteine, are interest in the relation of the metabolisms of vitamin B₆ and D-amino acid in clostridia.

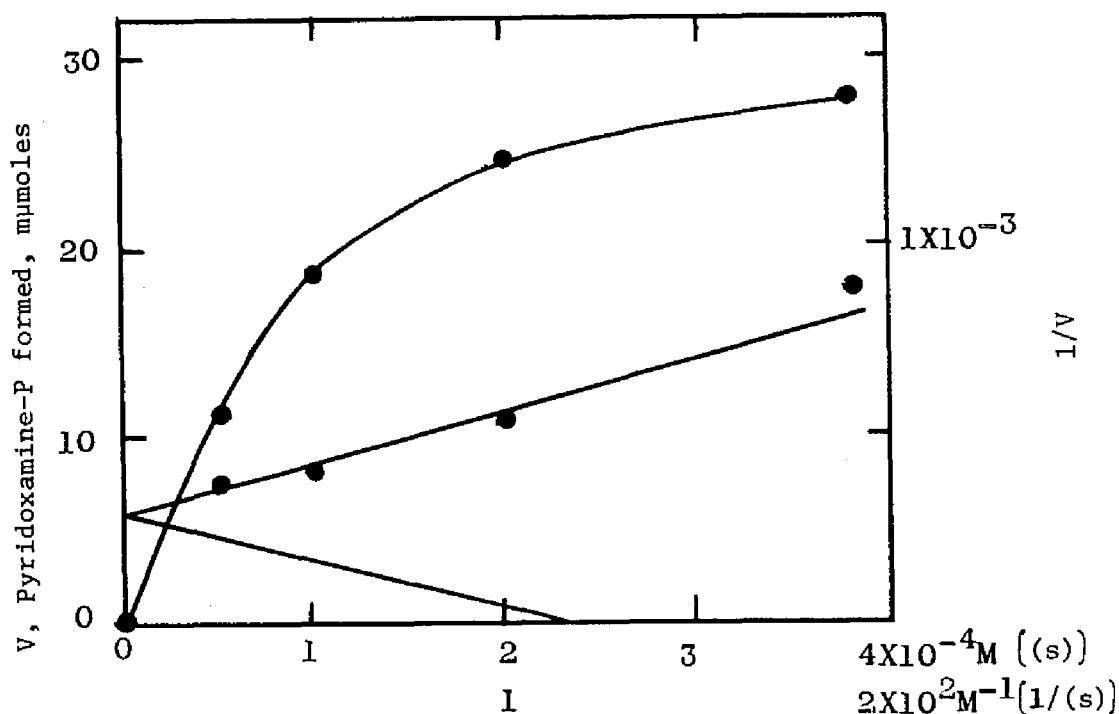


Fig. 8. Effect of D-Glutamic Acid Concentration on the Formation of Pyridoxamine-P by Pyridoxamine-P Transaminase

The reaction mixture containing 0.21 μ moles of pyridoxal-P. 50 μ moles of EDTA, 100 μ moles of potassium phosphate buffer, pH 8.0 and 2 μ g of purified enzyme protein in a total volume of 1.75 ml was incubated for 4 min at 37°C.

Formation of pyridoxamine-P from pyridoxal-P by the enzyme.

For the investigation of reversibility of pyridoxamine-P transaminase, the formation of pyridoxamine-P from pyridoxal-P was tested by the determination of pyridoxal-P in the reaction mixture. The more decrease of pyridoxal-P was observed by an addition of D-glutamic acid as amino donor substrate than L-glutamic acid. The formation of pyridoxamine-P and α -ketoglutaric acid was also confirmed by paper

chromatography. The effect of D-glutamic acid concentration on the inverse reaction was shown in Fig. 8. The Michaelis constant for D-glutamic acid as calculated by the method of Lineweaver and Burk¹⁴³⁾ was found to be $9 \times 10^{-3} \text{M}$. Though, the K_m value for D-glutamic acid is higher than that for pyridoxamine-P ($4.0 \times 10^{-6} \text{M}$), α -ketoglutaric acid ($1.5 \times 10^{-5} \text{M}$) under the standard assay conditions and pyridoxal-P (*ca.* 10^{-5}M) under the same condition of Fig. 8 with 3.5 μmoles of D-glutamic acid, pyridoxamine-P formation from pyridoxal-P and D-glutamic acid by the enzyme may give a significant problem in the vitamin B₆ metabolism.

SUMMARY

The enzyme which catalyzed the transamination between pyridoxamine-P and α -ketoglutaric acid was purified from the cell-free extract of *Clostridium kainantoi*. The purification procedures involved ammonium sulfate fractionation, protamine sulfate treatment and DEAE-cellulose, hydroxylapatite, DEAE-sephadex and Sephadex G-200 column chromatographies. The purified enzyme which had approximately 2700-fold specific activity against the original extract showed the single schlieren pattern in ultracentrifuge. Several properties of the purified enzyme were also investigated. It was recognized that the reaction was accelerated by the addition of amino acid and inhibited by diisopropylphosphofluoride. The formation of pyridoxamine-P from pyridoxal-P was observed by an

addition of D-glutamic acid as amino donor substrate. It was also demonstrated that the purified pyridoxamine-P transaminase seemed not to contain pyridoxal-P as the prosthetic group.

Chapter III. Microbial Formation of a New Vitamin B₆ Derivative, Pyridoxine-G

Section 1. Formation of Pyridoxine-G with *Sarcina lutea*

INTRODUCTION

Recently, the anabolic and catabolic transformation of vitamin B₆ have been investigated with microorganisms and mammalian tissues by several groups of workers. On the enzymatic conversion of pyridoxine to the active form of vitamin B₆, pyridoxal-P, the presences of several enzymes have been demonstrated^{54,155)} On the other hand, the bacterial degradation of pyridoxine has been studied by Snell and coworkers⁶⁹⁾ with certain soil pseudomonads which were grown in the medium containing pyridoxine as the sole source of carbon and nitrogen.

However, the work has scarcely been reported on the biological conversion of pyridoxine or its family to their derivatives which conjugated with other organic compounds.

During the course of the investigations on the metabolism of vitamin B₆ in microorganisms, one derivative of vitamin B₆ was detected in the cultural filtrate of certain bacteria which were grown in the medium containing sucrose as carbon source and pyridoxine. It seemed that the derivative has been synthesized neither biologically nor chemically.

This section describes the isolation of the new compound and its characteristic to be consisted of pyridoxine and glucose. The several conditions on the formation of pyridoxine-G were also investigated with *Sarcina lutea*.

EXPERIMENTAL

Materials. The bacteria used were the strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University. All chemicals were obtained from commercial sources.

Basal Medium. The basal medium for the organisms consisted of 2.0 g sucrose, 1.0 g peptone, 0.5 g K_2HPO_4 , 0.1 g KH_2PO_4 , 0.05 g $FeSO_4 \cdot 7H_2O$, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot xH_2O$, 0.005 g $CaCl_2 \cdot 2H_2O$, and 0.05 g yeast extract in 100 ml of tap water, pH 7.0. Cultures were carried out with 5 ml of the medium placed in the test tubes and kept on a reciprocal shaker at 28°C for 3 to 5 days.

Reaction with Intact Cells of *Sarcina lutea*. *Sarcina lutea* IFO 3232 was grown in 500 ml of the basal medium placed in 2-liter flask under the shaking for 20-40 hr at 28°C. The cells were harvested by centrifugation and washed twice with 0.85% potassium chloride solution, and then used as intact cell preparation. The standard reaction mixture contained 100 mg of neutralized pyridoxine hydrochloride (ca. 600 μ moles as pyridoxine), 250 mg of sucrose, 800 μ moles of potassium phosphate buffer, pH 8.0, and 140 mg as dry

weight of intact cells in a total volume of 5.0 ml. The reaction was carried out with shaking for 25 hr at 28°C and terminated by heating in a boiling water bath for 5 min. The reaction products were determined after removing the cells by centrifugation.

Paper Chromatography and Paper Electrophoresis of Vitamin B₆. Solvent mixtures used for the paper chromatography of vitamin B₆ had the following composition percentage (volume per volume): *n*-propanol, 70; water, 20; acetate buffer of pH 5.0, 10 (Solvent A); or 28% ammonium hydroxide solution, 10 (Solvent B); *n*-butanol saturated with 0.2 M acetate buffer of pH 5.0 (Solvent C). Solvent C was mainly used in the following experiments with ascending chromatography on Toyo Roshi No. 53 filter paper. Paper electrophoretic analysis was carried out at 200 V/cm on Toyo Roshi No. 53 filter paper (15X54cm) with Shiraimatsu high voltage paper electrophoretic apparatus, using 0.2 M acetate buffer of pH 3.5. Vitamin B₆ on paper was detected by Manasuru Light (2535 Å filter) and developed by spraying either with diazotized *p*-AAP followed by drying for 10 min at 60°C¹⁶⁰⁾ or with 0.1% dichloroquinonechlorimide in ethanol followed by dilute ammonium hydroxide solution.¹²¹⁾

Assay of Vitamin B₆ with *p*-AAP Method. The sensitivity of color test with diazotized *p*-AAP was recognized to be greater than that with diazotized sulfanilic acid. The color formed by coupling diazotized *p*-AAP with vitamin B₆ is permanent, whereas the blue indophenol formed by vitamin B₆ and dichloroquinonechlorimide fades

rapidly upon exposure to light. After separation by paper chromatography or electrophoresis, vitamin B₆ compounds on the paper were determined by diazotized *p*-AAP method¹²⁰⁾ with slight modification as follows. The corresponding spot detected by Manasuru Light was cut out and eluted with 2 ml of 99% ethanol and 2 ml of 25% sodium acetate solution for 60 min at room temperature under occasional stirring in test tube. To the extract was added 0.2 ml of diazo reagent which was prepared by vigorously mixing 5 ml of *p*-AAP solution (3.18 g of *p*-AAP was dissolved in 45 ml of concentrated hydrochloric acid and then diluted to 500 ml with deionized water) and 25 ml of 4.5% sodium nitrate solution at room temperature, and then immediately mixed. The color was allowed to develop for over 3 min at room temperature before reading the absorbancy at 470 mμ in photoelectric photometer. The color development was stable for 60 min. The method was the most effective in determining pyridoxine on the paper chromatogram in the range 0 to 50 μg as shown in Fig. 1. The aqueous sample to be assayed was pipetted into test tube and diluted to 1.0 ml. To the tube was then added 2 ml of 99% ethanol followed by an addition of 1 ml of 50% sodium acetate. The diazo reagent was added and the color was developed as described above.

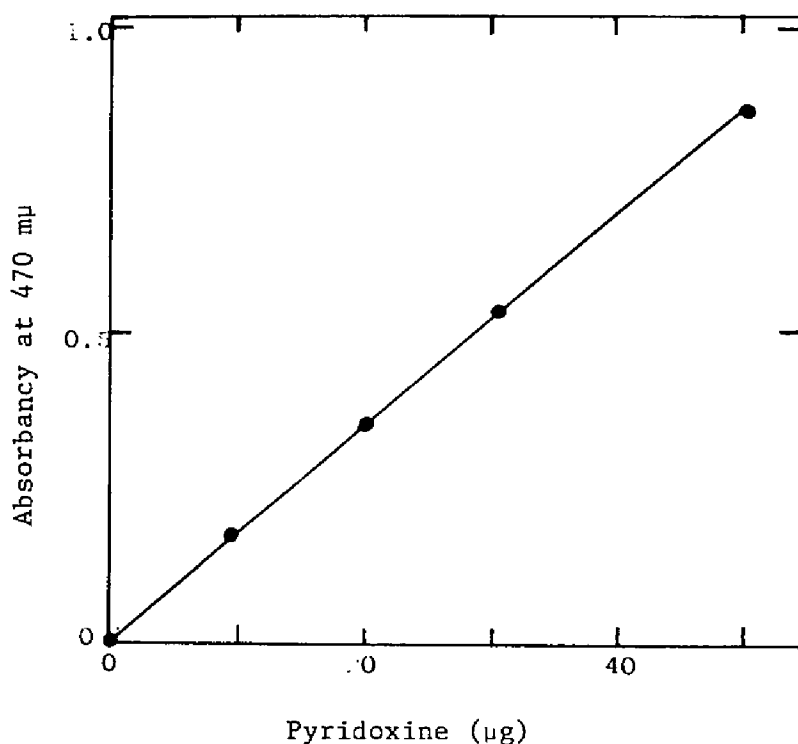


Fig. 1. Relation of Pyridoxine Concentration and
Color Development with *p*-AAP Method

RESULTS

Identification of a New Compound, Pyridoxine-G.

Firstly, it was found on paper chromatogram that a new derivative of vitamin B₆ was formed in the culture filtrate of certain microorganisms which were grown in the medium containing sucrose as carbon source and pyridoxine. The isolation and characterization of the compound were attempted as follows. Then the compound appeared to be a new derivative consisting of pyridoxine and glucose, pyridoxine-G.

Isolation of pyridoxine-G. The reaction mixture containing

20 g of neutralized pyridoxine hydrochloride, 50 g of sucrose, 100 mmoles of phosphate buffer, pH 8.0, and 28 g as dry weight of intact cells of *Sarcina lutea* IFO 3232 in a total volume of 1000 ml was incubated with shaking for 25 hr at 28°C. The reaction was terminated by heating in a boiling water bath for 5 min. After the removal of the cells by centrifugation, the supernatant solution was concentrated under reduced pressure below 50°C to about one-tenth of its original volume. The concentrated solution was subjected to a paper pile chromatography for the remove of most of pyridoxine. The lower part of the paper pile of Toyo Roshi No. 2 filter paper (9 cm in diameter) was submerged in the sample and then allowed to dry over phosphorus pentoxide under reduced pressure. The chromatography was performed in the solvent system C, which could sufficiently separate pyridoxine and pyridoxine-G on paper chromatogram. The fluorescent zone detected with Manasuru Light which located in 0.10 to 0.25 of R_f values was taken out and extracted with deionized water. The extracts were concentrated under reduced pressure. The condensed syrup was dissolved in a small amount of water and subjected to an active charcoal column chromatography for the remove of sugars and inorganic compounds. The refined absorbent was used to pack a column 2.7 x 32cm. The concentrated solution was placed onto a column after adjusting to pH 3.0 with hydrochloric acid and washed with 500 ml of 0.01 N hydrochloric acid and 1000 ml of deionized water, and then pyridoxine-G was eluted with 50% ethanol¹

containing 2% ammonium hydroxide at a flow rate of 2 ml per min. The active fractions were evaporated to dryness under reduced pressure at 35°C. The resulting residue was dissolved in 50 ml of water and subjected to a Dowex 1X2 (Cl⁻ form) column chromatography. The dissolved preparation was placed onto a column (2.7X32cm) and eluted with 1000 ml of deionized water. The pyridoxine-G fractions were condensed under reduced pressure to about 30 ml and subjected to a Sephadex G-15 column chromatography. The gel was used to pack a column 2.2X100cm. Every 3 ml of pyridoxine-G solution was applied to the column and eluted with deionized water at a flow rate of 12 ml per hr. A typical elution pattern is shown in Fig. 2. The contaminating pyridoxine and its unidentified derivative in small amount could be removed from pyridoxine-G in this procedure. The purified pyridoxine-G fractions were concentrated under reduced pressure to a syrup of about 10 ml, to which 30 ml of ethanol was added and warmed in a hot water bath. There appeared white precipitates on cooling, which were separated from mother liquor by filtration and dried over phosphorus pentoxide. This powder obtained had a purity of 70% and was found paper chromatographically to be free from pyridoxine and sugars. The powdered preparation was used for further characterization of pyridoxine-G.

Some attempts to isolate pyridoxine-G from the reaction mixture by cation exchange resins such as Amberlite IRC-50³⁰⁾ and Dowex 50W¹⁰⁰⁾ failed to play sufficient role for the separation of

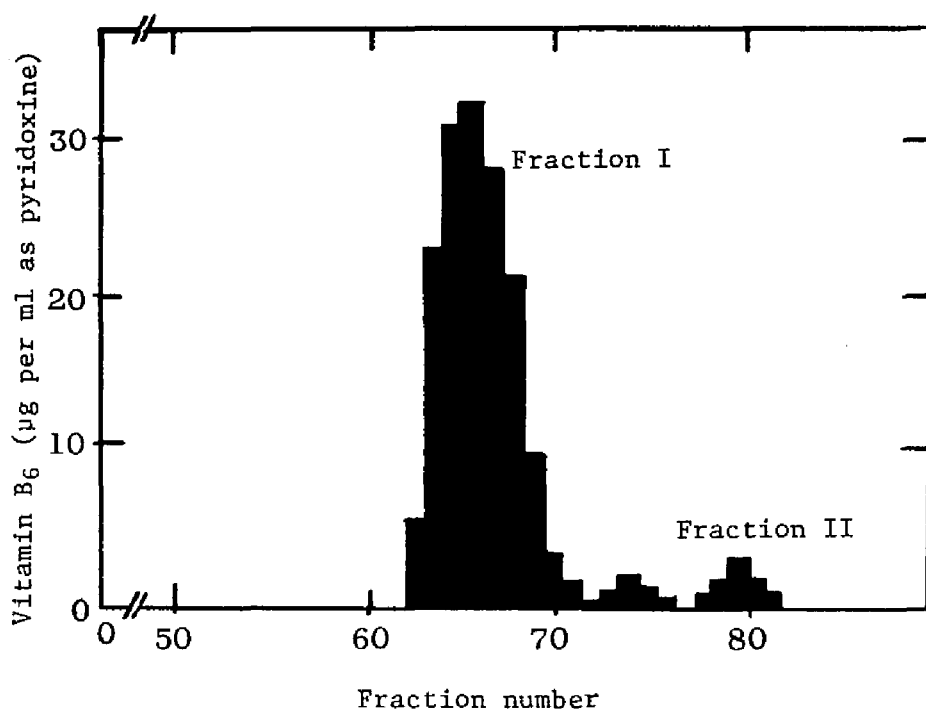


Fig. 2. Separation of Pyridoxine-G and Pyridoxine on
Sephadex G-15 Column Chromatography

Chromatography was performed as described in the text.
Fraction I; Pyridoxine-G, Fraction II; Pyridoxine.

pyridoxine-G from pyridoxine, because of an analogy of these electronic properties and of relative small amount of pyridoxine-G.

Characterization of pyridoxine-G. With the powder of pyridoxine-G, the identification of the moieties of sugar and vitamin B₆, and the brief characterization were performed.

The results with the employments of diphenylamine method¹⁸⁰⁾ cysteincarbazole-sulfuric acid method¹⁸¹⁾ and Somogyi-Nelson method¹⁸²⁾ revealed that the sugar moiety of pyridoxine-G may be not ketose but aldose, suggesting the possibility of glucose. Table I shows the

Table I. Paper Chromatography of Sugars

Sugar	Solvent system	
	Phenol:1% NH_4OH (4:1)	Pyridine: H_2O : NH_4OH (7:2:1)
Hydrolyzate of Pyridoxine-G*	0.41**	0.51
Glucose	0.41	0.52
Fructose	0.54	0.55
Galactose	0.45	0.51
Mannose	-	0.56

* The hydrolysis of pyridoxine-G was performed in 0.055 N sulfuric acid at 120°C for 2.5 hr and followed by neutralization with sodium carbonate solution.

** Sugars were detected by aniline hydrogen phthalate (0.93 g of aniline and 1.66 g of phthalic acid dissolved in water-saturated *n*-butanol) and presented with the R_f values.

result of paper chromatography of hydrolyzate. The R_f values of the sugar moiety favored with the presumption to be glucose. As shown in Fig. 3, the absorption spectrum of the sugar moiety by carbazole-sulfuric acid method¹⁸³) coincided with that of glucose. These results made possible to conclude that the sugar moiety of pyridoxine-G was glucose.

On the other hand, pyridoxine moiety of pyridoxine-G was detected and identified by paper chromatography with free forms of vitamin B₆. Results in Table II show that vitamin B₆ of hydrolyzed pyridoxine-G is pyridoxine.

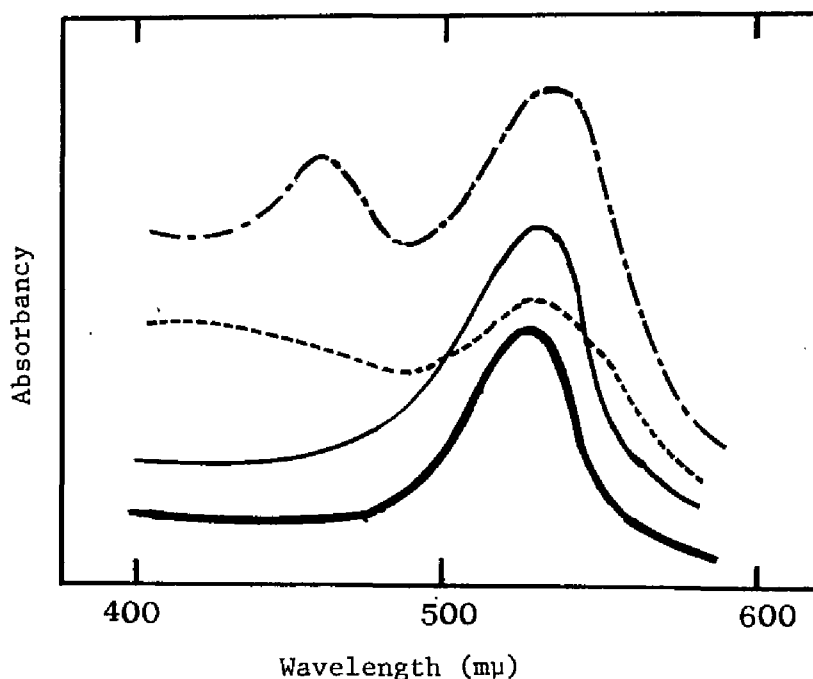


Fig. 3. Absorption Spectra of Sugars by Carbazole-Sulfuric
Acid Method.

The absorption spectrum was recorded on Shimadzu Model MPS-50 L recording spectrophotometer.

————; Pyridoxine-G, ———; Glucose, ———; Fructose, ———; Galactose.

The component of the new derivative of vitamin B₆ was estimated to be pyridoxine and glucose as described above. Subsequently, the amounts of each components were quantitatively estimated by Somogyi-Nelson method and Anthron method¹⁸⁴⁾ for glucose, and by diazotized *p*-AAP method for pyridoxine. These results indicated that glucose was conjugated with pyridoxine at a molar ratio of one to one. The position of glucose where pyridoxine conjugation took place was considered to be through glucosidic linkage, since pyridoxine-G had

Table II. Paper Chromatography of Pyridoxine and
Its Derivatives

Vitamin B ₆	Solvent	Solvent	Solvent
	A*	B	C
Hydrolyzate of Pyridoxine-G**	0.78***	0.77	0.69
Pyridoxine	0.78	0.77	0.69
Pyridoxal	0.80	0.79	0.55
Pyridoxamine	0.44	0.81	0.14
(Pyridoxine-G)	0.54	0.54	0.20

* Solvent systems were described in the text.

** The hydrolysis of pyridoxine-G was performed as same as Table I.

*** Vitamin B₆ was detected by diazotized *p*-AAP and presented the R_f values.

no reducing ability. While, there are four possible positions of pyridoxine involving the conjugation of glucose, *i.e.*, the 1 position, hydroxyl group at the 3 position, and hydroxymethyl groups at the 4 and 5 positions. As shown in Fig. 4, the ultraviolet absorption spectra of pyridoxine-G at acidic, neutral and alkaline pH, respectively, showed the changes of the position of maxima and minima in the resemblance with that of pyridoxine. The similarity of electrophoretic properties of pyridoxine-G to pyridoxine with various pH was also obtained. These data can be explained by assuming that the 1 and 3 positions of pyridoxine which have the electric charge may not be blocked by glucose. Furthermore,

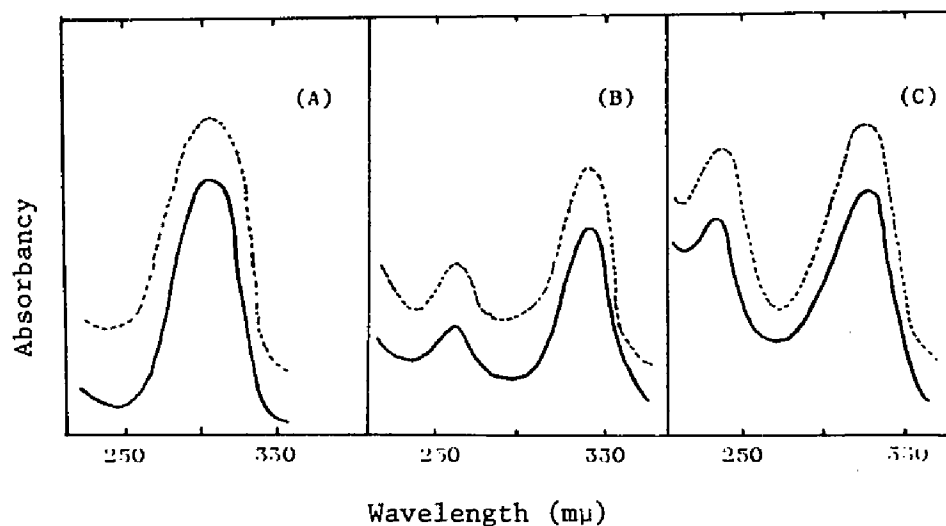


Fig. 4. Ultraviolet Absorption Spectra of Pyridoxine-G and Pyridoxine

The absorption spectrum was recorded by the same method in Fig. 3 in 0.1 N hydrochloric acid (A), 0.1 M potassium phosphate buffer, pH 6.8, (B) and 0.1 N sodium hydroxide (C).

—; Pyridoxine-G, -----; Pyridoxine.

pyridoxine-G gave a blue color with dichloroquinonechlorimide and reddish orange color with diazotized *p*-AAP, suggesting the existence of free hydroxyl group at 3 position. Then, the position where glucose conjugation takes place may be thought to be in the hydroxymethyl group at the 4 or 5 position of pyridoxine.

Formation of Pyridoxine-G.

Table III shows the distribution of the activity forming pyridoxine-G in several bacteria. Strains belonging to the genus *Sarcina* had higher activities. Several conditions of pyridoxine-G formation were investigated with the growing and resting cells of

Table III. Activity of Pyridoxine-G Formation in

Bacteria	
Strain	Pyridoxine-G formed (μ g per ml as pyridoxine)
<i>Escherichia coli</i> K 12 IFO 3208	0*
<i>Aerobacter aerogenes</i> IFO 3320	0
<i>Achromobacter aceris</i> IFO 3166	0
<i>Flavobacterium fuscum</i>	12
<i>Bacillus subtilis</i> IAM 1193	0
<i>Sarcina lutea</i> Schroeter IFO 3232	217
<i>Sarcina lutea</i> IAM 1099	202
<i>Sarcina marginata</i> IFO 3066	125
<i>Sarcina aurantiaca</i> IFO 3064	0
<i>Pseudomonas fragi</i> IFO 3458	0
<i>Proteus vulgaris</i> IFO 3167	0

* The values described as zero involved the formation of pyridoxine-G in trace amount.

The bacteria were grown in basal medium added pyridoxine of 1 mg per ml. After boiling for 5 min, the product was assayed as described in text.

Sarcina lutea IFO 3232 which was found to be the most active among the strains tested.

Effect of sort of carbon source. In order to know the effect of carbon source on the formation of pyridoxine-G, the cultivations were carried out with various sugars which supported the growth of this organisms. As shown in Table IV, sucrose was found the most effective glucosyl donor substrate, and maltose was about one-fifth as effective as sucrose. Whereas glucose, fructose and their mixture

Table IV. Effect of Carbon Source on Formation
of Pyridoxine-G

Carbon source	Pyridoxine-G formed (μg per ml as pyridoxine)
Sucrose	150
Glucose	0
Fructose	0
Glucose + Fructose	0
Maltose	35
Phenyl- α -glucoside	105
Lactose	0
Trehalose	0
Raffinose	0

The cultivation of *Sarcina lutea* and assay of product were performed by the same method described in Table III.

or other natural saccharides were inactive. Phenyl- α -glucoside which was a synthetic substrate could serve as a effective glucosyl donor substrate. It is apparent that the effective carbon sources were the glucoside consisted of α -D-glucose.

Effect of substrate concentration. The formation of pyridoxine-G with the intact cells was investigated with various concentrations of pyridoxine and sucrose (Fig. 5). The amount of pyridoxine-G formed in the reaction mixture increased with increasing addition of pyridoxine and reached a maximum value of 1300 μg per ml. The product increased linearly till the addition of sucrose at the concentration of 10 mg per ml. Further addition of sucrose resulted

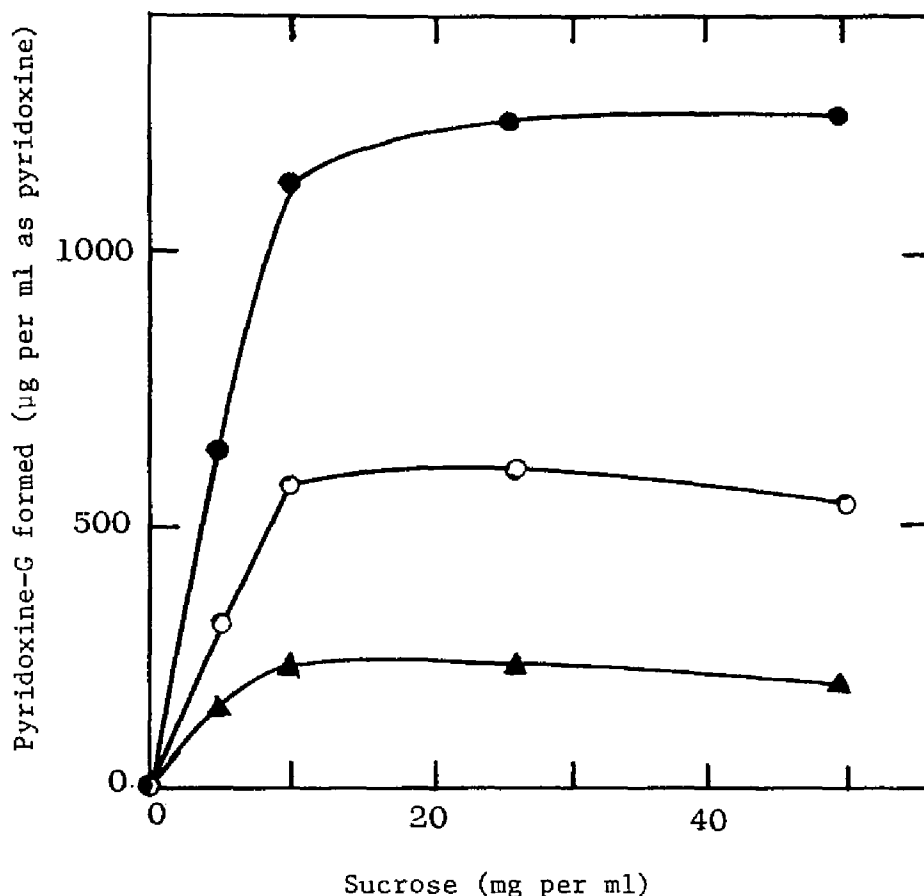


Fig. 5. Effect of Substrate Concentration on Pyridoxine-G Formation

The reaction was carried out under the standard assay conditions with intact cells of *Sarcina lutea* except for the concentration of substrate. Pyridoxine added 20 mg (—●—), 10 mg (—○—) and 5 mg (—▲—) per ml, respectively.

in no appreciable increase of its formation.

Effects of cell concentration and incubation time. The formation of pyridoxine-G with intact cells as functions of cell concentration and incubation time was shown in Fig. 6. The reaction pro-

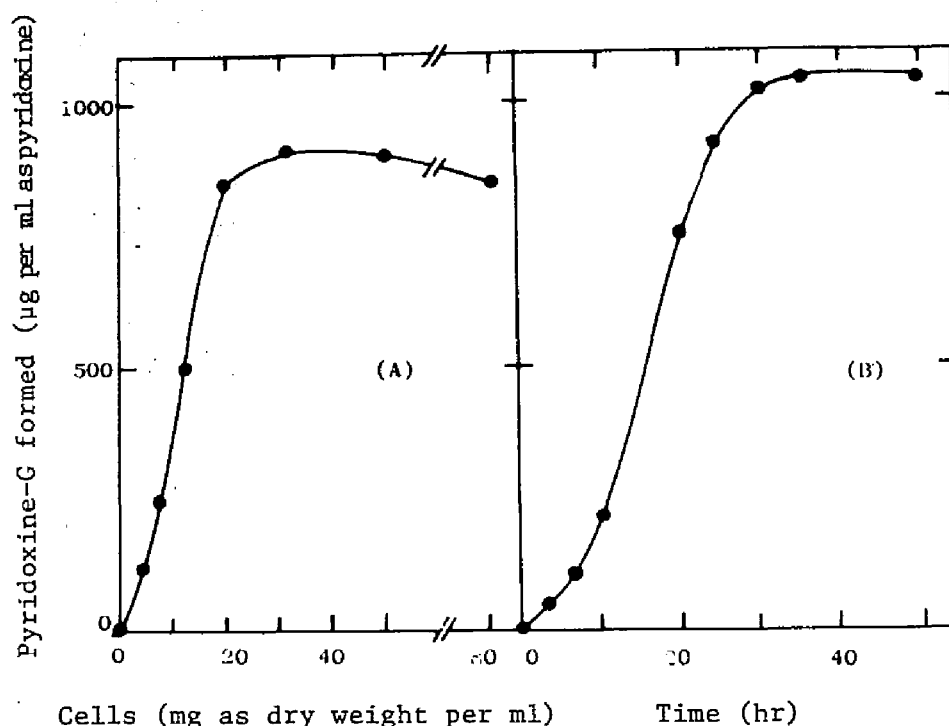


Fig. 6. Effects of Cell Concentration (A) and Incubation Time (B) on Pyridoxine-G Formation

The reaction was carried out under the standard assay conditions with intact cells of *Sarcina lutea* except for cell concentration (A) or incubation time (B).

ceeded in a linear function with the increase of cell concentration under 20 mg cells per ml. The higher cell concentration caused the slight decrease of the amount in the product. The accumulation of pyridoxine-G increased with incubation time and reached a maximum value at about 30 hr. From these data, it seemed that the enzymatic transglucosidation between pyridoxine and sucrose was scarcely accompanied with the hydrolyzation of the product under the conditions employed.

DISCUSSION

Pyridoxine-G, which is formed in the culture filtrate of *Sarcina lutea*, is a new derivative of vitamin B₆. The transglucosidation could be observed in the cultivation of the organism with sucrose or maltose as carbon source.

Until now, a large number of the derivatives of vitamin B₆ have been chemically synthesized. Recently, the chemical synthesis of the conjugated form of vitamin B₆ such as fatty acid esters,¹⁸⁵⁾ condensates with amino acid^{186,187)} and those with other vitamins^{188,189)} was also reported. On the other hand, pyridoxic acid¹⁰⁷⁾ and other oxidative products⁶⁹⁾ were found in the biological degradation of vitamin B₆. Several conjugates which include glucuronide of pyridoxine,¹⁹⁰⁾ pyridoxine- or pyridoxal-3-sulfate¹⁹¹⁾ and indole-pyridoxal complex,¹⁹²⁾ have also been reported as naturally occurring ones.

The sugar derivative of vitamin B₆, however, has been investigated neither chemically nor biologically. Many sugar esters with hydroxylated aromatic acids have been found in plants^{193,194)} and attention has been paid to the biosynthesis, natural distribution and biological function. Riboflavinyl glucoside is the only sugar derivative of vitamin, which has been found in rat liver¹⁹⁵⁾ and various microorganisms.¹⁹⁶⁻¹⁹⁸⁾ It has been suggested that riboflavinyl glucoside may play a physiological role in catalyzing the

successive transfer of glucosyl group through the formation of riboflavinyl compounds of oligosaccharides.

Though the physiological role of pyridoxine-G still remained to be elucidated, relatively wide distribution of the transglucosidation in microorganisms¹⁹⁹⁾ may suggest the possibility of the important role in the biological systems.

The suggestion of the structure of pyridoxine-G in which glucose may be conjugated with 4 or 5 hydroxymethyl group of pyridoxine, was obtained in this section. The definite structure is demonstrated in the next section.

SUMMARY

It was found that a new derivative of pyridoxine was formed in the culture filtrate of certain microorganisms which were grown in the medium containing sucrose as carbon source and pyridoxine. This compound was isolated and characterized its component to be pyridoxine and glucose. The activity of the formation of pyridoxine-G was observed in bacteria belonging to the genus *Sarcina*. Several conditions on pyridoxine-G formation with *Sarcina lutea* were also investigated. Sucrose was the most effective glucosyl donor substrate and maltose was about one-fifth as effective as sucrose, while glucose, fructose, their mixture and other sugars were inactive.

Section 2. Chemical Structure of Pyridoxine-G

INTRODUCTION

In a previous section, a new derivative of pyridoxine was found in the culture filtrate of *Sarcina lutea* and found to be composed of pyridoxine and glucose by using the powdered preparation isolated from the incubation mixture of the organism. But its definite structure could not be determined in that study.

Many derivatives of vitamin B₆ have been chemically synthesized which conjugated with other naturally occurring compounds. These include the esters with fatty acids^{185,186)} and the condensates with amino acid¹⁸⁷⁾ and nucleotides²⁰⁰⁾ However, the presence of these conjugates in nature has not been proved yet.

This section describes the structural studies of pyridoxine-G which include the enzymatic analysis and acetylation of isolated powder, and its chemical synthesis. The structure of main component of pyridoxine-G was identified as pyridoxine-5'- α -D-G.

EXPERIMENTAL AND RESULTS

Enzymatic Analysis of Pyridoxine-G.

As described in the previous section, the isolation of pyridoxine-G from the reaction mixture of intact cells of *Sarcina lutea* IFO 3232 was achieved to obtain the powdered preparation which had

the purity of 70%. Using this preparation, enzymatic hydrolysis and dehydrogenation of pyridoxine-G were attempted to determine the configuration and the position of the glucosidic linkage. From the results, it may be suggested that pyridoxine-G involved pyridoxine-5'- α -G.

Hydrolysis by β -glucosidase. *Aspergillus niger* was aerobically grown at 28°C for 4 days with the nutrient medium containing lactose which appeared to be more inducible carbon source for β -glucosidase production. The harvested cells in 0.01 M potassium phosphate buffer, pH 7.0, were crushed in a glass mortar with sea sand. The disrupted cells were then treated with Kaijo-Denki 19 Hz ultrasonic oscillator for 30 min. The supernatant solution obtained by centrifugation was fractionated by ammonium sulfate and tannin followed by alumina C_γ treatment according to the method of Niwa^{201,202)} with slight modifications. With the very active β -glucosidase in this preparation which hydrolyzed a wide range of β -glucosides, the hydrolysis of pyridoxine-G was examined. The results showed that pyridoxine-G could not be hydrolyzed by the enzyme preparation. Then it may be considered that pyridoxine-G is not β -glucoside.

Hydrolysis by α -glucosidase. α -Glucosidase of brewer's yeast which has been reported to attack not only holosides but also heterosides having α -glucosidic linkage²⁰³⁾ was employed in this experiment.

The washed cells of brewer's yeast which were the kind gift of Kirin brewer's Company were autolyzed and the debris was centrifuged off. The supernatant solution was partially purified by the treatment with calcium phosphate gel treatment and ammonium sulfate fractionation according to the method of Chiba *et al.*²⁰³⁾ The preparation hydrolyzed a few α -glucoside such as sucrose and phenyl- α -glucoside, but neither maltose nor β -glucosides. A slight breakdown of pyridoxine-G was observed. It has been considered that α -glucosidase was rather restricted in the substrate specificity than β -glucosidase. Then it seems possible to suggest that the configuration of pyridoxine-G is α -form. The strong hydrolysis of pyridoxine-G may be achieved by a specific α -glucosidase preparation.

Dehydrogenation by pyridoxine dehydrogenase. Existence of an NADP-specific enzyme which catalyze the oxidation of Pyridoxine at 4 hydroxymethyl group to pyridoxal, pyridoxine dehydrogenase, has been demonstrated for the first time by Morino and Sakamoto⁷⁰⁾ in baker's yeast. Recently, the formation of pyridoxal-semicarbazone from pyridoxine using baker's yeast cells in the presence of semicarbazide was found.⁷²⁾ It was also recognized that oxidation at 5 hydroxymethyl group of pyridoxine did not take place. Using the similar reaction conditions, the dehydrogenation of pyridoxine-G was examined to obtain a clue on the conjugated position at pyridoxine. The formation of pyridoxal glucoside semicarbazone was

recognized by paper chromatography and paper electrophoresis.

Thus, the presence of pyridoxine-5'-G in the preparation is confirmed.

Identification of Pyridoxine-4'-G and Pyridoxine-5'-G.

Pyridoxine has been shown to undergo complex formation with boric acid²⁰⁴⁾ *i.e.*, one molecule of boric acid is linked to 2 molecules of pyridoxine through the oxygen atoms in the 3 and 4 positions. Using the isolated pyridoxine-G, the formation of its borate complex was examined to clarify the position of the glucosidic linkage. It was observed that pyridoxine-G as well as pyridoxine on paper chromatogram formed the borate complex and gave negative Gibb's test¹²¹⁾ suggesting the glucose conjugation to take place at the 5 position of pyridoxine. But the ultraviolet absorption spectra showed a shoulder at 326 mμ besides an absorption maximum at 294 mμ, the latter of which resulted from the occurrence of pyridoxine-5'-G-borate complex²⁰⁴⁾ when the solution of pyridoxine-G was saturated with boric acid at pH 6.8 (Fig. 1). The shoulder at 326 mμ may be an indication that a part of pyridoxine is substituted with glucose at the 4 position.

Then, pyridoxine-G was applied to a Dowex 1X2 (borate form) column chromatography for the separation of 4'- and 5'-isomer. The elution pattern was shown in Fig. 2. Fraction I and II were assumed to be pyridoxine-4'-G and -5'-G, respectively. The ultraviolet spectra of each compound in saturated boric acid solution

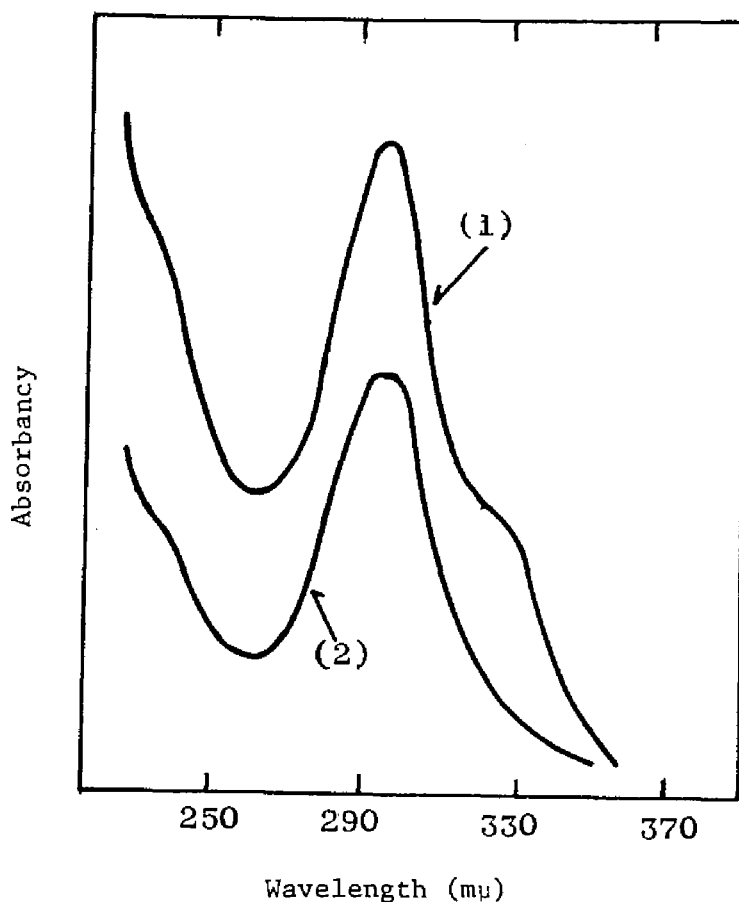
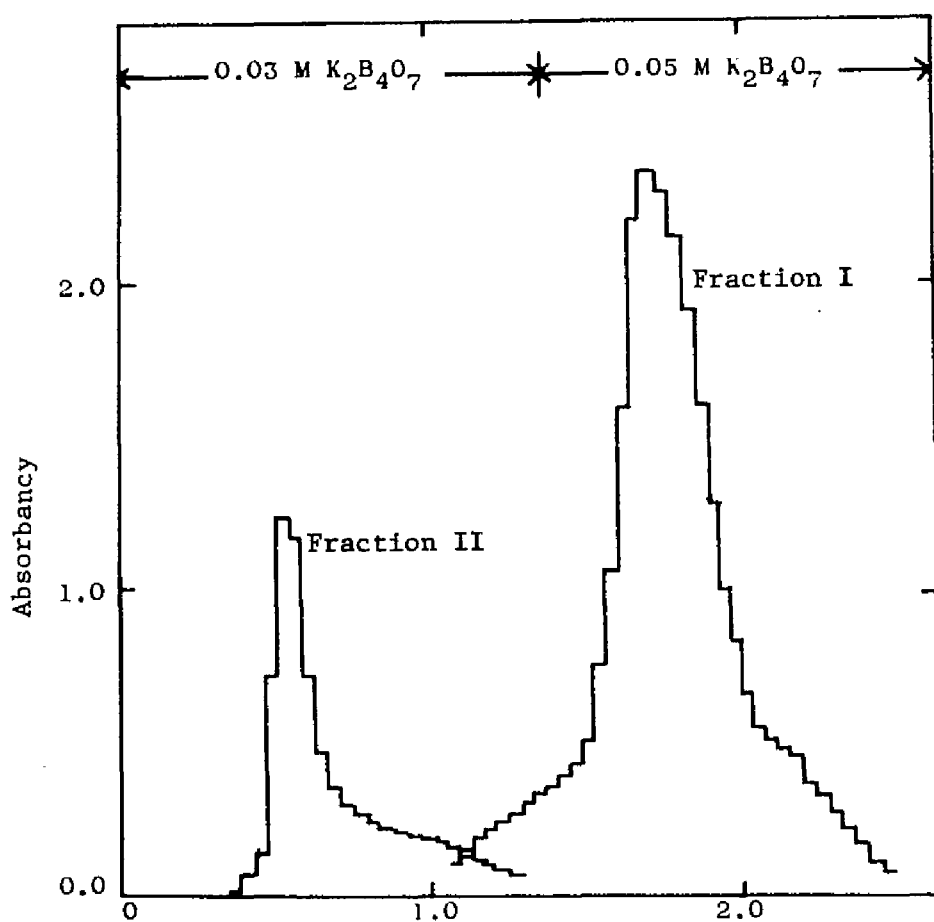


Fig. 1. Ultraviolet Absorption Spectra of
Pyridoxine-G and Pyridoxine in Saturated Boric
Acid Solution at pH 6.8

(1); Pyridoxine-G, (2); Pyridoxine.

at pH 6.8 emphasized the assumption as shown in Fig. 3.

From these results, it may be concluded that pyridoxine-G preparation isolated from the reaction mixture of *Sarcina lutea* consists of pyridoxine-5'-G mainly, containing a small amount of pyridoxine-4'-G.



Fraction (liters of eluate through column)

Fig. 2. Chromatography for Separation of Pyridoxine-4'-G
and Pyridoxine-5'-G as Borate

Complex on Dowex 1X2 Column

Chromatography was performed with a column, 1.5X20cm, and pyridoxine-4'-G (Fraction I) and pyridoxine-5'-G (Fraction II) were measured their absorbancies at 330 mμ and 290 mμ, respectively.

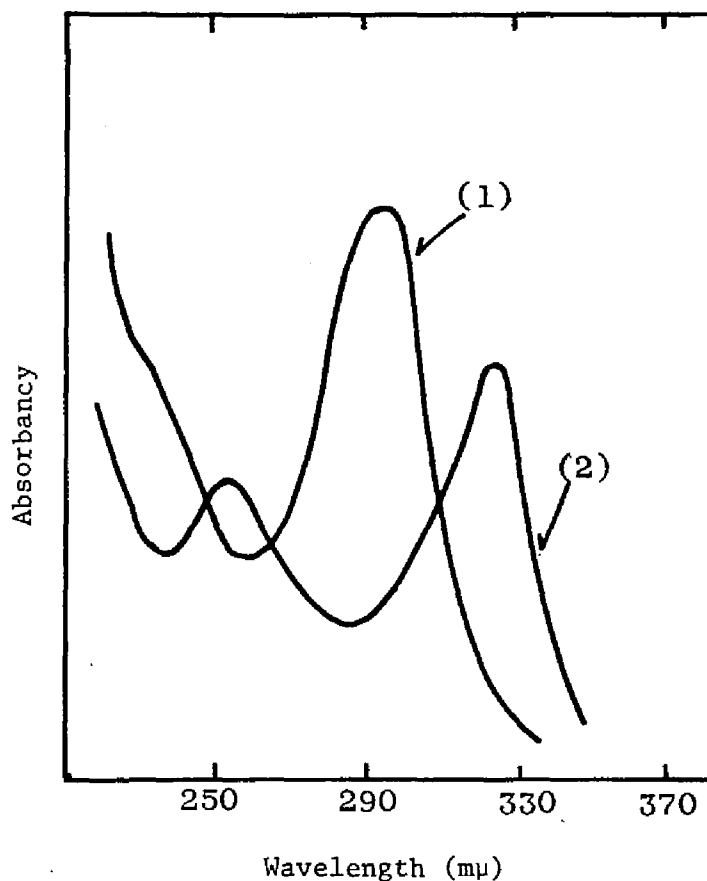


Fig. 3. Ultraviolet Absorption Spectra of
Pyridoxine-G in Saturated Boric Acid Solution
at pH 6.8

(1); Fraction II of Fig. 1. (pyridoxine-5'-G).

(2); Fraction I of Fig. 1. (pyridoxine-4'-G).

Isolation of Acetylated Pyridoxine-G.

Using the powder obtained previously, as starting material, the acetylation of pyridoxine-G was performed as follows.

Six g of powdered pyridoxine-G were dissolved in a mixture of 50 ml acetic anhydride and 30 ml dry pyridine. The solution was

allowed to stand overnight at room temperature and then poured into 200 ml of ice water. After neutralization with sodium bicarbonate, acetylated pyridoxine-G was extracted with ether and the extract was washed with water. Then it was dried with anhydrous sodium sulfate, and filtrated. The filtrate was concentrated under reduced pressure to dryness. The resulting preparation was dissolved in a small amount of benzene and applied to a Silica-gel column chromatography. The absorbent (Mall. AR-100 mesh) homogenized in benzene was used to pack a column of 4X40cm. After placing the solution of acetylated pyridoxine-G and washing the column by 200 ml of benzene, the elution was performed with a solvent which composed of benzene, 20; methanol, 1; ethylacetate, 1 (volume per volume). The fractions shown to contain acetylated pyridoxine-G by thin layer chromatography were collected and concentrated under reduced pressure to dryness. Pyridoxine-G hexaacetate obtained was then dissolved in anhydrous ether. Introduction of dry hydrogen chloride gas to the ether solution produced the precipitates of hydrogen chloride salt of pyridoxine-G hexaacetate. After recrystallization from ethanol, a small amount of crystals was obtained melting at 154-157°C, which was considered to be hydrogen chloride salt of pyridoxine-4'- α -G hexaacetate from its nuclear magnetic resonance spectrum as described below. The result of elemental analysis of the crystal was as follows. Found: C, 50.37; H, 5.79; N, 2.20. Calcd. for $C_{26}H_{34}O_{14}HCl$: C, 50.36; H, 5.52; N, 2.25%.

A major component of acetylated pyridoxine-G, which should be hydrogen chloride salt of pyridoxine-5'- α -G hexaacetate as described above, was obtained as the pure powder. It was also examined by nuclear magnetic resonance spectroscopy and was shown to be identical with the synthetic pyridoxine-5'- α -G hexaacetate as described below.

Chemical Synthesis of Pyridoxine-G.

In order to confirm the structure of naturally occurring pyridoxine-G, the chemical synthesis of pyridoxine-5'- α -D-G was studied according to the route shown in Fig. 4. $\alpha^4,3$ -O-Isopropylidene pyridoxine (I) was synthesized by the method of Korytnyk^{205,206}) The synthesis of 2,3,4,6-tetra-O-benzyl-D-glucopyranosyl chloride (II) was performed from methyl α -D-glucopyranoside by the method of Austin *et al*²⁰⁷) Königs-Knorr condensation of I and II was performed as follows. A mixture of aglycon (isopropylidene pyridoxine, 1.04 g), silver carbonate (11.0 g), and Drierite (23.0 g) in pure dry benzene (120 ml) was stirred for 5 hr in the dark. To the reaction mixture was added silver perchlorate (0.3 g) and 2,3,4,6-tetra-O-benzyl-D-glucopyranosyl chloride (2.0 g) in 40 ml of dry benzene. Stirring was continued till the spot of aglycon was no more detected on thin layer chromatogram. After filtration through 'Hyflo' silica, the reaction mixture was condensed *in vacuo* and applied to a Silica-gel column chromatography. The column was prepared as described above. Elution was performed with a solvent

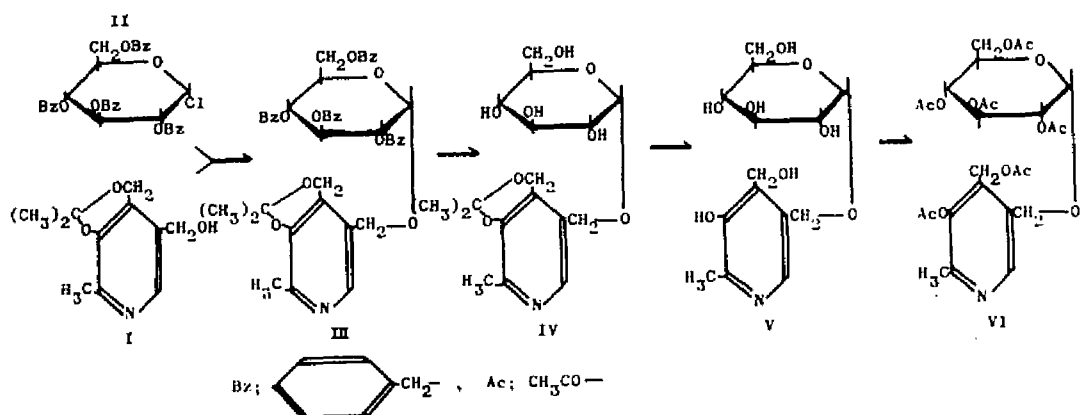


Fig. 4. Route of Chemical Synthesis of Pyridoxine-5'-α-D-G
Hexaacetate

which composed of benzene, 20; methanol, 1; ethyl acetate, 1 (volume per volume). Each fractions of 5 ml was: examined on thin layer chromatogram (Silica-gel G; solvent system; chloroform, 10; ethyl-acetate, 1). The fractions containing the glucoside were collected and evaporated to a syrup (470 mg). The benzyl groups of III were removed by the hydrogenation for 48 hr in 50 ml of 85% ethanol using palladium black (prepared by hydrogenation of 1 g of palladium chloride) as the catalyst. After filtration, the solution was evaporated to dryness. Isopropylidene pyridoxine-5'-α-G (IV) obtained was heated with 80% acetic acid in a boiling water bath for 2 hr. This compound, pyridoxine-5'-α-G (V), showed the same ultraviolet absorption spectra and the R_f values as those of naturally occurring pyridoxine-G. β-Anomer mixing in this preparation was removed by the exhaustive hydrolysis with β-glucosidase of *Aspergillus niger*.

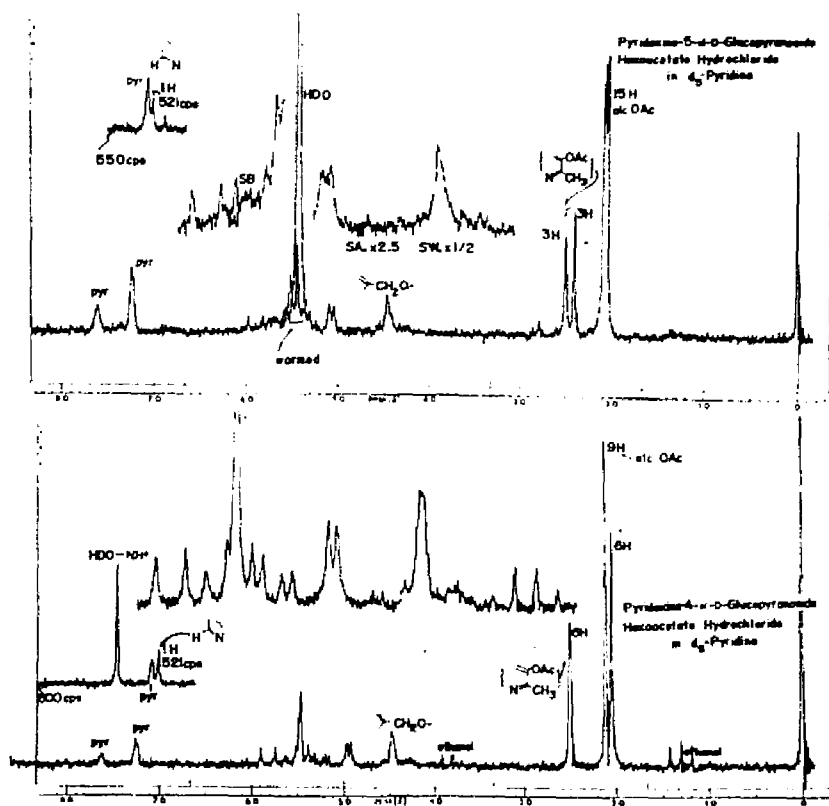


Fig. 5. Nuclear Magnetic Resonance Spectra of
Isolated and Synthesized Pyridoxine-5'-α-G,
and Isolated Pyridoxine-4'-α-G

The result indicated that α-anomer may be involved about 80% of the preparation. Acetylation of pyridoxine-5'-α-G and purification by Silica-gel column chromatography gave a colorless syrup of pyridoxine-5'-α-G hexaacetate (VI).

Nuclear magnetic resonance spectra of synthesized acetate of pyridoxine-5'-G, and of isolated pyridoxine-4'-G and -5'-G were shown in Fig. 5. These results also support the presumed structure of naturally occurring pyridoxine-G.

DISCUSSION

The enzymatic and spectral analysis of pyridoxine-G and its acetylation, and the chemical synthesis of pyridoxine-5'-G were described herein. The pyridoxine-G isolated from the reaction mixture of *Sarcina lutea* was presumed to be pyridoxine-5'- α -G involving a small amount of pyridoxine-4'-G (Fig. 6).

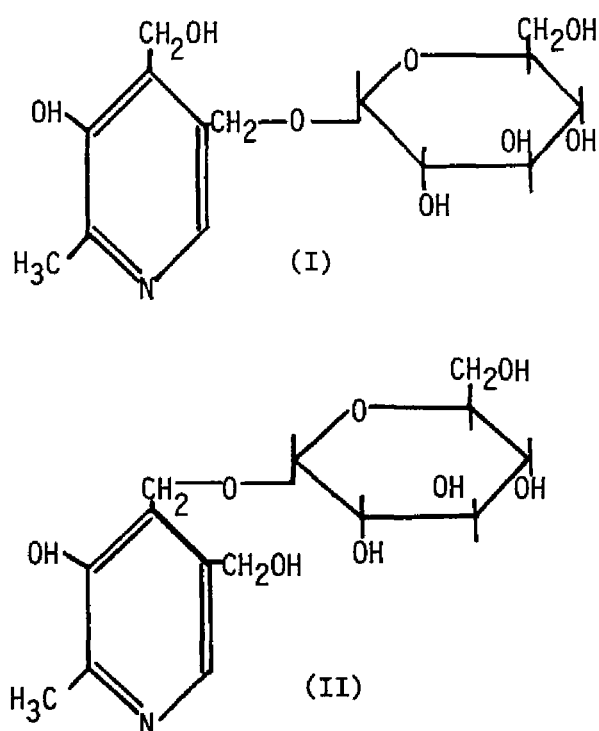


Fig. 6. Structure of Pyridoxine-5'-G (I)
and Pyridoxine-4'-G (II)

The enzymatic hydrolysis of pyridoxine-G could not be completed by glucosidase preparation employed here. The properties of yeast α -glucosidase have been extensively studied by several workers who reported the enzyme to be inactive on isomaltose^{208,209)} Helferich and Johannis;²¹⁰⁾ and Chiba *et al.*²⁰³⁾ have been demonstrated that the partially purified preparation of yeast α -glucosidase could attack aryl- α -glucosides such as phenyl- and cresyl- α -glucoside. On the hydrolysis of riboflavin- α -glucoside, the slow breakdown has been reported by α -glucosidase preparation from baker's yeast¹⁹⁵⁾ Recently, Suzuki²¹¹⁾ reported its remarkable hydrolysis with α -glucosidase preparation from riboflavinyl glucoside forming bacterium, *Leuconostoc mesenteroides*. The degradation of pyridoxine-G is observed in growing culture of *Micrococcus* sp.¹⁹⁹⁾ The specific or nonspecific glucosidase which strongly attack the α -glucosidic linkage of pyridoxine-G may be presented in such microorganisms.

SUMMARY

The definite structure of pyridoxine-G which was isolated from the reaction mixture of the intact cells of *Sarcina lutea* was investigated.

The enzymatic analyses which involved the hydrolysis by yeast α -glucosidase and *Aspergillus* β -glucosidase, and the oxidation by yeast pyridoxine dehydrogenase were attempted. It was observed

that pyridoxine-G was slowly hydrolyzed by α -glucosidase and oxidized by pyridoxine dehydrogenase. The spectral change in saturated boric acid solution was also recognized. From these data, the isolated pyridoxine-G was presumed to be pyridoxine-5'- α -G involving a small amount of pyridoxine-4'- α -G.

The acetylation of pyridoxine-G was performed for the isolation of pure preparation of pyridoxine hexaacetate.

Pyridoxine-5'- α -D-G was chemically synthesized by the condensation of isopropylidene pyridoxine and tetra-O-benzyl-glucopyranosyl chloride. The synthetic product was identical with naturally occurring one, supporting its presumed structure.

CONCLUSION

From the first work for the estimation of vitamin B₆ utilizing the nutritional requirement, a number of investigations on the metabolism of the vitamin by microorganisms have been attempted by various groups of workers. These include the discovery of several compounds belonging to the group of the vitamin and their enzymatic interconversion referring with the formation of the coenzyme form, pyridoxal-P. Moreover, the studies on the degradation and synthesis of the vitamin by microorganisms have recently arrested the attention. Although still far from adequate, the results from these investigations have contributed to various areas of biological philosophy such as biochemistry, pharmacology, clinical medicine and biotechnology.

Present study has been performed to develop the knowledge on the microbial metabolism of vitamin B₆ concerning to the expanding the ability of microorganisms. Then, it was found a few new metabolic pathways which involved the phosphorylation of vitamin B₆ by the phosphotransferring reaction differed from the kinase system participating of adenine nucleotidic compounds, the conversion of pyridoxamine-P to pyridoxal-P by the transamination with α -ketoglutaric acid and the formation of pyridoxine-G by transglucosidation between pyridoxine and sucrose. These results will be summarized below.

On the phosphorylation of vitamin B₆ group, phosphokinase system, capable of transferring the phosphoryl moiety of ATP to the free forms of vitamin B₆ has been known to be the common system. The phosphotransferring reaction being different from pyridoxal kinase was found and searched in wide ranges of microorganisms using pyridoxine and *p*-NPP as phosphoryl acceptor and donor substrates. The enzymatic properties of the transphosphorylation were inquired with the enzyme extracted from one bacterial isolate. Subsequently, the enzyme was purified employing the bacterial extract as starting material and crystallized. Then it was pointed out that the transphosphorylation of vitamin B₆ was catalyzed by the intrinsic and inherent function of an acid phosphatase. The results obtained here on the transphosphorylation gave not only the knowledge of the new system on the enzymatic phosphorylation of vitamin B₆, but also the clue for the clarification on the mechanism of general enzymatical transphosphorylation. That is, the transphosphorylations between a variety of organic phosphates not involved ATP and other phosphoryl acceptor substrates have been known to be catalyzed by the action of phosphatases and that of phosphotransferases. The mechanisms of these reactions, however, have not still been elucidated satisfactorily, especially in regard to the identity of both the enzymes. The study on the transphosphorylation of vitamin B₆ presented here provided the first clear-cut evidences for the identity of the activities of transphosphorylation and phosphohydrolyzation.

Using the cells of organisms having the higher activity of the transphosphorylation, the accumulation of phosphate esters of vitamin B₆ was studied, resulted in the formation of the considerable amount of pyridoxine-P and pyridoxal-P from pyridoxine. The producibility of the phosphate esters showed the possibility of the reaction for the application to the industrial object.

Pyridoxamine-P transaminase which was contained in the cells of the clostridia lacking pyridoxamine-P oxidizing enzyme, was purified from *Clostridium kainantoi*. Although the purification did not result in a complete purification such as crystallization, the purified enzyme was shown to have a single component by ultracentrifugal analysis and gel filtration. Spectral analysis of the purified enzyme demonstrated no involvement of pyridoxal-P as the prosthetic group in the enzyme. Transaminase concerning to amino acid and amine involves vitamin B₆ as the coenzyme and the elucidation of the reaction mechanism has been investigated on the relation between the vitamin and apotransaminase. The transaminase presented here is a more simple transaminase and may give the suitable sample for the elucidation of transamination mechanism. It was also found that pyridoxamine-P was formed with the additions of pyridoxal-P and D-glutamic acid as amino acceptor and donor substrates to the reaction mixture by the inverse activity of the enzyme, suggesting some relation of the enzyme to the D-amino acid metabolism in clostridia.

Recently, several derivatives of vitamin B₆ which are combined with other organic compounds have been chemically synthesized in view point of their application to pharmacological use. But the natural occurrence of such conjugated derivatives of the vitamin has scarcely been discovered. A new compound, pyridoxine-G, was found and characterized the definite structure together with the chemical synthesis of the compound. The activity of the formation of pyridoxine-G was especially observed in bacteria belonging to the genus *Sarcina*, and it was found that pyridoxine-G formed by *Sarcina lutea* consisted of two isomers in which pyridoxine conjugated with glucose at its 4 or 5 hydroxymethyl group. Sucrose was the most effective glucosyl donor substrate and maltose was about one-fifth as effective as sucrose, while monosaccharides such as glucose and fructose were inactive. Such a transglucosidation of vitamin is only known with riboflavin. The finding of the natural occurrence of pyridoxine-G may introduce the new metabolic role of vitamin B₆. Furthermore, the more water-solubility of pyridoxine-G than pyridoxine and its relative stability may give the further availability of the vitamin for various areas of biology.

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