NEW REACTIONS ON

MICROBIAL METABOLISM OF VITAMIN B$_6$

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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
p-NPP	p-Nitrophenyl phosphate
p-NP	p-Nitrophenol
Phenyl-P	Phenyl phosphate
p-AAP	p-Aminoacetophenone

INTRODUCTION

Vitamin B_6 was first defined and delineated to be a distinct entity in the vitamin B_2 complex as the rat pellagra preventive factor[1]. Subsequently, the isolation of pure crystalline vitamin B_6 was reported by Lepkovsky[2] and several other groups[3-8]. Within a year, the structure and the synthesis were demonstrated by Harris and Folkers[9] and others[10-12]. Then, the term "pyridoxine" was proposed by György and Eckhardt[13] 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 B_6-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. Followingly, the additional members of pyridoxine were searched in a wide range of natural product[18-23] and defined the structure along with the synthesis[24-30]. Then, it was established that the biological

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activity of the vitamin B_6 group was displayed by pyridoxine (pyri-
doxol), pyridoxal, pyridoxamine, and their 5'-phosphate esters.[31]

In various investigations on the function of the vitamin B_6
group, it has been made evident that pyridoxal-P was the main bio-
catalytically active form of the vitamin, and that the other com-
pounds 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-
lyases,[41~46] isomerases,[47,48] and ligases.[49]   And these enzymes
have been available on the elucidation of the coenzymatic action of
vitamin B_6 on a molecular level from the first nonenzymatic dupli-
cation by Snell *et al.*[50~52] and Braunstein.[53]

On the other hand, the knowledges of the enzymatic intercon-
version of various forms of free vitamin B_6 and of their conversion
to pyridoxal-P have been expanded considerably in recent years.[54]
The number of known reactions that vitamin B_6 may undergo in tissues
of one or another organism is already large as summarized below.

Pyridoxine⟷Pyridoxal⟷Pyridoxamine

Pyridoxine-P⟶Pyridoxal-P⟷Pyridoxamine-P
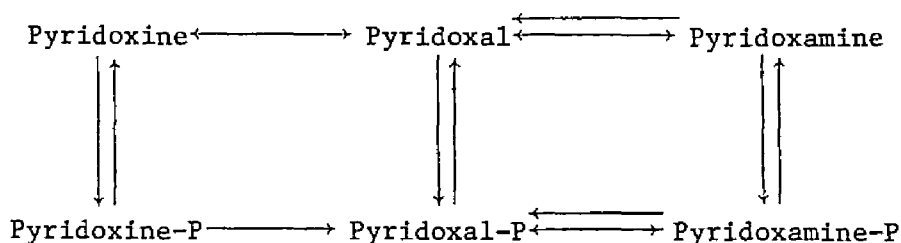
The enzyme, pyridoxal kinase, which catalyzed the phosphoryla-
tion of vitamin B_6 was first purified partially from brewer's yeast
by Hurwitz[55] He established the stoichiometry of the reaction
between ATP and pyridoxal, together with the facts that the kinase
phosphorylated pyridoxine, pyridoxamine and several other pyridine
derivatives in addition to pyridoxal[56] 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 sources[57-59]

The dephosphorylation of phosphorylated forms of vitamin B_6
has been clarified to be catalyzed by various phosphatase prepara-
tions[60-66] In these cases, non-specific acid or alkaline phospha-
tase 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 alde-
hyde oxidase, which catalyzed the oxidation of pyridoxal to 4-pyri-
doxic acid, to the reaction mixture[67,68] Snell *et al.* purified
pyridoxine oxidase from pseudomonad which had FAD as the prosthetic
group[69] While, the existence of a NADP-specific pyridoxine dehy-
drogenase 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 cata-
lyzed 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 irrever-
sible 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 pyrido-
xamine 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 establish-
ment 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 Happold,[84] and Pogell[85] demonstrated
the occurrence of an enzyme catalyzing the deamination of pyridoxa-
mine-P. Separately, Morisue *et al.* reported a pyridoxine-P oxidiz-
ing 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 micro-
organisms suggested the significance in vitamin B_6 metabolism[88,89]

and a large amount production of pyridoxal-P.[90] Furthermore, a transamination between pyridoxamine-P and α-ketoglutarate has recently been recognized in strict anaerobe, clostridia, whicn lacked the system of pyridoxamine-P oxidation.[91]

Since the discovery of vitamin B_6, 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 cells[92~95] and that the stimulating effect of carbon and nitrogen compounds on the biosynthesis could be observed.[96~99] The incorporation study of ^{14}C-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 mechanism.[102] 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 B_6 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 B_6 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 B_6 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 B_6 have been revealed.[69]

As described above, great many investigations have been available on the metabolism of vitamin B_6. 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 B_6. Firstly, a new phosphotransferring reaction on the phosphorylation of free forms of vitamin B_6 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 B$_6$ through a New Phosphotransferring Reaction

Section 1. Distribution in Microorganisms and Isolation of Phosphorylated Product

INTRODUCTION

On the enzymatic phosphorylation of vitamin B$_6$, 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 B$_6$ 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 compounds[117] Recently, Kumar and Vaidyanathan[118] 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 K_2HPO_4 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 $MgSO_4 \cdot 7H_2O$, 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 phenyl-
hydrazine 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 Microorga-
nisms.

The phosphotransferring activity which catalyzed the phospho-
rylation 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, Neuro-
spora, 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 B_6 Phosphates Formation by Molds

Strains	p-NPP**	Pyridoxine-P* +	Pyridoxine-P* −	Pyridoxal-P* +	Pyridoxal-P* −
Mucor javanicus Wehmer IFO 4570		1.6	1.2	0.9	1.2
Mucor javanicus Wehmer IFO 4572		2.7	0.4	1.1	1.3
Mucor fragilis Bainier IFO 6449		1.5	1.2	1.1	1.4
Rhizopus oryzae M-21		1.1	0.9	0.9	1.1
Rhizopus batatas M-24		0.0	0.4	0.9	0.4
Rhizopus javanicus Takeda IFO 5442		0.0	1.4	0.8	1.1
Aspergillus oryzae M-61		14.0	0.4	3.2	1.2
Aspergillus niger No. 4416		5.2	0.0	1.2	1.2
Aspergillus awamori M-66		1.6	0.0	0.8	1.2
Aspergillus candidus M-70		29.2	1.2	4.4	1.4
Aspergillus oryzae var. *globosus* M-71		28.4	0.0	3.6	1.2
Aspergillus usamii IFO 4388		0.8	0.3	1.6	0.8
Aspergillus flavus Link IFO 5839		37.8	0.9	5.4	1.3
Aspergillus nidulans IFO 5719		2.8	0.0	1.4	1.4
Aspergillus oryzae Cohn IFO 4117		8.8	1.0	0.0	0.0
Aspergillus terreus Thom IFO 6123		2.6	1.1	1.4	1.2
Penicillium chrysogenum IFO 4626		1.8	0.7	0.6	0.9
Penicillium oxalicum IFO 5750		4.1	0.0	0.2	0.0
Penicillium notatum IFO 4640		2.0	0.2	1.0	0.8
Monascus purpureus IAM 8010		0.4	0.2	0.2	0.4
Monascus anka IAM 8001		0.5	0.2	0.0	0.0
Neurospora crassa IFO 6068		2.7	0.3	1.7	0.8
Pullularia pullulans IFO 4464		0.5	0.0	0.0	0.0
Fusarium oxysporum IFO 5942		0.2	0.2	0.8	0.8
Fusarium culmorum IFO 5902		0.5	0.2	0.1	0.6
Gibberella fujikuroi 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 B_6 Phosphates Formation by Yeasts

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

*Pyridoxine-P or pyridoxal-P formed (µg) per 4 ml of reaction mixture.
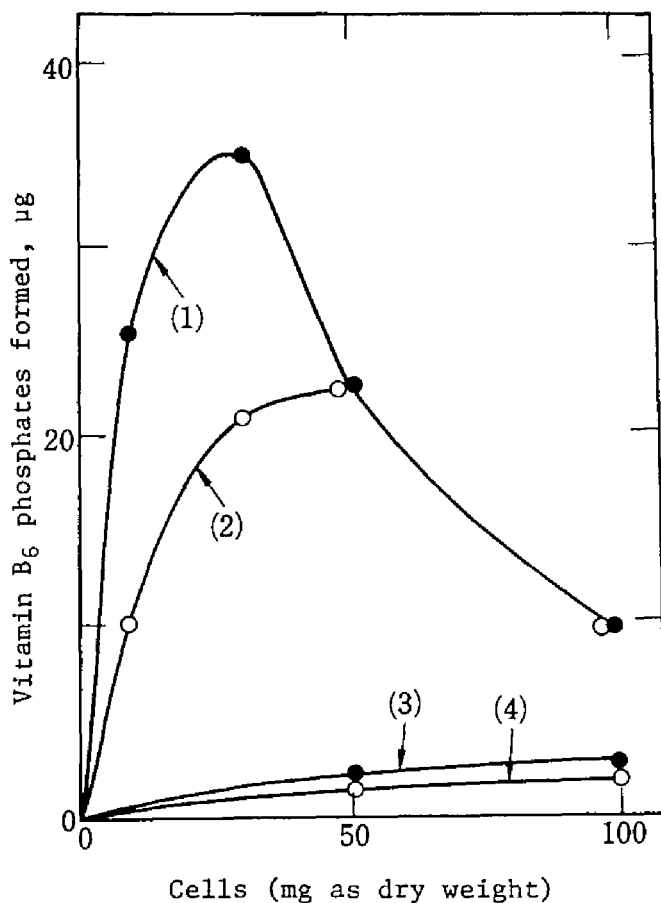** p-NPP added (+) or not added (−).

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 B_6-P formed with dried cells. (2); Pyridoxal-P formed with dried cells. (3); Total vitamin B_6-P formed with intact cells. (4); Pyridoxal-P formed with intact cells.

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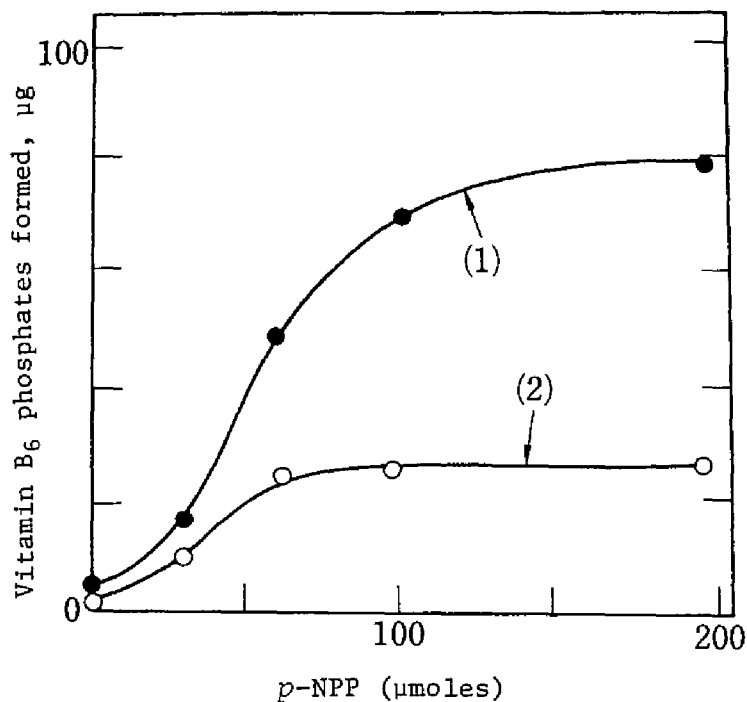
Fig. 2. Effect of p-NPP Concentration on the Reaction

The reaction was carried out under the standard conditions with 20 mg of dried cells of *Saccharomyces cerevisiae*. (1); Total vitamin B_6-P formed. (2); Pyridoxal-P formed.

tivity. 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 B_6 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 B_6 Phosphates

with *Saccharomyces cerevisiae* Dried Cells

| | Total vitamin B_6-P | Pyridoxal-P |
	(μg)	
Complete system	200.0	32.5
Omitted pyridoxine	0.0	0.0
Omitted *p*-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 filtrated. 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 (apotryptophanase 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
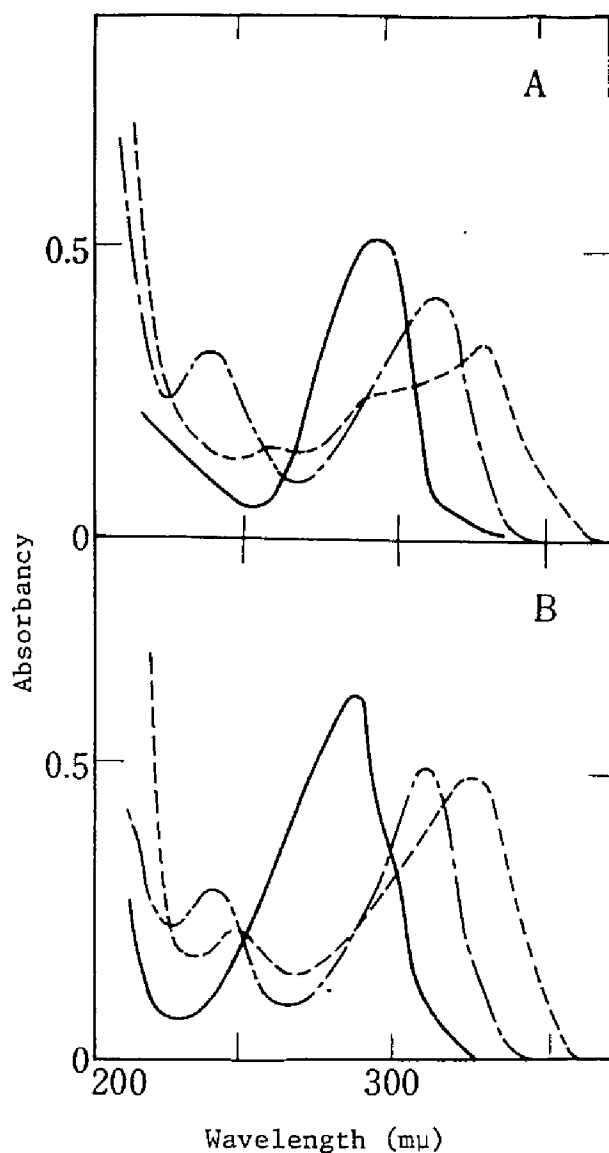
Fig. 3. Ultraviolet Spectra of Isolated Pyridoxine-P

(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 mµ in 0.1 N hydrochloric acid, at 310 mµ in 0.1 N sodium hydroxide and at 325 mµ 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;[20] Gibbs reagent[121] and ferric chloride[4] 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 microorganims 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
n-Propanol 70, NH_4OH 10, H_2O 10	0.14	0.14
2,4-Lutidine 55, Ethanol 20, H_2O 15, Diethylamine 1	0.21	0.21
$tert$-Butanol 70, H_2O 15, 90% Formate	0.44	0.44
Color Reagents		
Diazotized p-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 ATP.[58]

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.[117,122] 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 protein (mg)	PTase*		Pase**	
			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 μmoles 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 μmoles of pyridoxine, 50 μmoles of p-NPP, 200 μmoles 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 μmoles of pyridoxine, 50 μmoles of p-NPP, 200 μmoles 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., 1957.[28)]

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 μmoles of pyridoxine, 50 μmoles of p-NPP. 250 μmoles 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 μmoles of pyridoxine, 50 μmoles of p-NPP, 200 μmoles 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

-25-

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 method[75]. Para-nitrophenol was measured with the optical density at 430 mµ with addition of saturated sodium carbonate solution[129]. 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	PIN-P*	*p*-NP
		(μmoles)			
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
Asp. tamarii M-68	3.7	0.27	18.6	0.28	41.2
Asp. candidus M-70	0.8	0.16	15.0	0.06	8.3
Asp. oryzae var. *globosus*	1.5	0.08	6.0	0.02	3.8
Asp. flavus IFO 5839	2.5	0.13	9.0	0.23	30.9
Pen. chrysogenum IFO 4626	2.3	0.02	10.5	0.03	3.8
Pen. oxalicum IFO 5750	2.8	0.04	15.8	0.03	3.8
Neu. crassa IFO 6068	3.5	0.03	14.3	0.02	3.8
End. hordei IFO 0104	1.2	0.00	3.2	0.00	1.2
End. lindneri IFO 0106	1.7	0.00	2.4	0.00	0.9
Pi. polymorpha 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, raffi-

nose, lactose, maltose, glycerol, mannitol, sucrose. Dulcitol, sta-

rch, 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.

Accodding 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 transphosphory-lation 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 Mannual.

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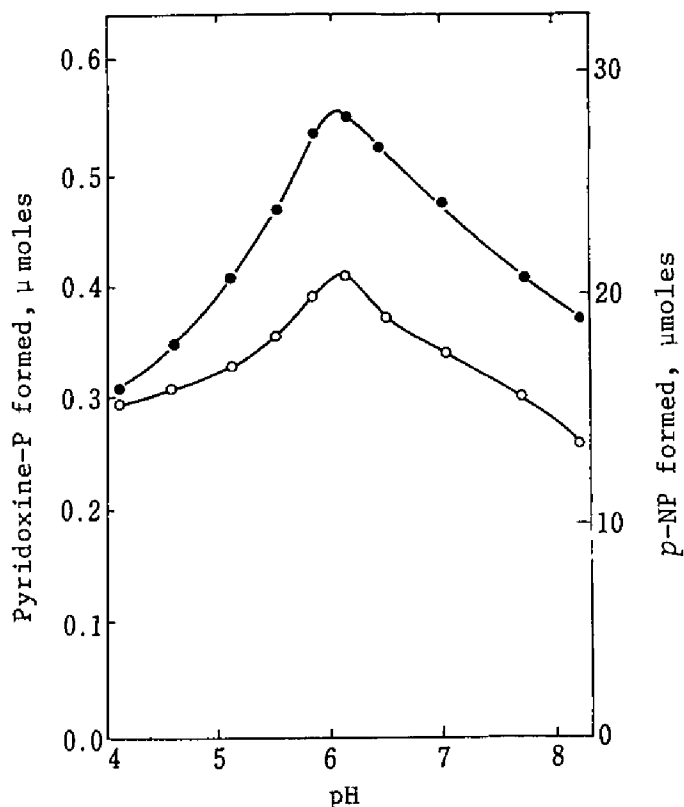
Fig. 1. pH-Activity Curves of Phosphatase and

Phosphotransferase Activities

(●) Pyridoxine-P formed, (O) 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
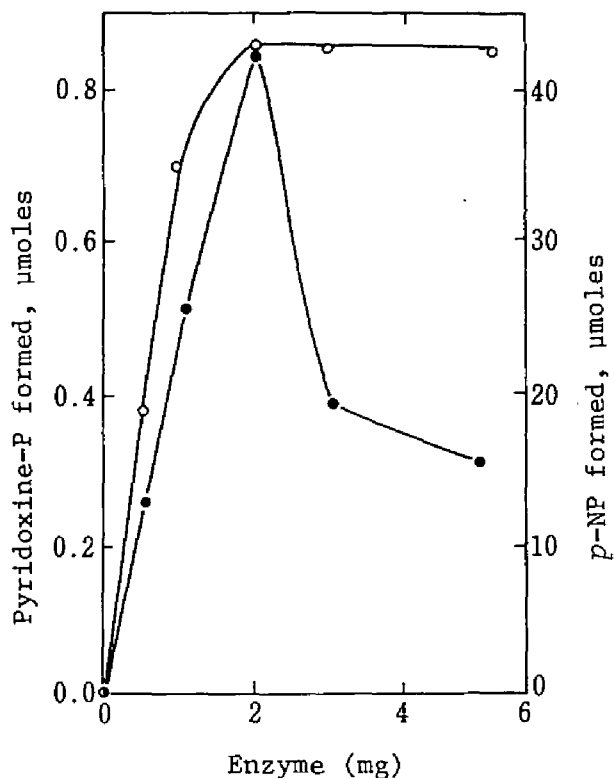
Fig. 2. Effect of Enzyme Concentration on Phos-

phatase 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
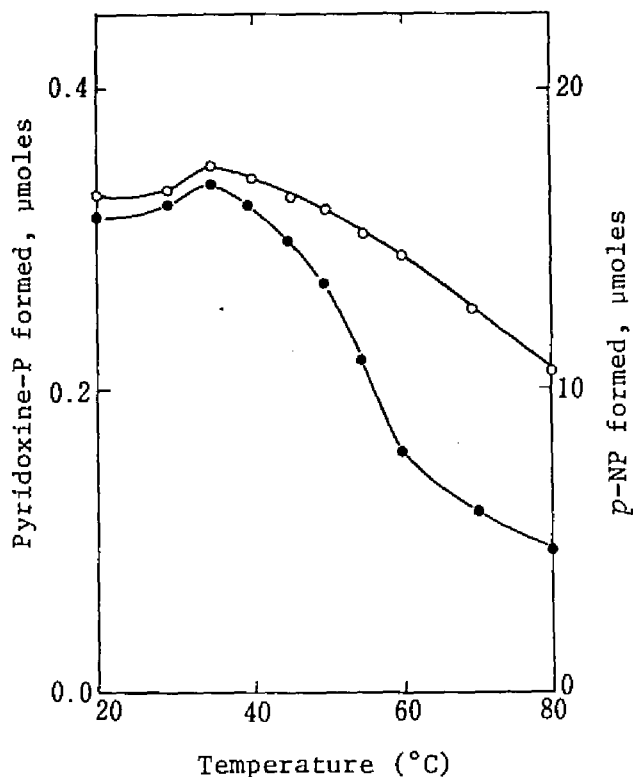the inhibition against the activity of the enzyme used for the assay,

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.

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pyridoxine-P oxidase. The stimulating effect of several metal ions

on nucleoside phosphotransferase has been demonstrated in detail.[131]

The inhibitory or accelerating effect of metal ions on phosphatase

has also been reported.[132,133] 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	p-NP formed
	(Relative activity)	
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 diff-
erent 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 $10^{-2}M$. Hg^{2+} 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 µmoles except for
PCMB (0.3 µmoles), and NaF, KH$_2$PO$_4$ and HgCl$_2$ (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 B_6. 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 B_6 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 (μmoles)
p-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'-Uridyric 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 μmoles.

ate[58])

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

Table VI. Phosphoryl Acceptor Specificity

of Vitamin B_6

Acceptor substrate	Vitamin B_6-P formed μmoles	p-NP formed 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 B_6 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 Axelrod[123] and Appleyard[135] 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 Chargaff[124] 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 bacteria[136] A few phosphotransferases, which were considered to be different from phosphatase, such as glucose-1-phosphate phosphotransferase have been also discovered[137]

The phosphorylation of pyridoxal by enzymes other than pyrido-

xal kinase was attempted by Axelrod and resulted in no success

using plant phosphatase.[23] Recently, Kumar and Vaidyanathan have

been shown that the preparation of the enzyme hydrolyzing FMN

from green gram extracts possessed the phosphotransferase

activity.[18] 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 B_6 presented here provides the

first clear-cut evidences for the formation of phosphorylated coen-

zymes 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 inorga-

nic phosphorus and sodium fluoride on phosphatase activity has been

investigated by several groups of workers. The phosphatase described

-38-

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 B_6, pyridoxine was the most excellent substrate followed by pyridoxamine. Pyridoxal phosphokinases from various enzyme sources could phosphorylate the three forms of vitamin B_6, though individual kinases differ greatly in their affinities for these compounds[58] Pyridoxal-P is known as active coenzyme form of vitamin B_6 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 a enzyme, pyridoxine-P oxidase, which is capable of conversion of pyridoxine-P and pyridoxamine-P to pyridoxal-P, exists widely in various aerobic bacteria[88] 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[38] Though it is considered to be not necessary to assume the existence of a separate class of phosphotransferase in the phosphatase preparation[39] 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 B_6, 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 aerobica-
lly 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 μmoles of pyri-
doxine, 50 μmoles of *p*-NPP, 200 μmoles 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 per-
formed 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 μmoles 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 deter-
mined according to the method of Takahashi[40] Sedimentation velo-
cities were measured with a Spinco model E ultracentrifuge opera-
ting 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*.[41] Other chemicals were used the same as in the
previous section.

RESULTS

Purification of Acid Phosphatase Having Pyridoxine-Phosphory-
lating Activity.

All operations were performed at 0–5°C throughout the purifi-
cation 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 centri-
fugation 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 10,000xg for 30
min. The supernatant solution was then dialyzed against deionized
water overnight.

Step III. First ammonium sulfate fractionation. To the dialy-
zed enzyme solution was added solid ammonium sulfate to 35% satura-
tion, adjusting pH to 7.0 with 10% ammonium hydroxide solution.
After stirring for 30 min, the precipitated protein was removed by
centrifugation at 10,000xg for 20 min and discarded. The ammonium
sulfate concentration was then increased to 65% saturation by fur-
ther addition of solid ammonium sulfate. After stirring for 30 min,
the precipitate was collected by centrifugation at 10,000xg 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 dissol-
ved 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 cen-
trifugation at 12,000xg 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 dialy-
zed enzyme solution from step III and allowed to stand for 15 min.
The precipitate formed was removed by centrifugation at 12,000xg
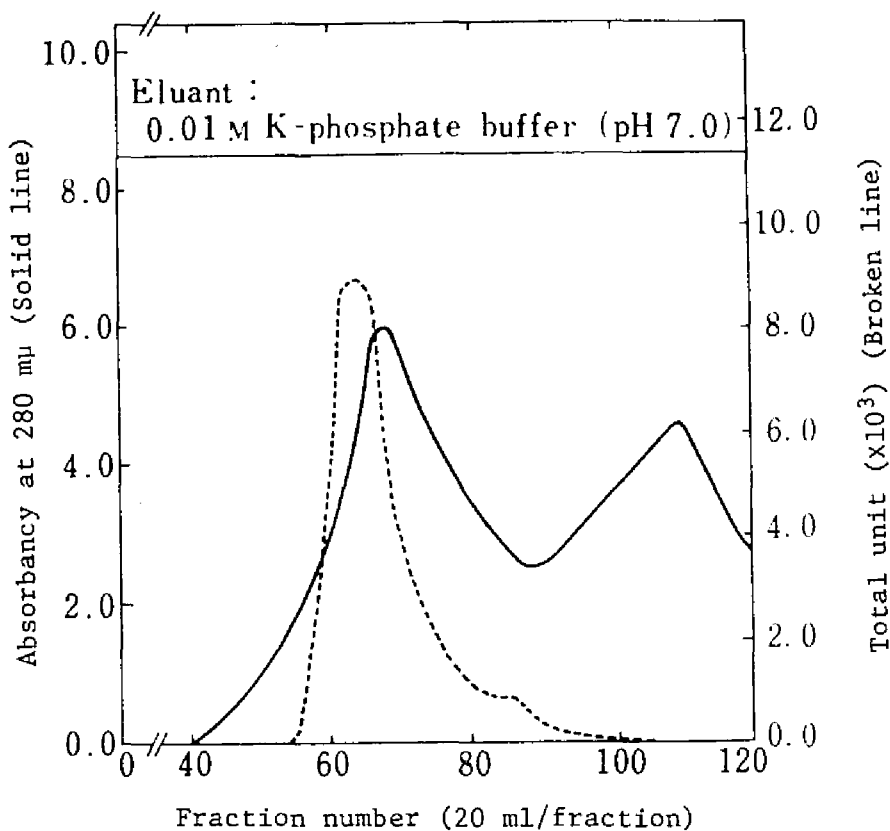
Fig. 1. Chromatography of Phosphatase on DEAE-Cellulose

Column

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

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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 concen-
tration was then increased to 55% saturation by the further addition
of solid ammonium sulfate. After stirring for 30 min, the precipi-
tate was collected by centrifugation at 12,000xg for 20 min and di-
ssolved 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 phos-
phate 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
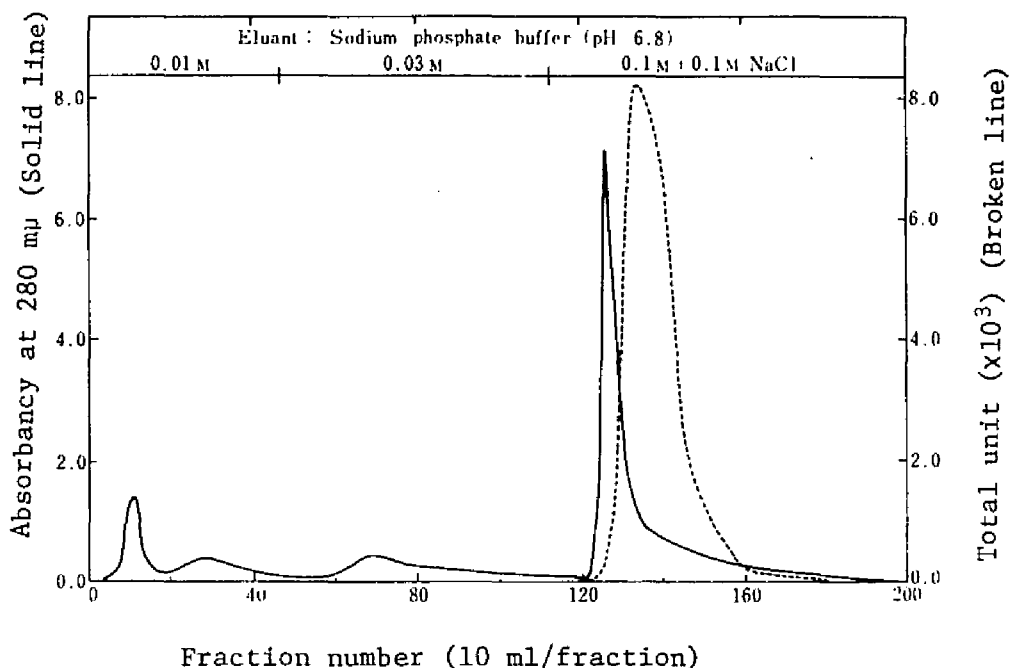with 0.1 M of the same buffer containing 0.1 M sodium chloride and

Fig. 2. Chromatography of Phosphatase on Hydroxylapatite

Column

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

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ombined to give 150 ml of solution. The elution pattern is shown

n Fig. 2. The active fraction was concentrated by the addition of

mmonium sulfate to 80% saturation. The precipitate obtained by

entrifugation at 12,000xg for 30 min was dissolved in 0.01 M potas-

ium phosphate buffer, pH 6.6, and dialyzed against two changes of

he same buffer, each 5 liters, overnight.

Step VIII. CM-Sephadex column chromatography. The dialyzed

nzyme solution (9.4 ml) was subjected to CM-sephadex column chro-

atography. The absorbent equilibrated with 0.01 M potassium phos-

hate buffer, pH 6.6, was used to pack a column 2.2X90 cm. The

-47-
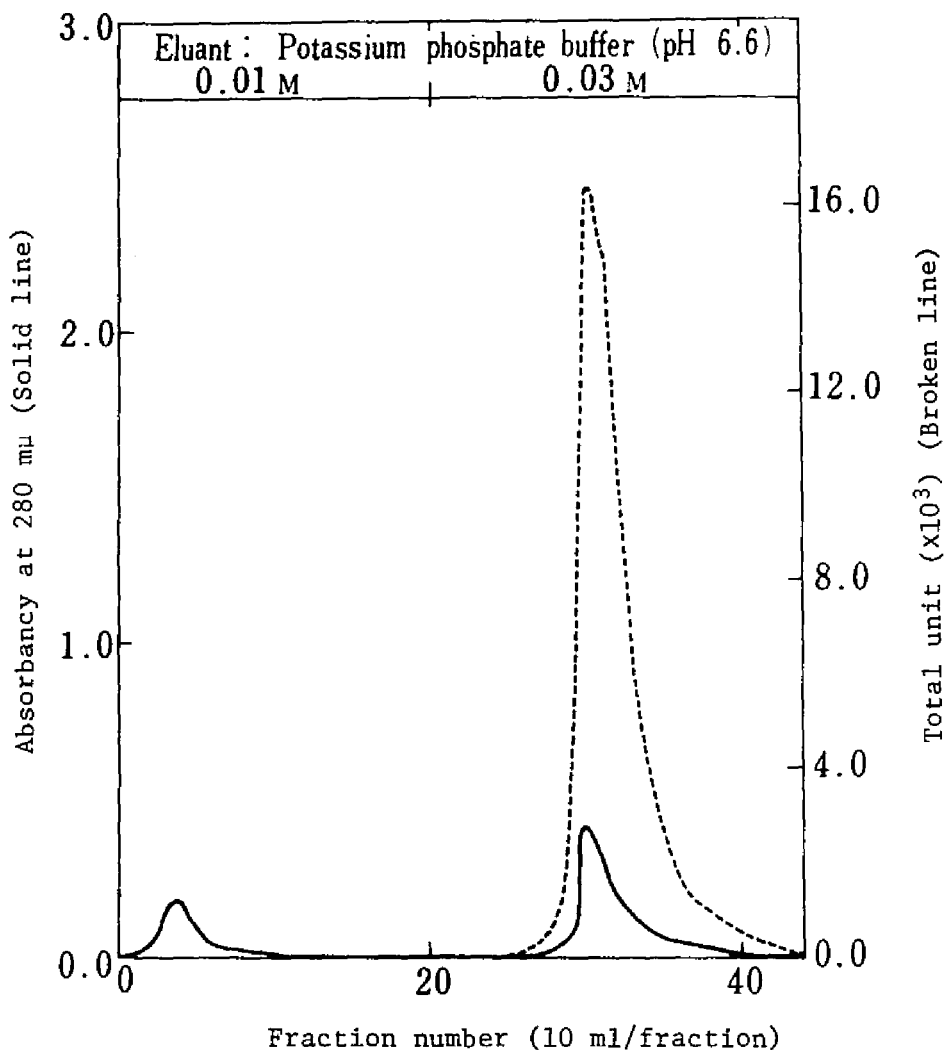
Fig. 3. Chromatography of CM-Sephadex Column

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

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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 15,000Xg 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 refractvie 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 p-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 $6.15 \times 10^{-7} cm^2/sec$. From these values, the molecular weight of

the enzyme was calculated to be about 120,000 according to the

equation of Svedberg and Erikson[42])

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 mμ. The shoulders at 285 and

293 mμ were also noted in the spectrum. The ratio of 260 mμ/280 mμ

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

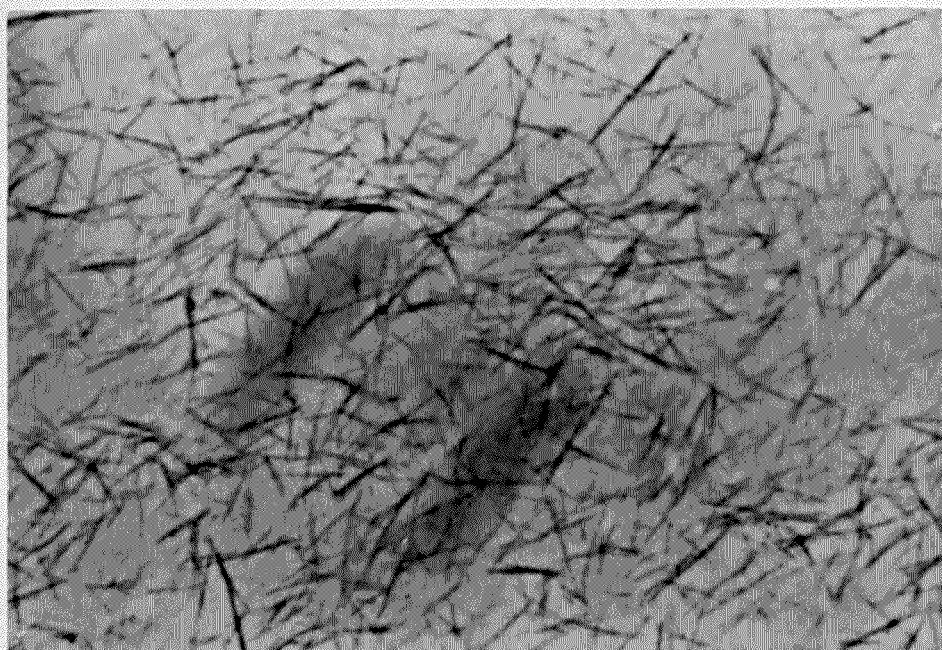was also observed that both the enzyme activities of phosphatase

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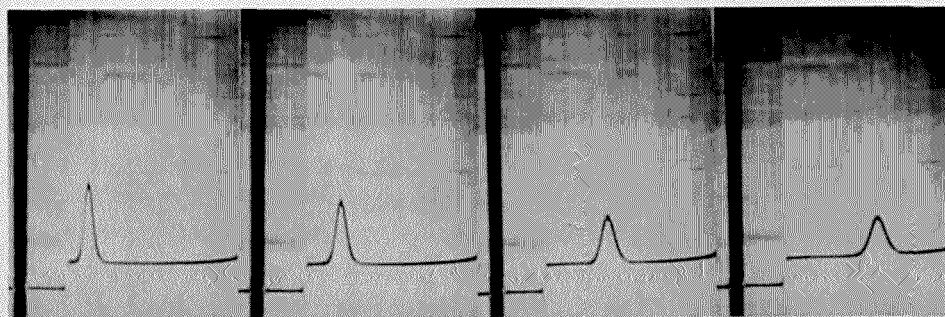
of *Escherichia freundii* K-1

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.
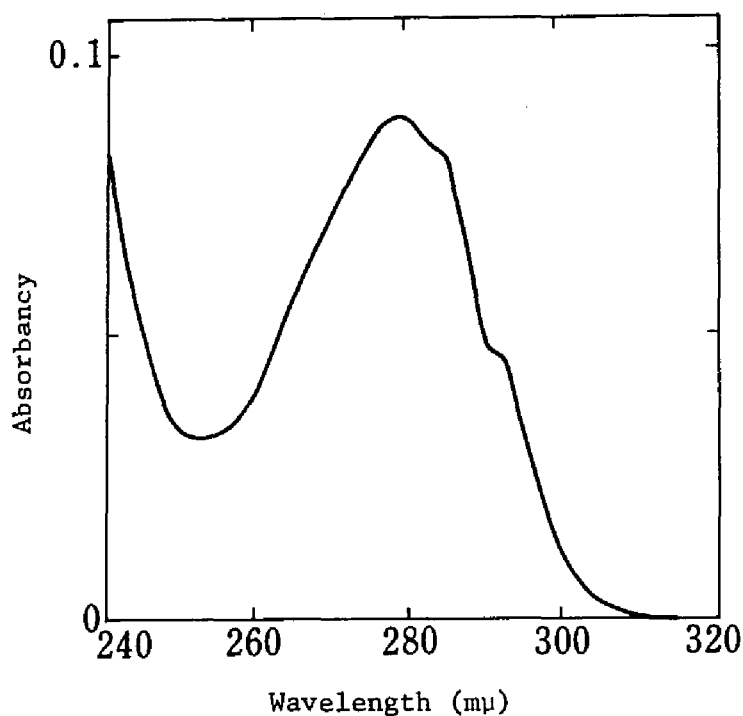
Fig. 6. Ultraviolet Spectrum of Phosphatase

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 propertly.

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.

-52-
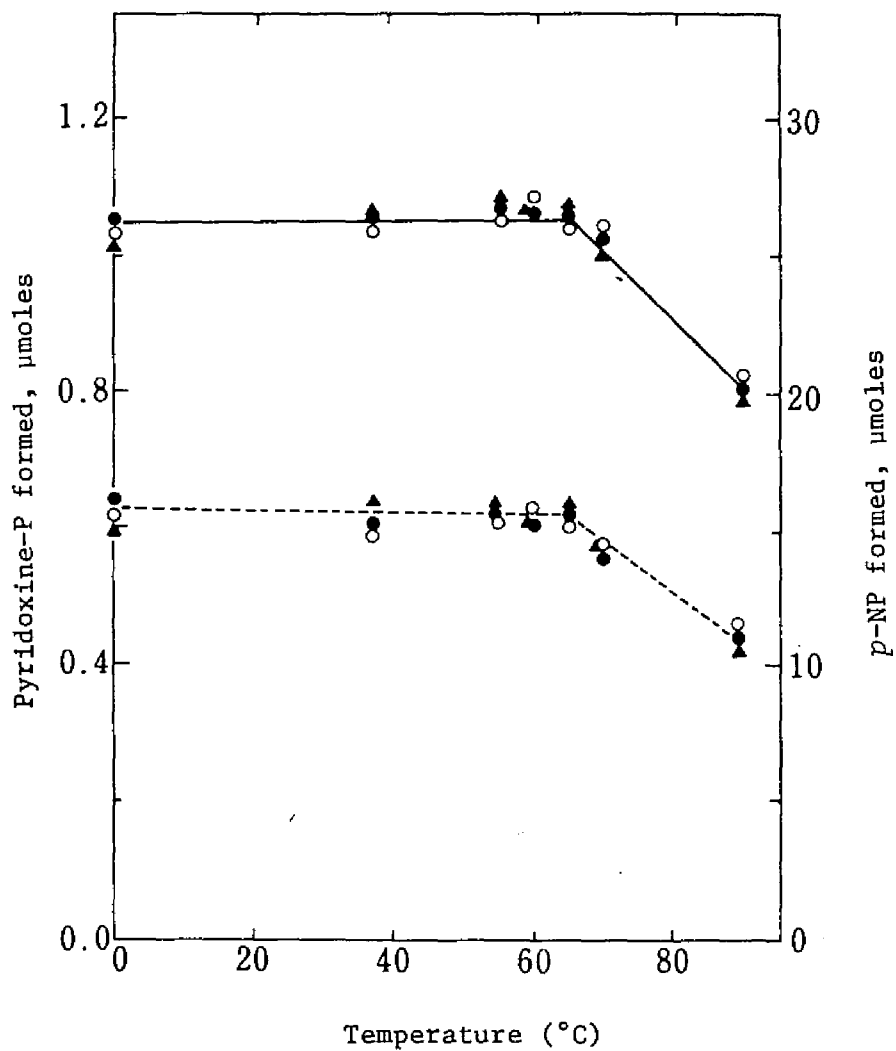
Fig. 7. Heat Stability of the Phosphatase

The enzyme (18 μg) was heated for 10 min at each temperatures 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 formed	Inorganic-P formed
	(μmoles)	
p-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'-Uridyric 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')-Uridyric 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 μg of protein) without p-NPP. The phosphoryl donor substrate added was 50 μmoles.
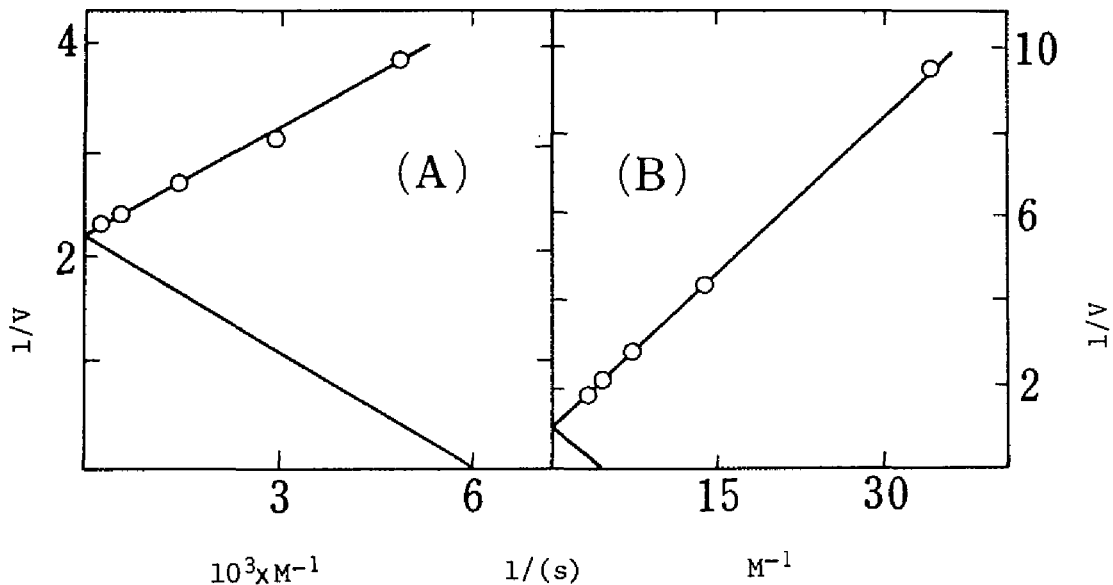
Fig. 8. Effect of Substrate Concentration, (S), on the Reaction Rate, V, by Phosphatase

The enzyme assay was carried out under the standard conditions with purified enzyme (18 μg of protein) without substrate concentration. The results are plotted by the method of Lineweaver and Burk.[43] (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 Burk.[43] 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*.[151)

The purified enzyme preparation also hydrolyzed various phos-

phates 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,[152)

the *Escherichia* enzyme utilized the substrates with a somewhat dif-

ferent 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[151)] 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 meta-

bolic function. A more anabolic function of the enzyme is, however,

not excluded in view of the transferase activity observed for various

phosphatases.[122)] which might occur with a competition at the enzyme

between the acceptor alcohol and water for the phosphoryl moiety

from the donor substrate.[153)] 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 B_6 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 B_6, the study on the accumulations of the vitamin and its phosphate esters by microorganism has been scarcely attempted. The microbial transphosphorylation between vitamin B_6 and an organic phosphate may produce a considerable amount of the phosphorylated product of vitamin B_6, 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 *Aspergi-
llus flavus*, the following standard condition was used: The assay
mixture which contained 25 μmoles of pyridoxine, 125 μmoles of *p*-NPP,

300 μmoles of potassium phosphate buffer of pH 6.6 or 250 μmoles 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 μmoles of pyridoxine, 300 μmoles of p-NPP, 200 μmoles 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 B_6-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.

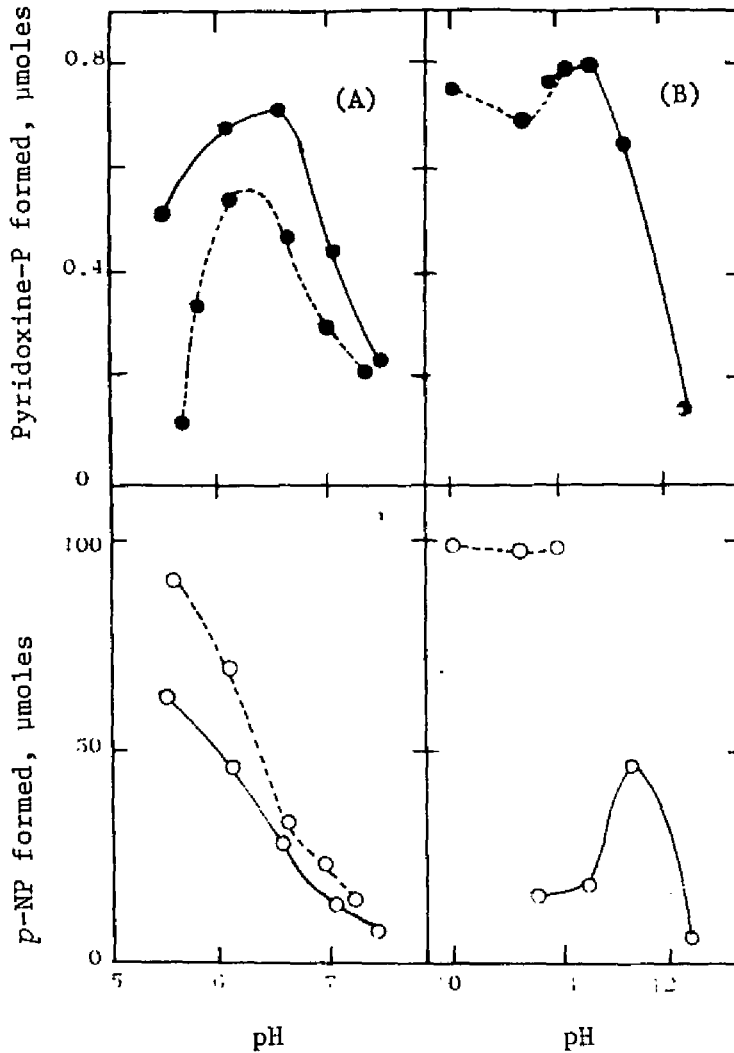Time course of the reaction. The time course of pyridoxine-P

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 μmoles per 4 ml.

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

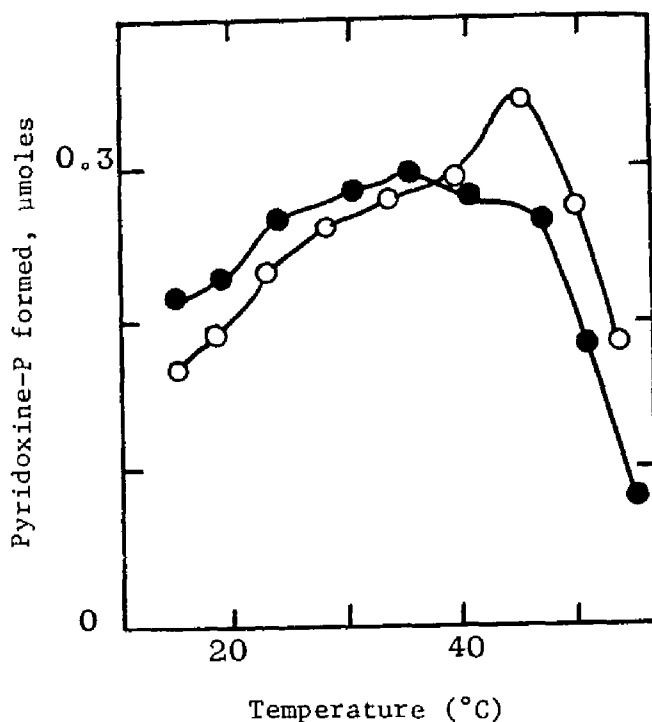————;Phosphate buffer, —————;Citrate buffer (A) or Sodium-carbonate buffer (B).

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.

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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.
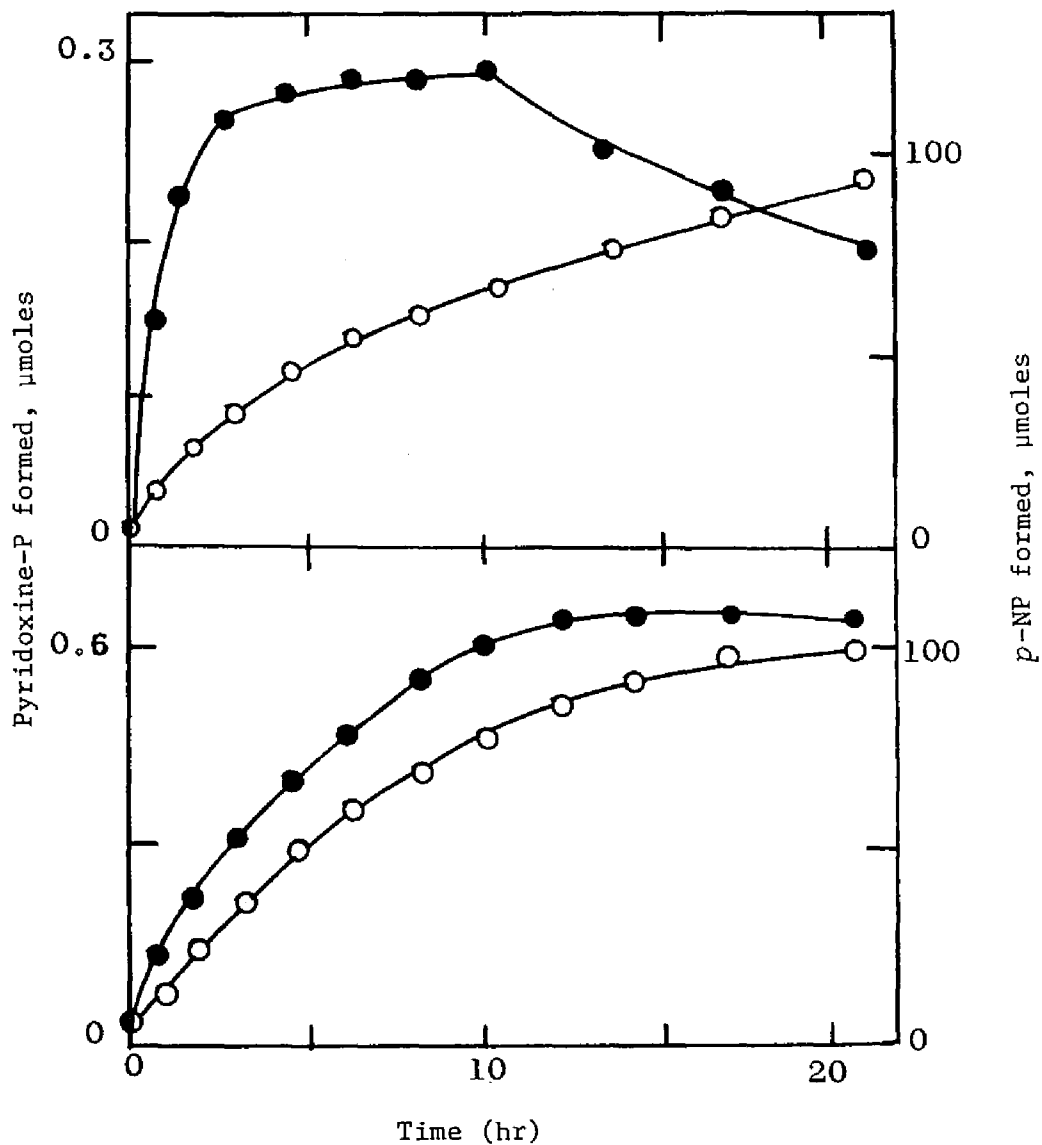
Fig. 3. Time Course of the Reaction

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, μmoles	
	(A)	(B)
p-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'-Uridyric 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 μmoles.

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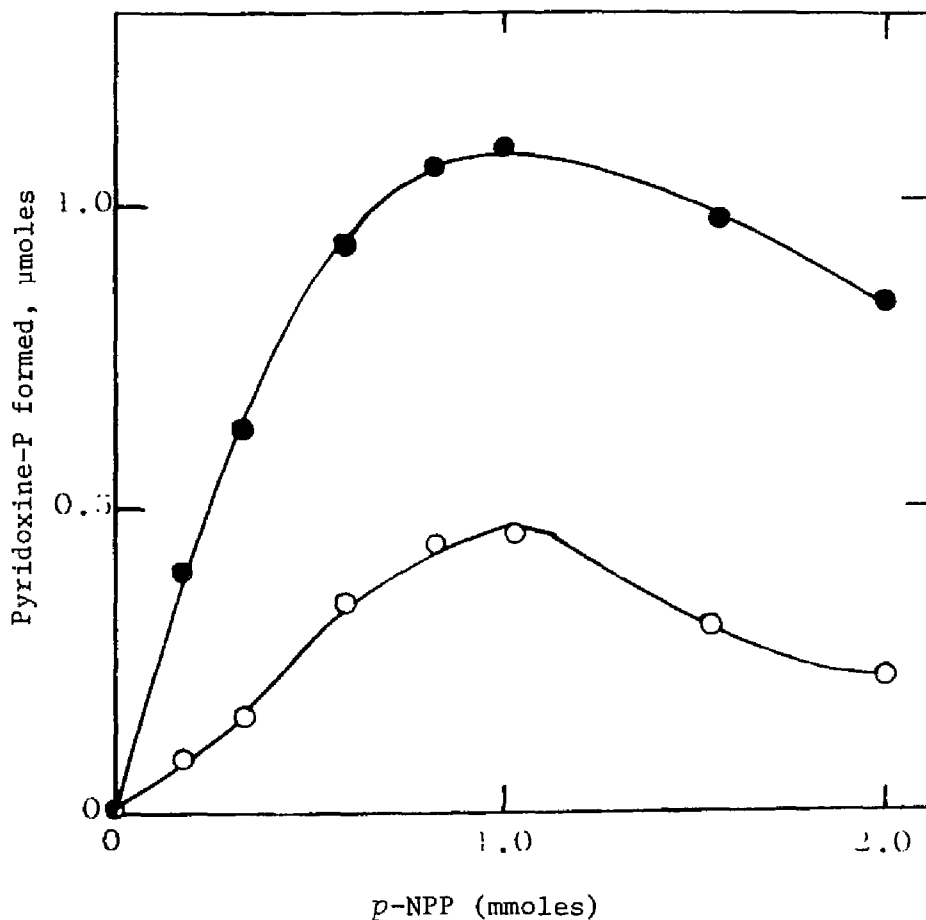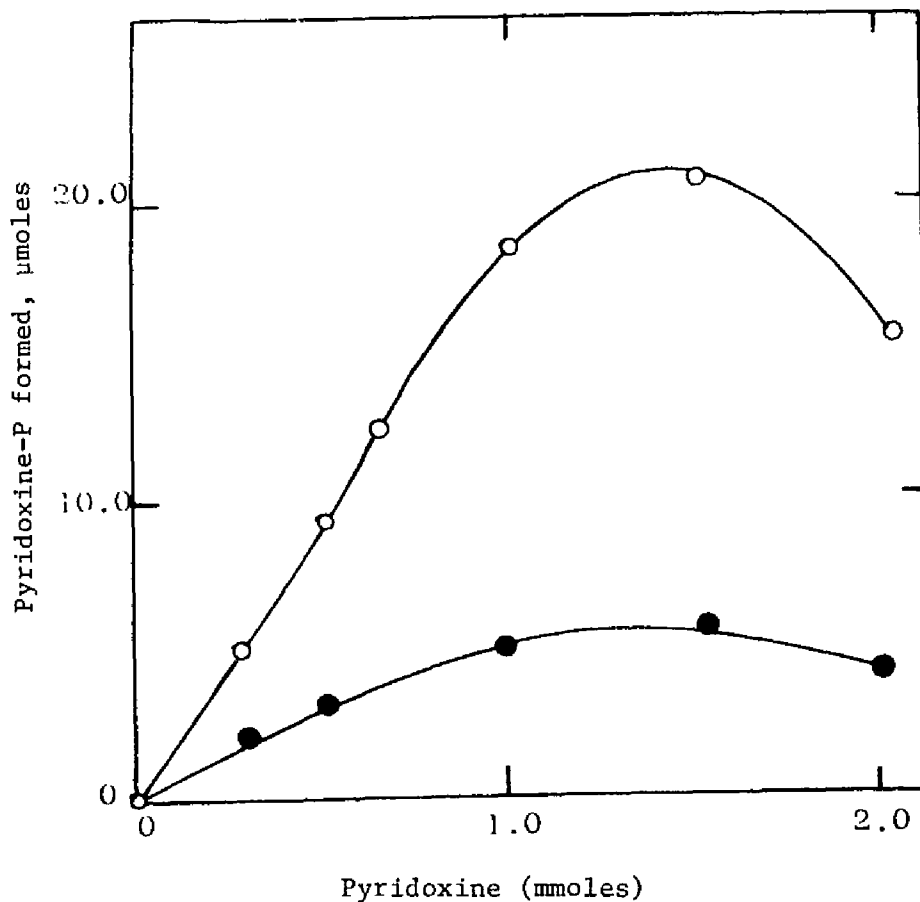
Fig. 4. Effect of p-NPP Concentration on Pyridoxine-P

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, (O) at pH 11.0.

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

-67-

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

Formation

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

(●) at pH 6.6, (○) at pH 11.0.

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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 µmoles (*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 B_6-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^{-3}M.

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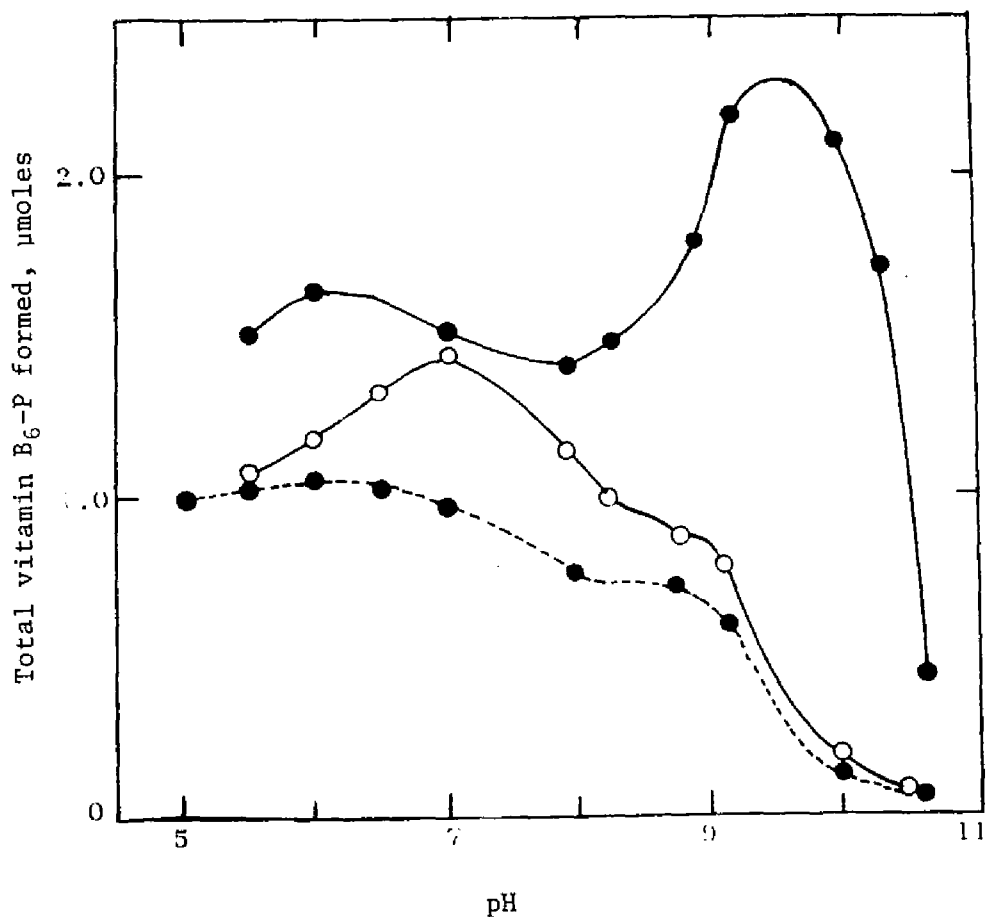
Fig. 6. pH-Activity Curves of Phosphorylation of Pyridoxine

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 B_6-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
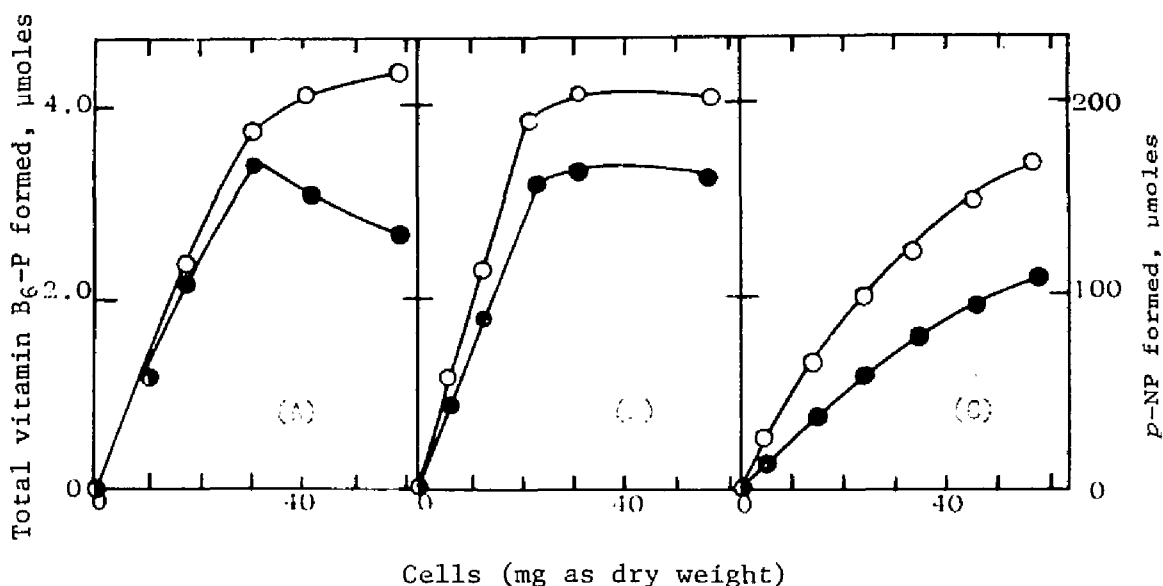
Fig. 7. Effect of Cell Concentration on Phosphorylation

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 B_6-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 phos-

phorylated product and the maximal amount of total vitamin B_6-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

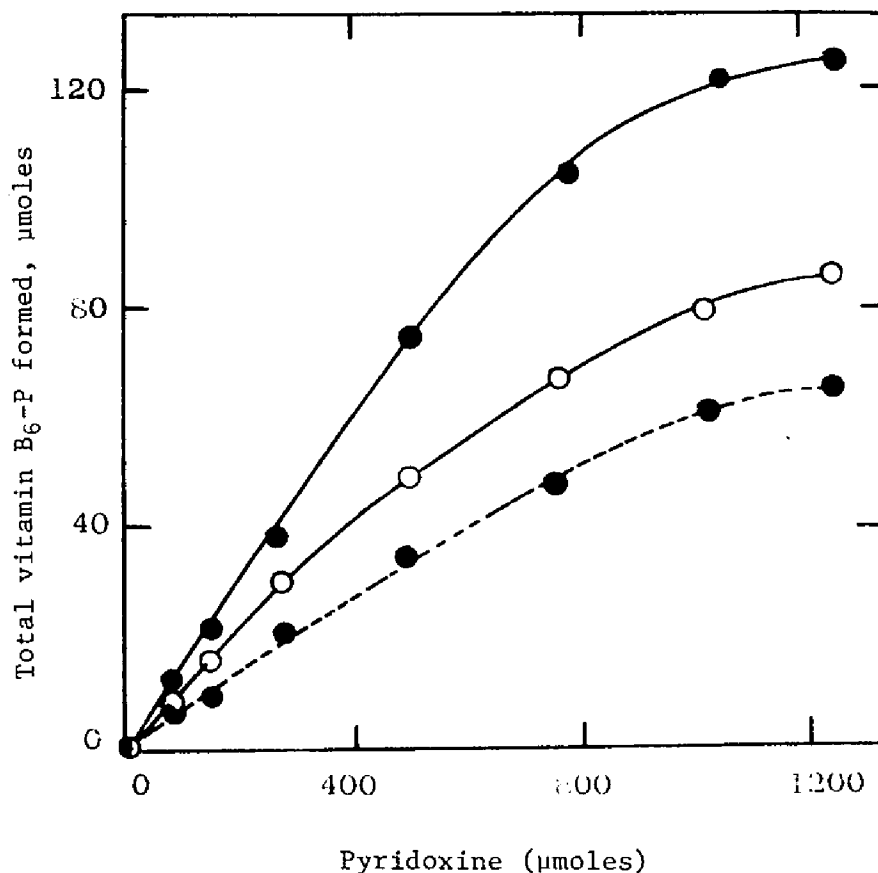*via* phosphorylation by ATP of pyridoxal, pyridoxamine and pyridoxine

-72-

Fig. 8. Effect of Pyridoxine Concentration on

Vitamin B_6-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 (--●--).

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to the corresponding 5'-phosphates, followed by oxidation of the phosphates of pyridoxamine and pyridoxine to pyridoxal-P by pyridoxine (pyridoxamine)-P oxidase.[56] 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
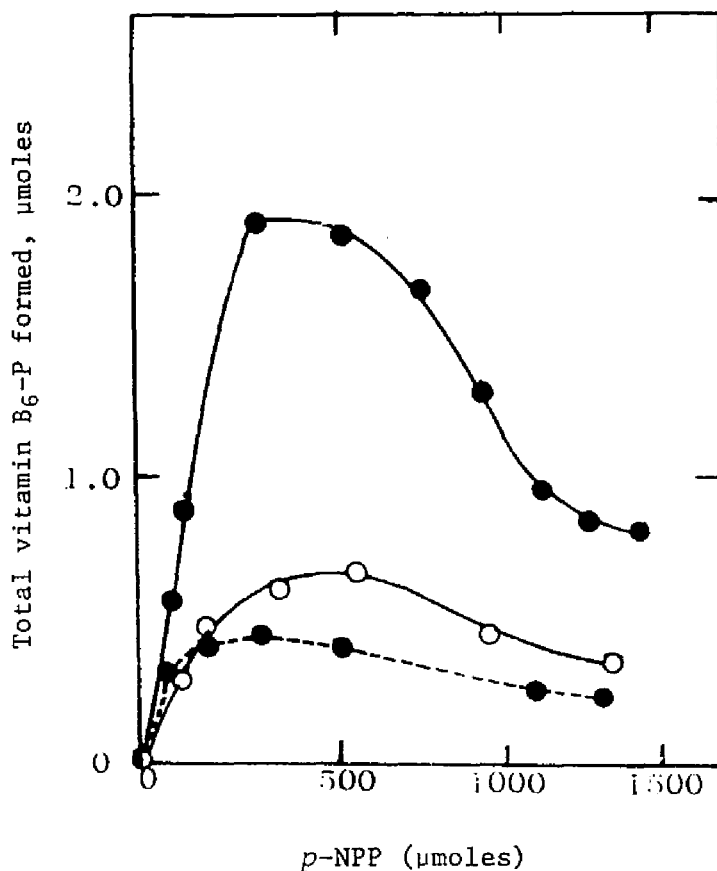
Fig. 9. Effect of p-NPP Concentration on Phospho-

rylation of Pyridoxine

The reaction was carried out under the standard con-
ditions 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 (--●--).

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to pyridoxal also exists.[67,157]. The presence of pyridoxine-P *in
vivo* was demonstrated in tissues[158] and microorganisms.[102]

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 ions[145] 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 B_6-P in the reaction mixtu-

re 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 B_6 phosphates was obtained in about 40%

yield using a suitable amount of bacterium in the reaction mixture.

The most excellent activity of vitamin B_6 as phosphoryl accep-

tor 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 B_6,

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 phos-

phatases could catalyze the phosphoryl group transfer to various

compounds which have alcoholic group[122)] Pyridoxine has two hydro-

xymethyl group which may be phosphorylated by the action of phospha-

tase. 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 B_6, pyridoxal-P, was performed through two pathways.[54] 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 12,000Xg 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 esti-
mate 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 µmoles of pyridoxine,
200 µmoles of phenyl-P, 300 µmoles 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 phenylhydra-
zine and apotryptophanase. In the following experiments, the term
mentioned as "total vitamin B_6-P" involved pyridoxine-P in addition
to pyridoxal-P. Pyridoxal was determined by phenylhydrazine method
reported by Wada and Snell.[75] 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 sys-
tems 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	Total vitamin B_6-P
			(μmoles)	
p-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
Phenyl-P	Intact cells	6.0	0.10	0.54
		8.6	0.21	0.25
	Cell free extract	6.0	0.17	0.50
		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.

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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
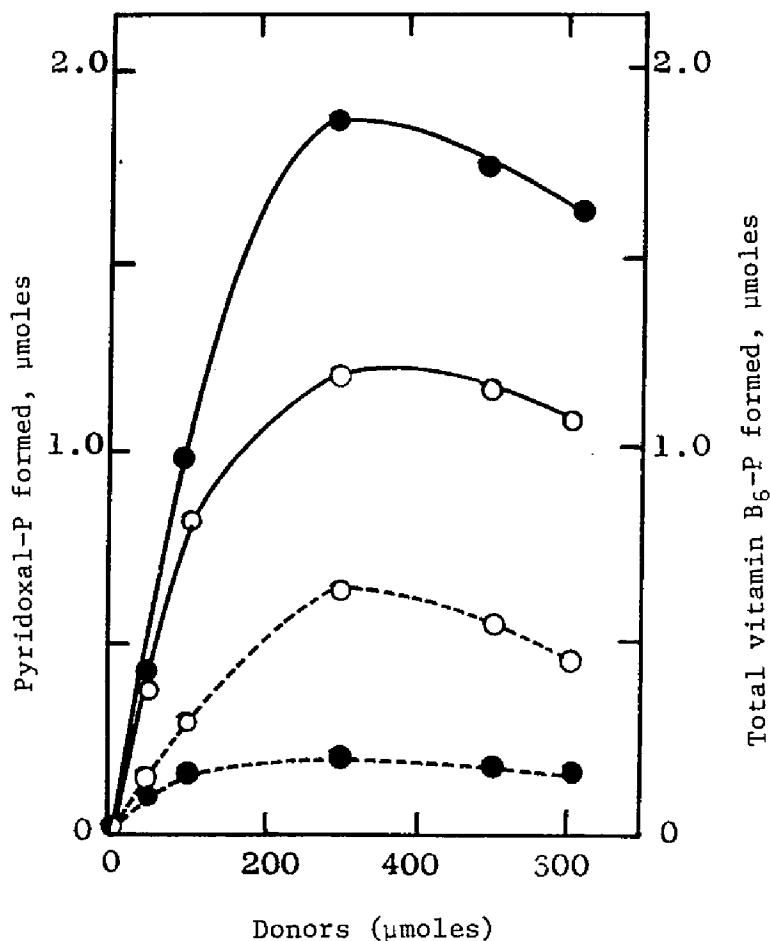
Fig. 1. Comparative Effects of Donor Concentration

on Phosphorylation of Pyridoxine

The reaction was carried out under the standard con-
ditions with 20 mg as dry weight of intact cells.
(●) p-NPP added, (○) phenyl-P added as phosphoryl donor
substrate.
------;pyridoxal-P formed. ————;total vitamin B_6-P formed.

of total vitamin B_6-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
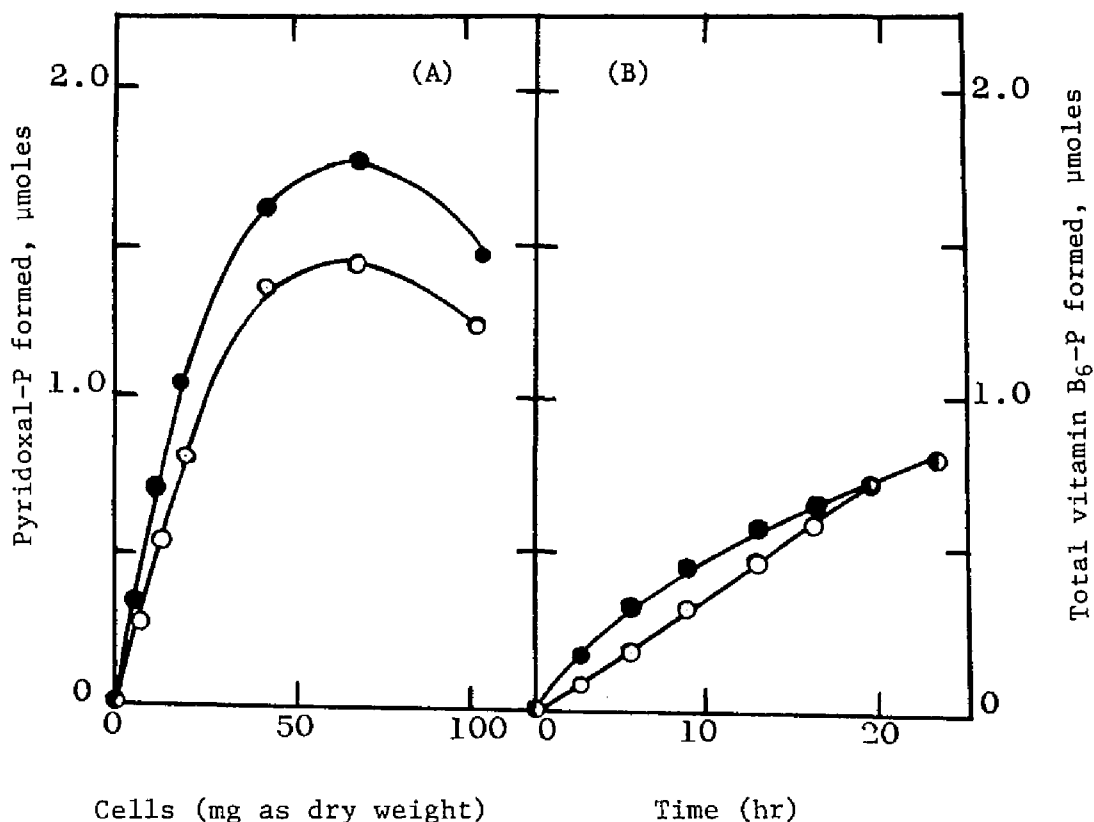
Fig. 2. Effects of Cell Concentration (A) and Reaction Time

(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 B_6-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

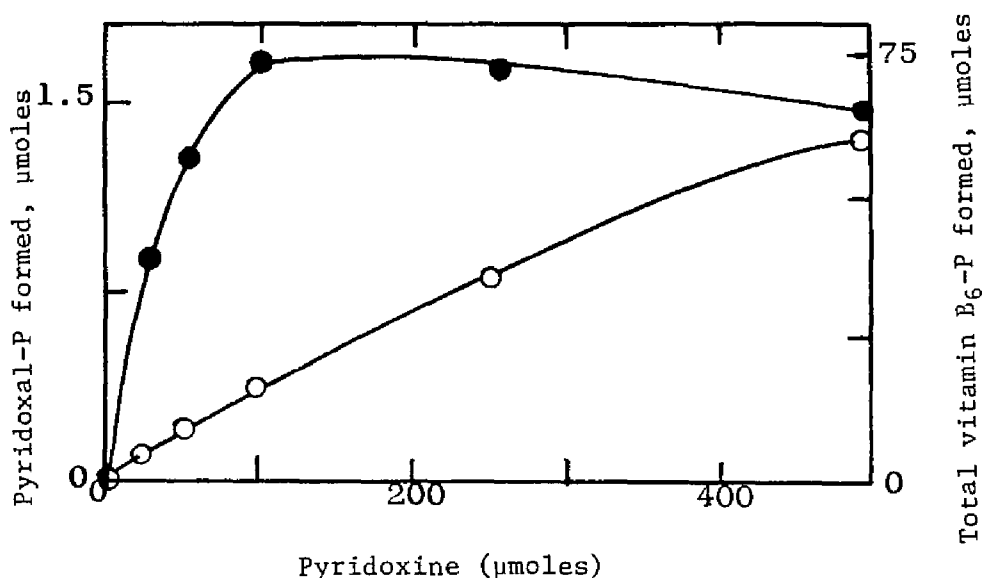in reaction mixture resulted in the decomposition of both the phos-

Fig. 3. Effect of Pyridoxine Concentration on Phosphoryla-

tion 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 B_6-P formed.

---

phorylated products. The formations of pyridoxal-P and total vita-

min B_6-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 B_6 phosphates is shown in Fig. 3. The amount of pyridoxal-P

formed increased with the increased addition of pyridoxine as phos-

phoryl 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
$3 \times 10^{-3}M$	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 $10^{-3}M$ 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 B_6-P were examined (Table II). Cupric ion showed the stimulating effect only on pyridoxal-P formation; Hg^{2+}, Zn^{2+} 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 frac-

tions 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 per-

formed 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

chromatogram[174] and paper electrophoregram[160] and to coincide with

the Rf value of authentic specimen. Further investigation with

infrared analysis[88] gave additional confirmation. The ultraviolet

spectra were also identical with those of authentic pyridoxal-P,

which had λ_{max} at 330 mμ and 388 mμ in 0.1 M phosphate buffer, pH

7.0, at 307 mμ and 388 mμ in 0.1 N sodium hydroxide, and at 295 mμ

in 0.1 N hydrochloric acid.

Propeties of Enzymes Concerning to Conversion of Pyridoxine

to Pyridoxal-P.

From the results mentioned above, it was apparent that pyrido-

xal-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 oxida-

tion of pyridoxine to pyridoxal and that of pyridoxine-P to pyrido-

xal-P were catalyzed by the two distinct enzymes, pyridoxine dehydro-

genase 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 oxidatiors

of pyridoxine and pyridoxine-P were investigated using the cell-free

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

a) Pyridoxine-P Oxidase.

Partial purification of enzyme. For the separation of pyrido-
xine-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-mercaptoetha-
nol. 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 addi-
tion 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
p-NPP		118	130	119
Phenol		101	96	6
p-NP		97	100	5

The reaction mixture containing 0.5 μmole of pyrido-xine-P, 200 μmoles of Tris-HCl buffer of pH 8.6, 100 μmoles 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.

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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[61] 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 μmole
Omitted pyridoxine	0.00
Omitted NADP	0.04
Omitted Enzyme	0.00
Boiled enzyme	0.01

The complete system contained 10 μmoles of pyrido-xine, 200 μmoles of Tris-HCl buffer of pH 8.6, 1 μmole of NADP and 27 mg protein of cell free extract in a to-tal 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.

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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 alka-line 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 trans-phosphorylation with the intact cell systems might be differentiated
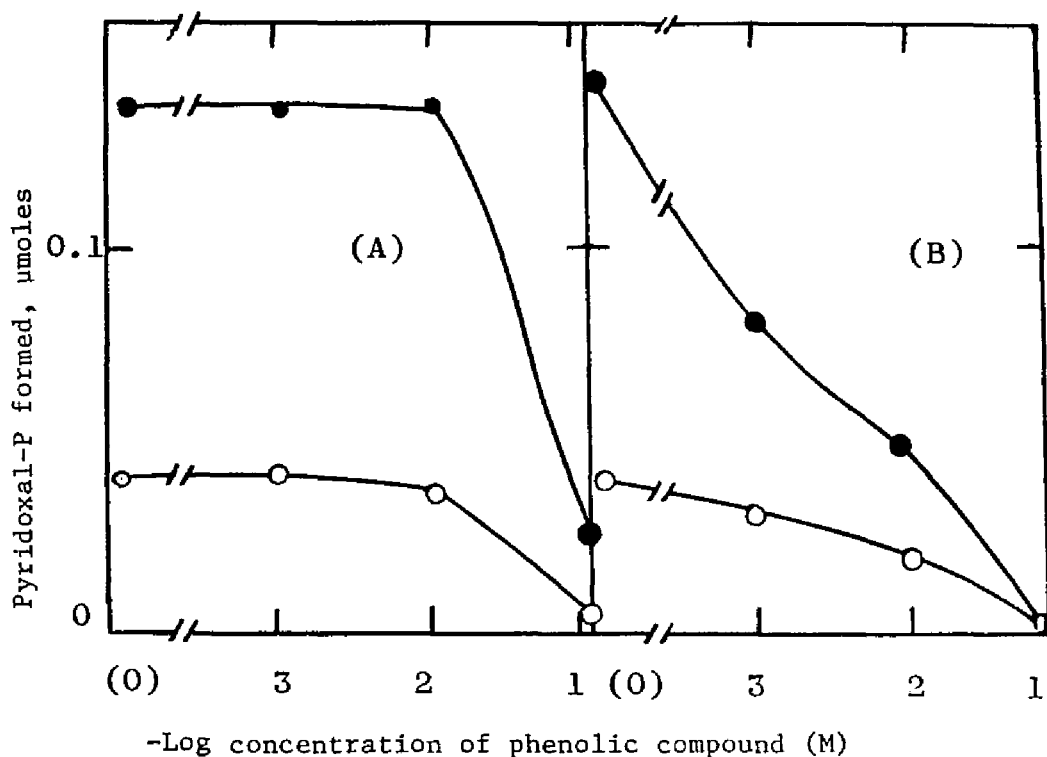
Fig. 4. Effect of Phenolic Compound on Pyridoxine Dehydrogenase

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.

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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^{-3}M
metal ion and 11.3 mg protein of cell-free extract.

DISCUSSION

The enzymatic conversion of free forms of vitamin B_6 to the
active form was demonstrated in the primitive state of the studies
on vitamin B_6 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 differenti-

ate the effect of p-NPP and phenyl-P on the pyridoxal-P formation

from pyridoxine through the transphosphorylation. Though the acti-

vity 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 pro-

blem for the control mechanism of pyridoxal-P formation from pyri-

doxine.

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 phosphoryl donor 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 cource of investigation on the micro-

biological determination of vitamin B_6.[21,22,165] The enzyme, which

catalyzed the irreversible oxidation of pyridoxamine-P forming pyri-

doxal-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 transaminases[166-168] 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 transami-

nation.[52,170,171]

Recently, an enzyme "pyridoxamine-P transaminase (pyridoxamine-

P: 2-oxoglutarate aminotransferase)" which catalyzed the transamina-

tion 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 partial-
ly purified from *Clostridium kainantoi* and some properties were
investigated roughly.[91)]

Further purification of pyridoxamine-P transaminase was attemp-
ted from *Clostridium kainantoi*, and, then, the further characteriza-
tion 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 gluco-
se, 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 commer-
cial sources.

Activity Measurement. Enzymatic activity was routinely measu-
red in a system with a final volume of 1.75 ml containing 5 μmoles
of pyridoxamine-P, 5 μmoles of α-ketoglutaric acid, 250 μmoles of
Tris-HCl buffer, pH 7.0, 50 μmoles 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 precipi-
tate 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 mμmoles of pyridoxal-P in 60 min at 37°C.

Analytical Method. Pyridoxal-P was determined by phenylhydra-
zine 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 purifi-
cation procedures.

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

500 liters of the culture medium. The cells were harvested by a
continuous centrifugation at 10,000xg and washed twice with deioni-
zed 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 12,000xg 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 cen-
trifugation at 10,000xg for 20 min and discarded. The ammonium
sulfate concentration was then increased to 75% saturation by fur-
ther addition of solid ammonium sulfate. After stirring for 30 min,
the precipitate was collected by centrifugation at 10,000xg 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
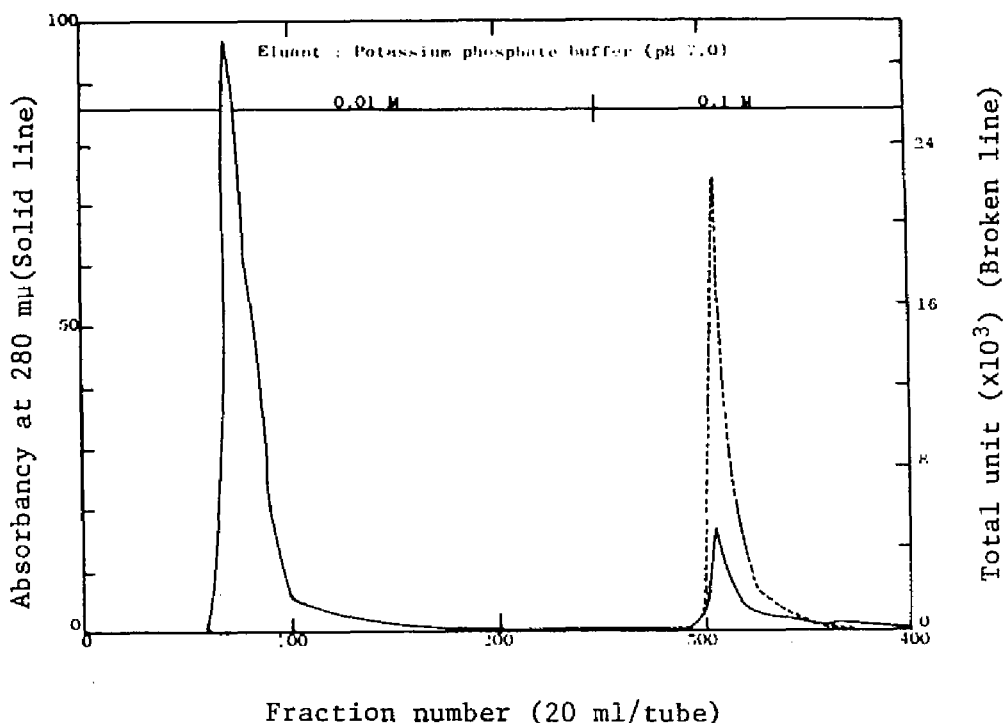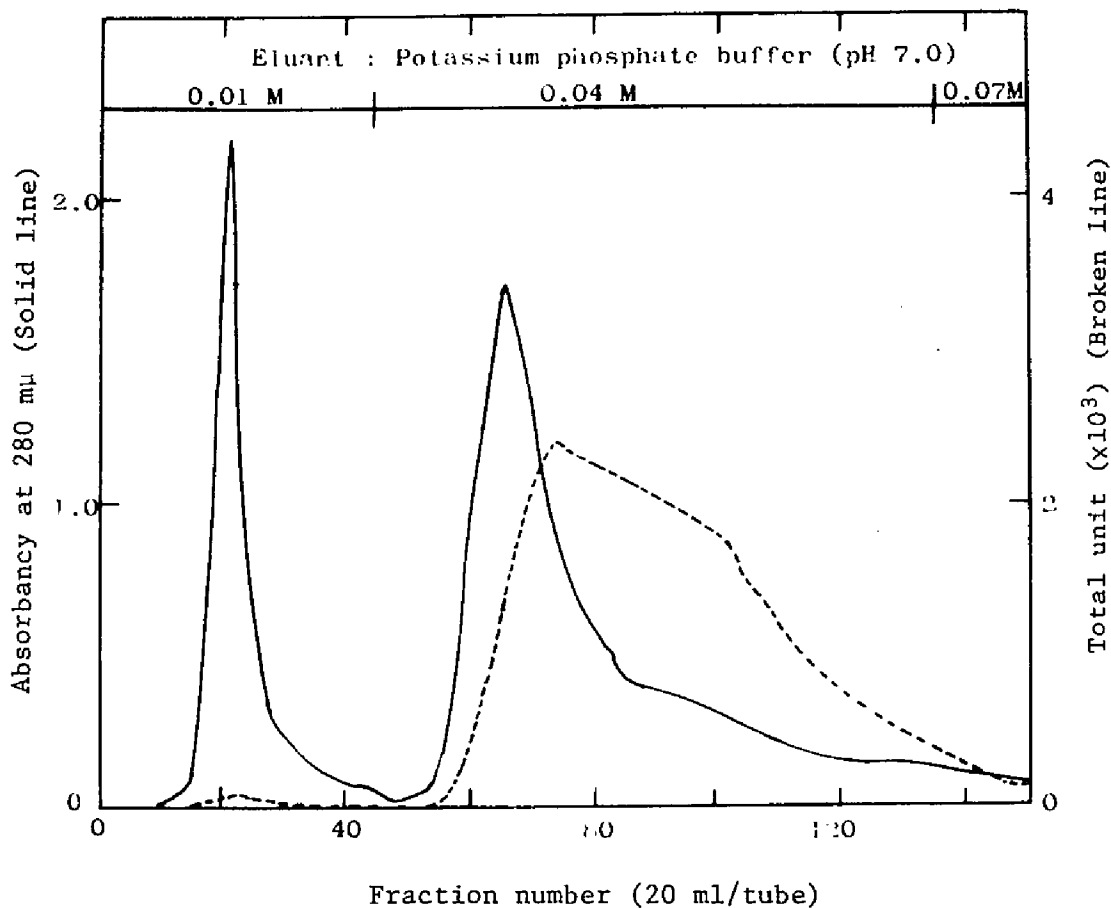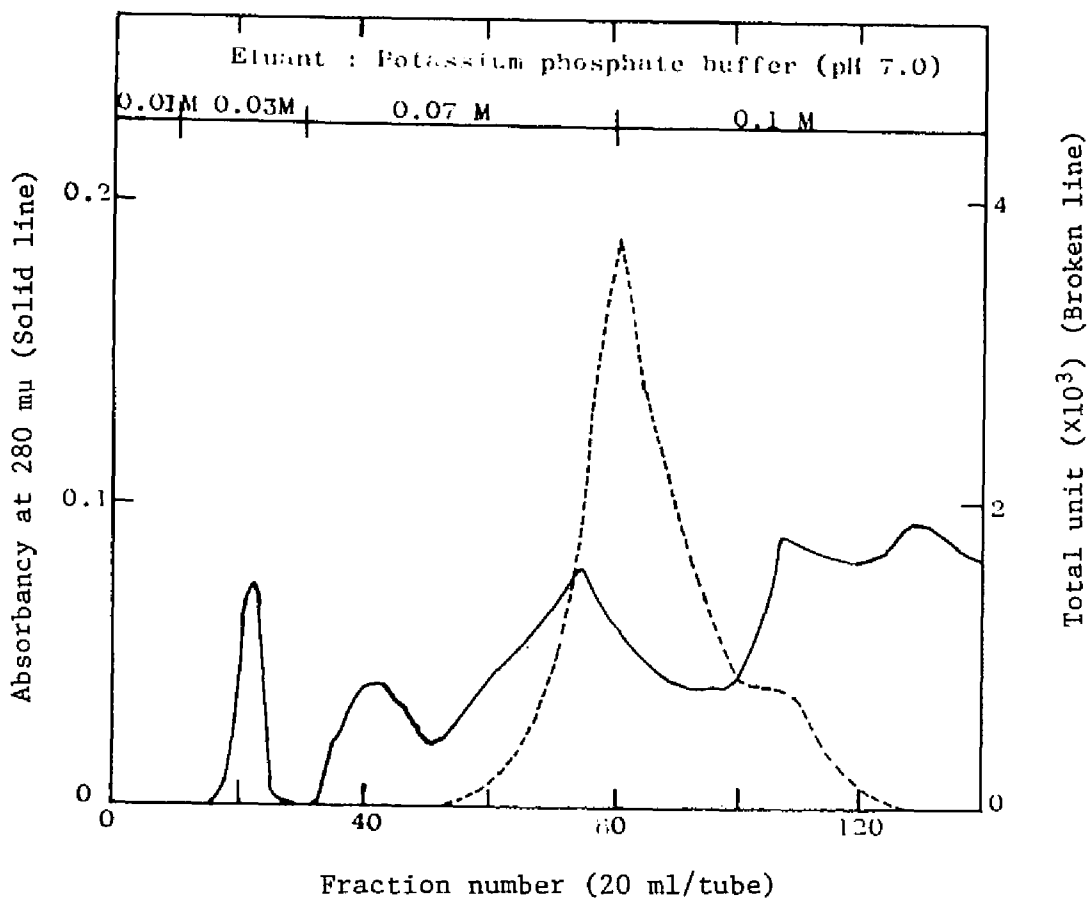
Fig. 1. Chromatography of Pyridoxamine-P Transaminase on

DEAE-Cellulose Clumn

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

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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.

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solution was concentrated by the addition of solid ammonium sulfate

to 80% saturation. The precipitate obtained by centrifugation at

12,000x*g* 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-satura-
ted 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 pota-
ssium 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 solu-
tion. 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 chromatogra-
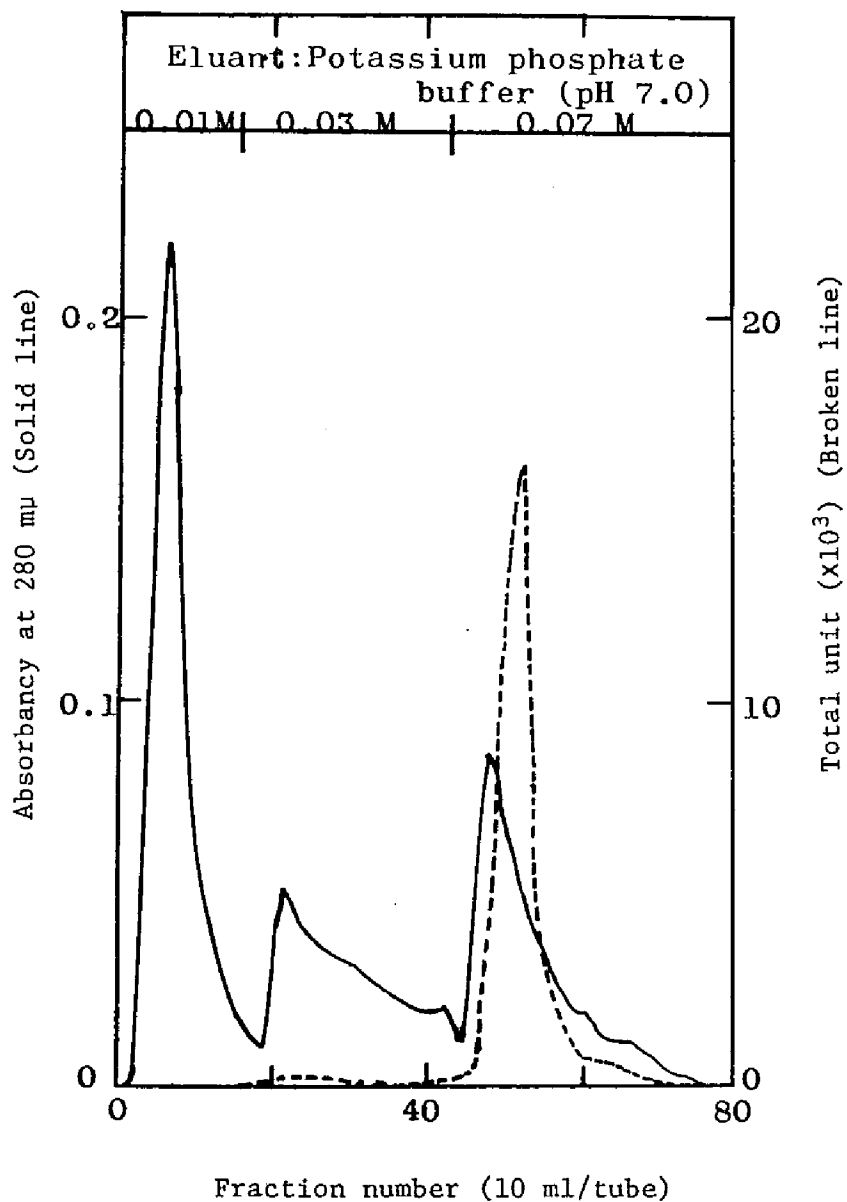phy. The absorbent equilibrated with 0.01 M potassium phosphate

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
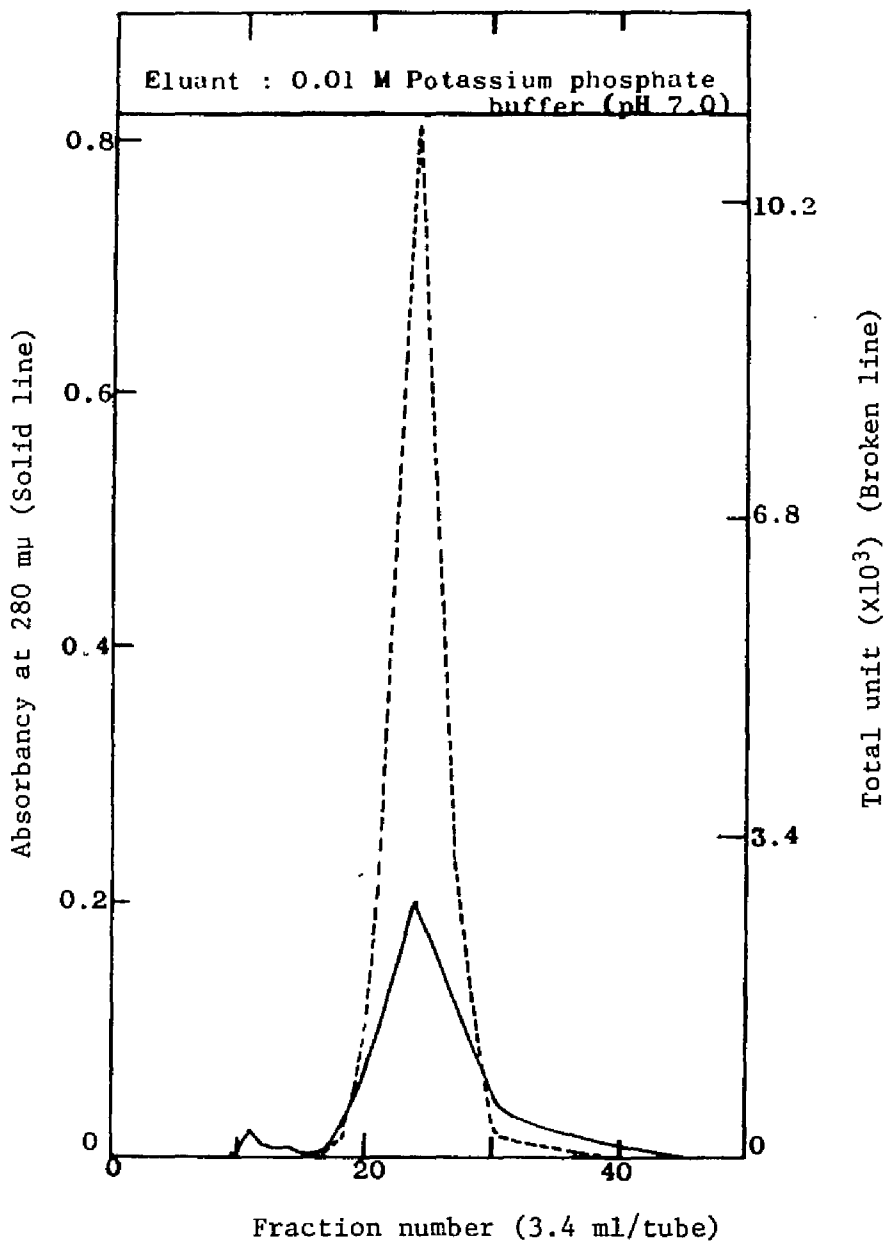assay conditions.

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 collo-

dion bag for 4 hr to 0.7 ml. The purified enzyme preparation obtain-

ed 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 pre-

paration.

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 ana-

lysis 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 mμ and 280 mμ 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
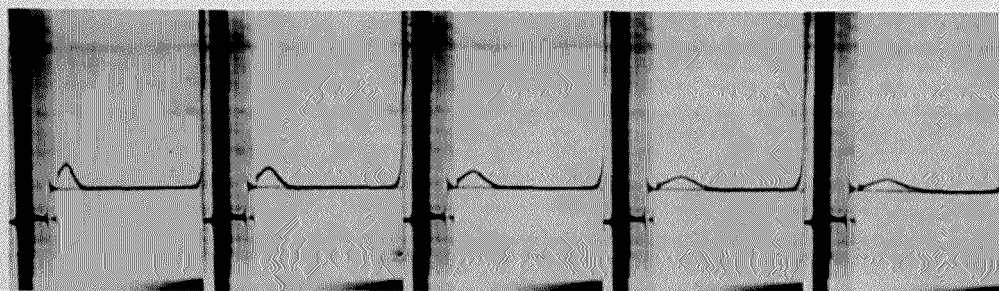
Fig. 6. Ultracentrifugal Analysis of Purified Pyridoxamine-P

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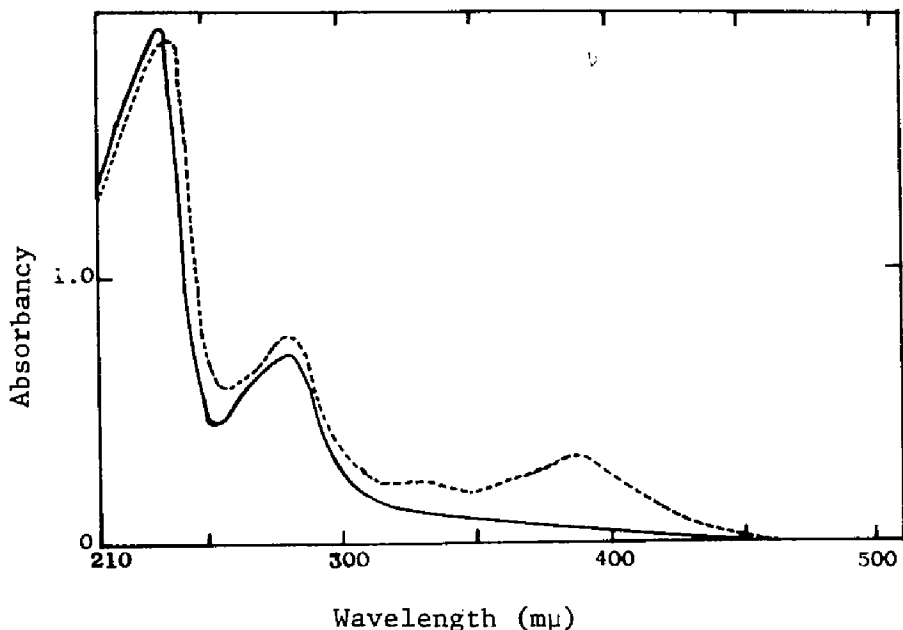
Fig. 7. Absorption Spectrum of Purified, Pyridoxamine-P

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 mµ and 388 mµ. It has been recently demonstrated that

the enzymes contained pyridoxal-P as the prosthetic group showed

the typical absorption maximum near at 410 mµ by the formation of

aldimine bond between pyridoxal-P and lysine in apoenzyme[175)]

These pyridoxal-enzymes were also classified to three groups[176)]

which were 1) cystathionase type[177)] 2) glutamic acid-oxalacetic acid

transaminase type[178)] and 3) phosphorylase type[179)] 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 mμ 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 mμ 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 Cu^{2+} and Ni^{2+}, 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 Hg^{2+} 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 3×10^{-4}M. 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,

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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 μmoles of pyridoxamine-P, 5 μmoles of α-ketoglutaric acid, 250 μmoles of Tris-HCl buffer, pH 8.0, 3 μmoles of amino compound and 1 μg 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 B_6 and D-amino acid in clostridia.
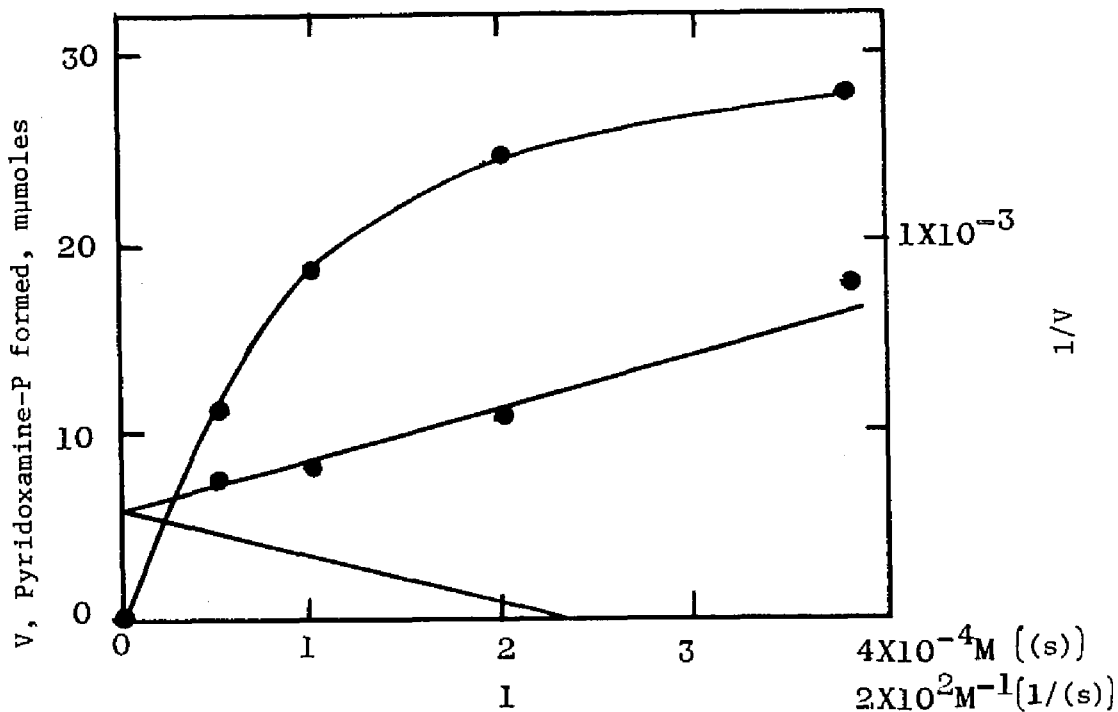
Fig. 8. Effect of D-Glutamic Acid Concentration on the
Formation of Pyridoxamine-P by Pyridoxamine-P Transaminase

The reaction mixture containing 0.21 μmoles of pyridoxal-P.
50 μmoles of EDTA, 100 μmoles of potassium phosphate buffer, pH 8.0
and 2 μg 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 dec-

rease 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 Burk[143] was found to be 9×10^{-3}M. Though, the Km value for D-glu mic acid is higher than that for pyridoxamine-P (4.0×10^{-6}M), α-keto-glutaric acid (1.5×10^{-5}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 pyri-doxal-P and D-glutamic acid by the enzyme may give a significant problem in the vitamin B_6 metabolism.

SUMMARY

The enzyme which catalyzed the transamination between pyrido-xamine-P and α-ketoglutaric aicd was purified from the cell-free extract of *Clostridium kainantoi*. The purification procedures involved ammonium sulfate fractionation, protamine sulfate treat-ment 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 B$_6$ Derivative,

Pyridoxine-G

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

INTRODUCTION

Recently, the anabolic and catabolic transformation of vitamin

B$_6$ have been investigated with microorganisms and mammalian tissues

by several groups of workers. On the enzymatic conversion of pyri-

doxine to the active form of vitamin B$_6$, 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 coworkers[69] 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 B$_6$ in microorganisms, one derivative of vitamin B$_6$ was

detected in the cultural filtrate of certain bacteria which were

grown in the medium containing sucrose as carbon source and pyrido-

xine. 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 inves-.
tigated 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 har-
vested 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 pyridoxi-
ne hydrochloride (*ca.* 600 µmoles as pyridoxine), 250 mg of sucrose,
800 µmoles 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 B_6.
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 satu-
rated with 0.2 M acetate buffer of pH 5.0 (Solvent C). Solvent C
was mainly used in the following experiments with ascending chroma-
tography 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°C[160] or with 0.1% dichloroquinonechlorimide in ethanol
followed by dilute ammonium hydroxide solution.[121]

Assay of Vitamin B_6 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 in-
dophenol formed by vitamin B6 and dichloroquinonechlorimide fades

rapidly upon exposure to light. After separation by paper chroma-
tography or electrophoresis, vitamin B_6 compounds on the paper
were determined by diazotized p-AAP method[120] with slight modifi-
cation 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 concent-
rated 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
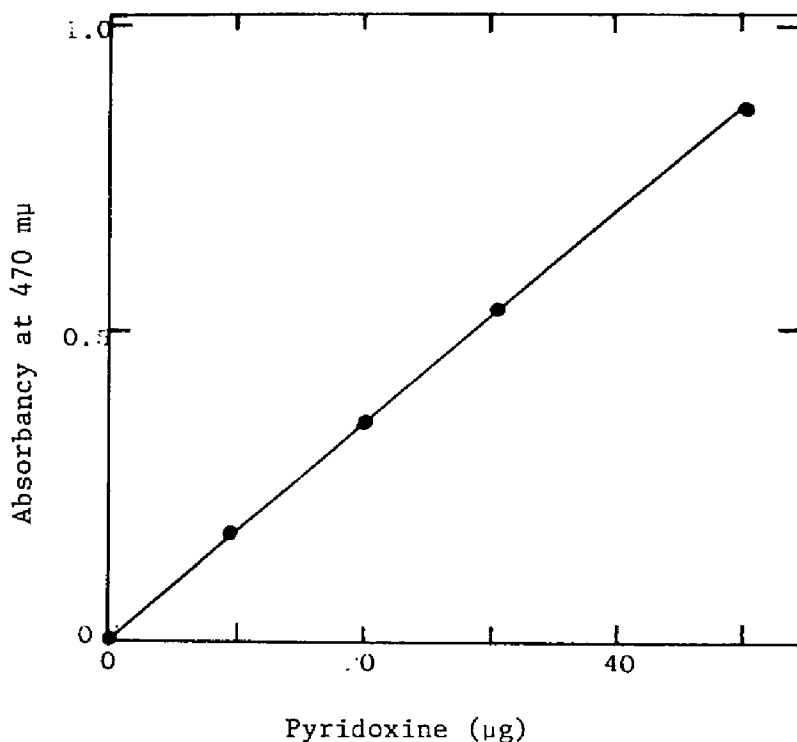diazo reagent was added and the color was developed as described
above.

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 deriva-

tive of vitamin B_6 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 Rf 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% ethanol

containing 2% ammonium hydroxide at a flow rate of 2 ml per min.
The active fractions were evaporated to dryness under reduced pressu-
re 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 preci-
pitates on cooling, which were separated from mother liquor by fil-
tration 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 mixtu-
re by cation exchange resins such as Amberlite IRC-50[30]) and Dowex
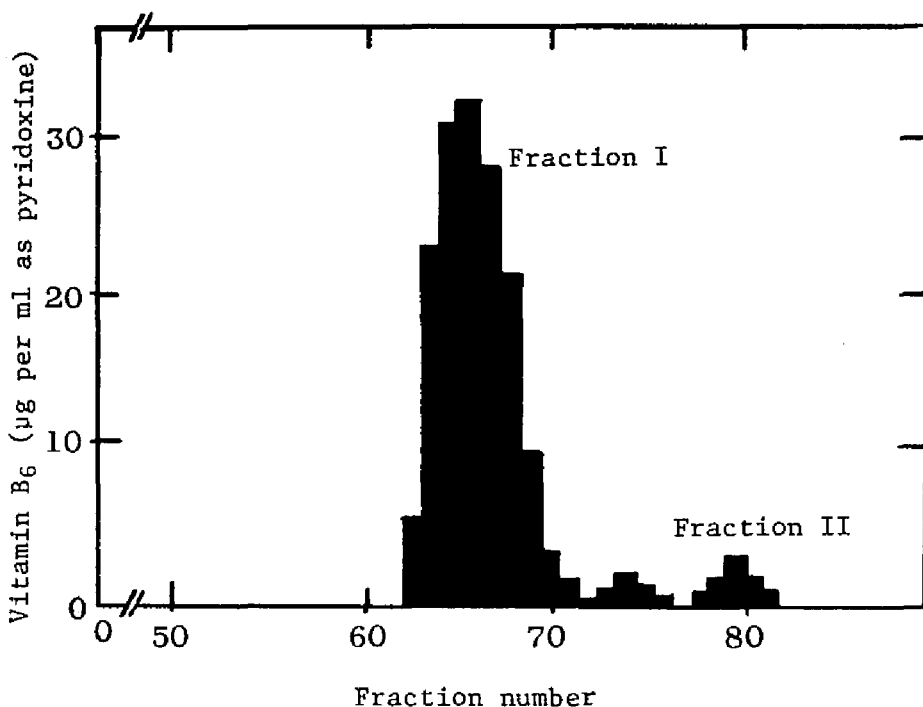50W[100]) failed to play sufficient role for the separation of

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 elec-

tronic properties and of relative small amount of pyridoxine-G.

Characterization of pyridoxine-G. With the powder of pyrido-

xine-G, the identification of the moieties of sugar and vitamin B_6,

and the brief characterization were performed.

The results with the employments of diphenylamine method,[180]

cysteincarbazole-sulfuric acid method[181] and Somogyi-Nelson method[182]

revealed that the sugar moiety of pyridoxine-G may be not ketose but

aldose, suggesting the possibility of glucose. Table I shows the

-122-

Table I. Paper Chromatography of Sugars

| Sugar | Solvent system | |
	Phenol:1% NH₄OH (4:1)	Pyridine:H₂O: NH₄OH (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 Rf values.

result of paper chromatography of hydrolyzate. The Rf 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 method[183] 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 B_6. Results in Table II show that vitamin B_6 of hydrolyzed pyridoxine-G is pyridoxine.
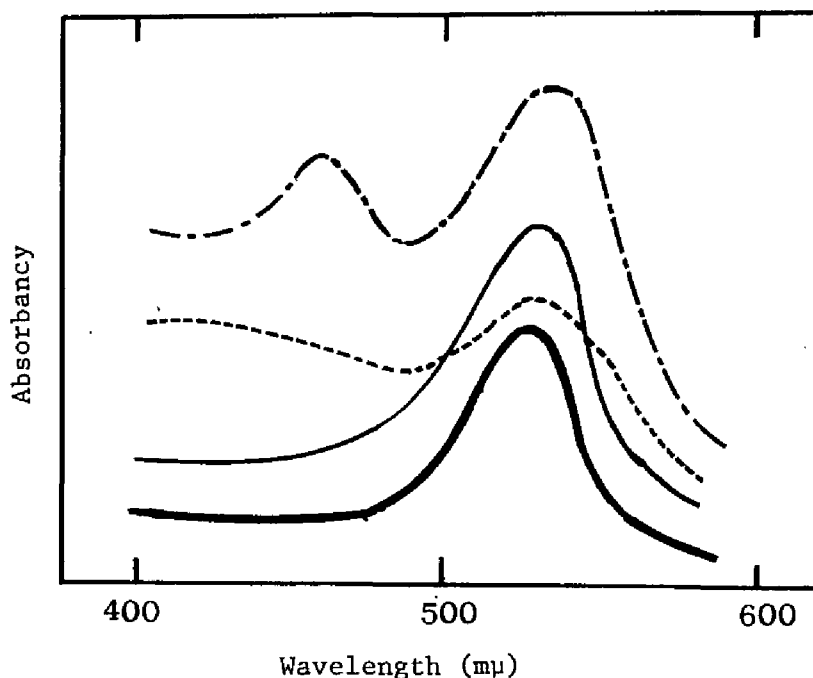
Fig. 3. Absoption Spectra of Sugars by Carbazole-Sulfuric

Acid Method.

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

━━━; Pyridoxine-G, ───── ; Glucose, ─·──·─ ; Fructose, ------- ; Galactose.

The component of the new derivative of vitamin B_6 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 method[184] 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 A*	Solvent B	Solvent 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 B$_6$ was detected by diazotized p-AAP and presented the Rf values.

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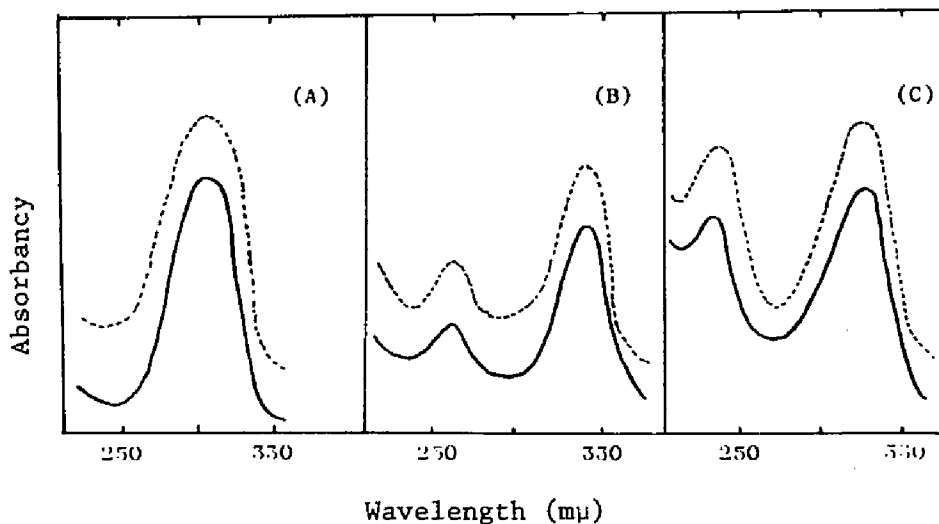
Fig. 4. Ultraviolet Absorption Spectra of Pyridoxine-G and

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.

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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

-126-

Table III. Activity of Pyridoxine-G Formation in

Bacteria

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

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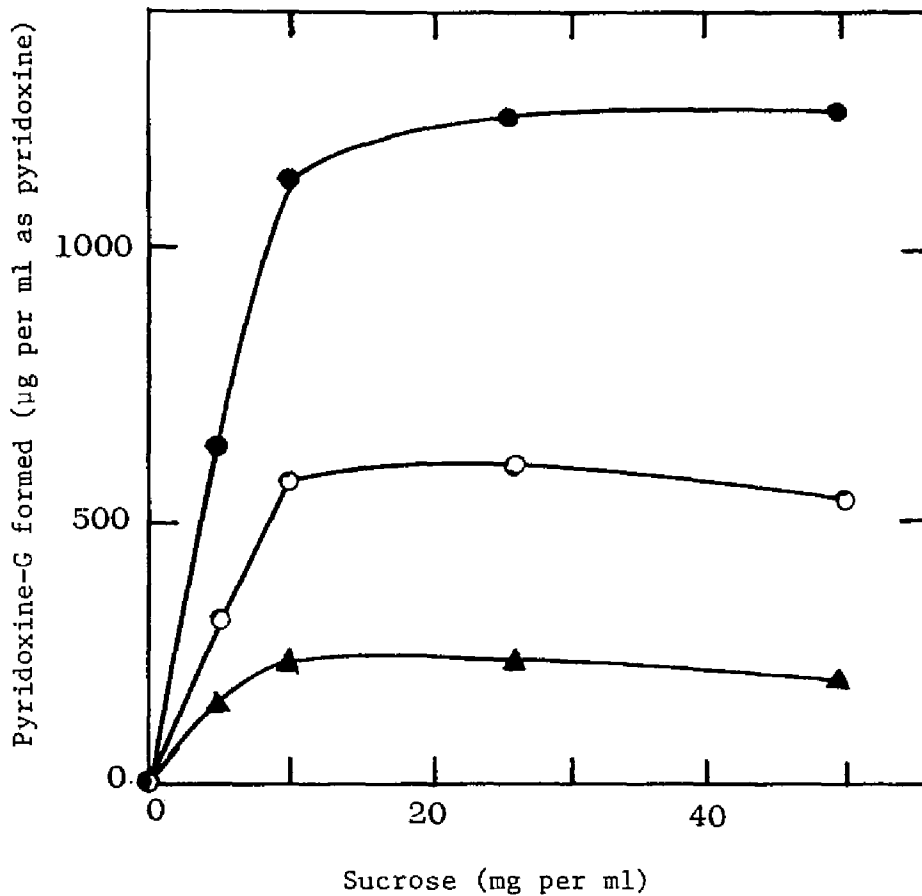
Fig. 5. Effect of Substrate Concentration on Pyridoxine-G

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.

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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-
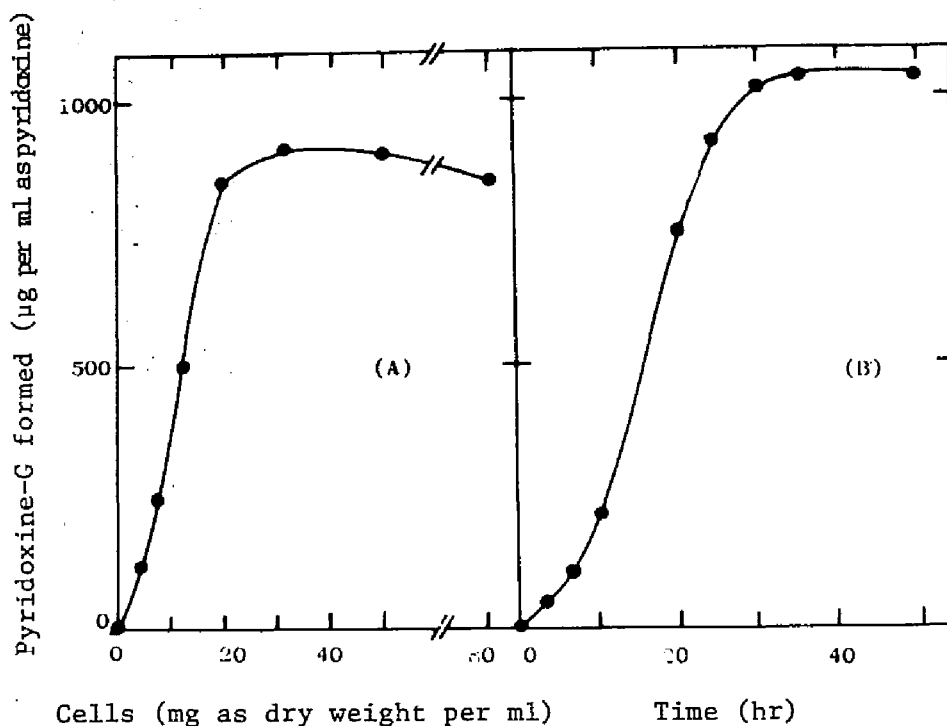
Fig. 6. Effects of Cell Concentration (A) and Incubation Time

(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).

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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 B_6. The transgluco-
sidation 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 B_6
have been chemically synthesized. Recently, the chemical synthesis
of the conjugated form of vitamin B_6 such as fatty acid esters,[185]
condensates with amino acid[186,187] and those with other vita-
mins[188,189] was also reported. On the other hand, pyridoxic acid[107]
and other oxidative products[69] were found in the biological degra-
dation of vitamin B_6. Several conjugates which include glucuronide
of pyridoxine,[190] pyridoxine- or pyridoxal-3-sulfate[191] and
indole-pyridoxal complex,[192] have also been reported as naturally
occurring ones.

The sugar derivative of vitamin B_6, however, has been investi-
gated 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 liver[195] and
various microorganisms.[196~198] It has been suggested that ribofla-
vinyl 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 microorganisms[199] 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 B_6 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 acid[187] and nucleotides[200] 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 pyrido-xine-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 linkage[203] 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 Sakamoto[70] 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 acid[204] $i.e.$, 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 test[121] 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 complex[204] 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 soltution
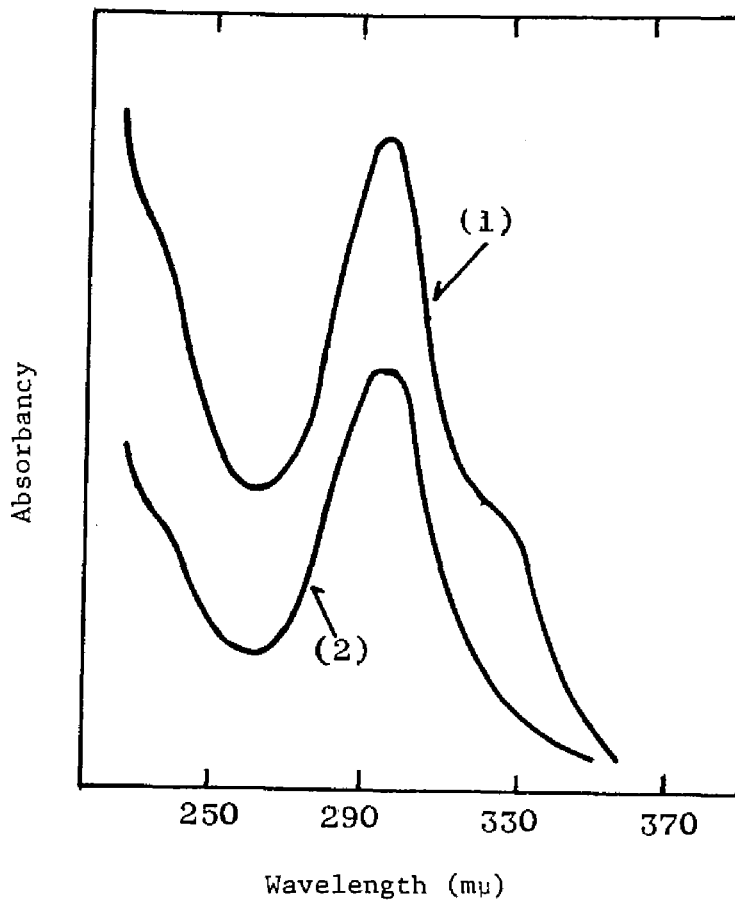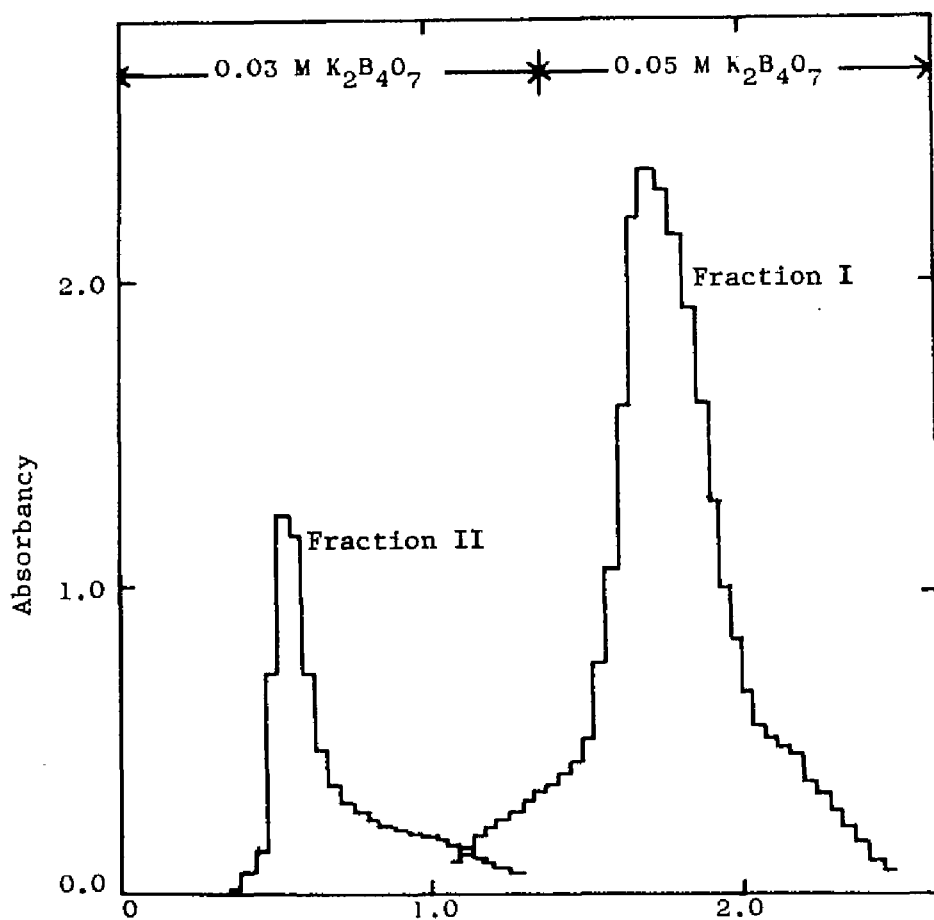
Fig. 1. Ultraviolet Absorption Spectra of

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, respective-
ly.
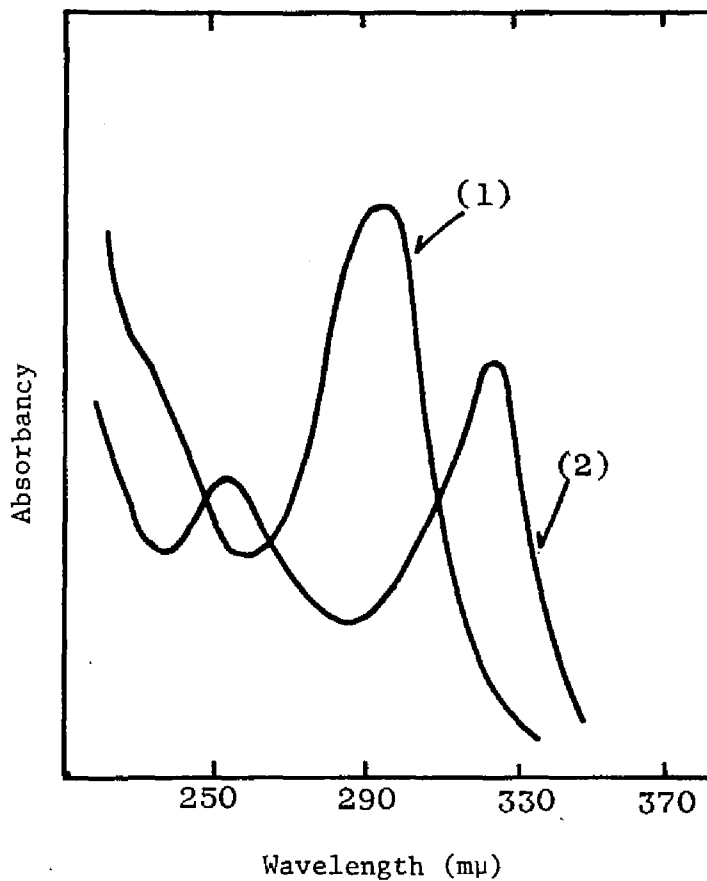
Fig. 3. Ultraviolet Absorption Spectra of

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 bicar-

bonate, 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) homoge-

nized 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 dissovled in anhydrous ether. Introduction of dry hydro-

gen chloride gas to the ether solution produced the precipitates of

hydrogen chloride salt of pyridoxine-G hexaacetate. After recrys-

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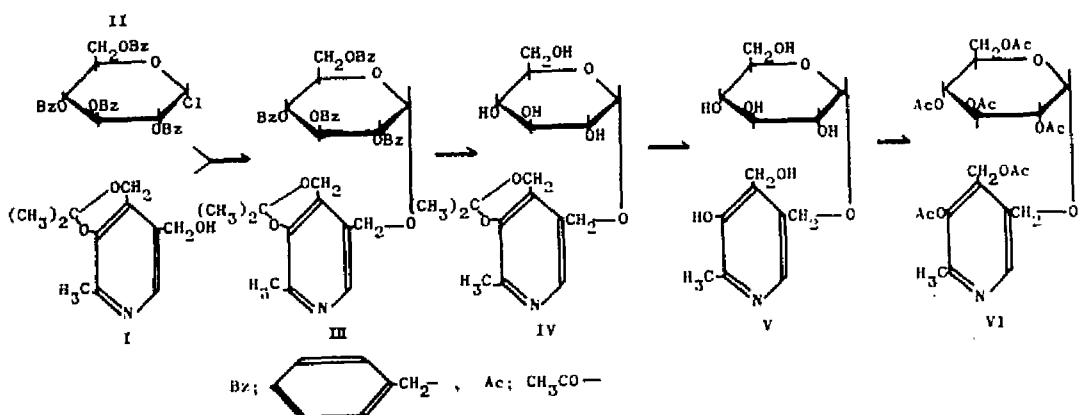
Fig. 4. Route of Chemical Synthesis of Pyridoxine-5'-α-D-G

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 Rf 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*.
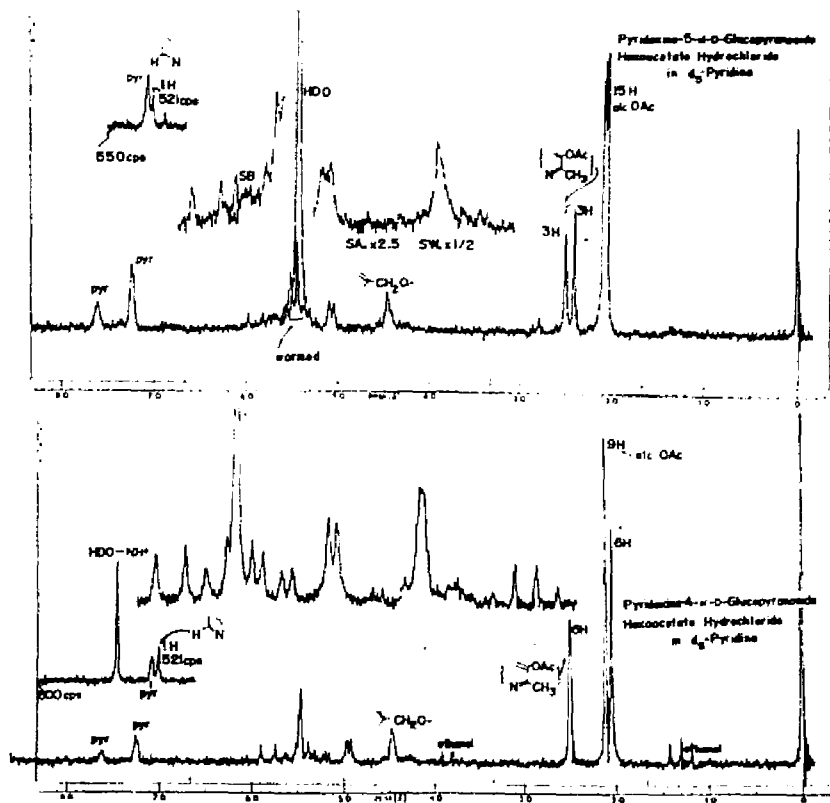
-142-

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 pyrido-

xine-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.

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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 involv-
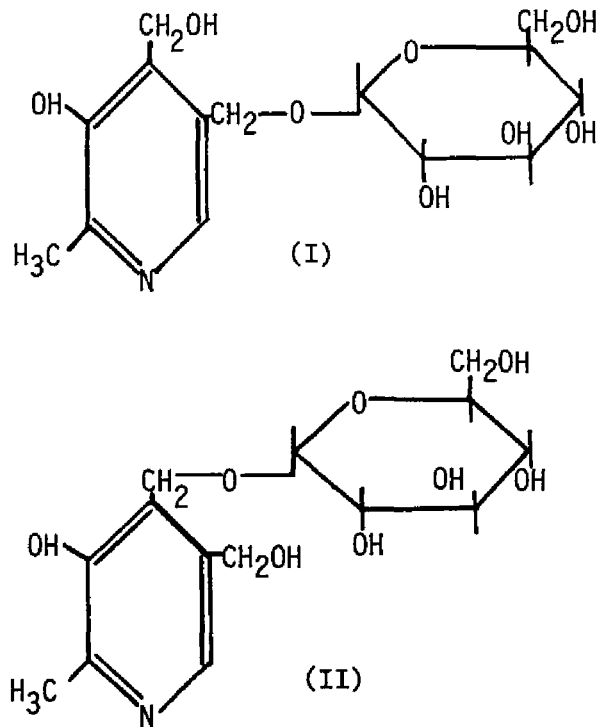ing a small amount of pyridoxine-4'-G (Fig. 6).

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

and Pyridoxine-4'-G (II)

The enzymatic hydrolysis of pyridoxine-G could not be comple-
ted 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 demonstra-
ted that the partially purified preparation of yeast α-glucosidase
could attack aryl-α-glucosides such as phenyl- and cresyl-α-gluco-
side. On the hydrolysis of riboflavin-α-glucoside, the slow break-
down has been reported by α-glucosidase preparation from baker's
yeast.[195)] Recently, Suzuki[211)] reported its remarkable hydrolysis
with α-glucosidase preparation from riboflavinyl glucoside forming
bacterium, *Leuconostoc mesenteroides*. The degradation of pyridoxi-
ne-G is observed in growing culture of *Micrococcus* sp.[199)] The
specific or nonspecific glucosidase which strongly attack the α-glu-
cosidic linkage of pyridoxine-G may be presented in such microorga-
nisms.

SUMMARY

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

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 B_6 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 atten-

tion. 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 B_6 concerning to the expanding

the ability of microorganisms. Then, it was found a few new meta-

bolic pathways which involved the phosphorylation of vitamin B_6 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-ketoglu-

taric acid and the formation of pyridoxine-G by transglucosidation

between pyridoxine and sucrose. These results will be summarized

below.

On the phosphorylation of vitamin B_6 group, phosphokinase system, capable of transferring the phosphoryl moiety of ATP to the free forms of vitamin B_6 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 B_6 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 B_6, 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 B_6 presented here provided the first clear-cut evidences for the identity of the activities of transphosphorylation and phophohydrolyzation.

Using the cells of organisms having the higher activity of the transphosphorylation, the accumulation of phosphate esters of vitamin B_6 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 resulted 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 B_6 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 B_6 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 B_6. 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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