Studies on Vitamin B₆ Metabolism in Microorganisms

Part. II. Pyridoxine-P and Pyridoxamine-P Oxidation (2)

Enzyme purification and properties

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Pyridoxine-P and pyridoxamine-P oxidase in the extract of *Alcaligenes faecalis* was purified and some properties of the enzyme were investigated. Several lines of evidence indicated that both pyridoxine-P oxidation and pyridoxamine-P oxidative deamination were catalyzed with a single enzyme. The enzyme is a flavoprotein, and the treatment of the enzyme with acid ammonium sulfate resolved the enzyme into apo- and coenzyme. Flavin mononucleotide reactivated the apoenzyme for the oxidation of both substrates. Physiological role of the pyridoxine-P and pyridoxamine-P oxidase was suggested in relation to the transformation of vitamin B₆ in microorganisms.

INTRODUCTION

In the previous paper,¹⁾ the distributions of pyridoxine-P and pyridoxamine-P oxidase in various strains and genera of bacteria were investigated. From the results of these investigations demonstrating the existence of the pyridoxine-P and pyridoxamine-P oxidases, it would be interesting to examine whether these activities are catalyzed by single enzyme, or by two different enzymes. Wada and Snell²⁾ reported that the two enzymatic activities in rabbit liver are characteristic of a single enzyme from their results of comparative studies of purification, pH optima, and rates of heat inactivation.

In the present paper, pyridoxine-P and pyridoxamine-P oxidase in *Alcaligenes faecalis* was purified to a great extent and the properties were compared with that obtained by the previous workers in order to approach

the problem of the identity of pyridoxine-P and pyridoxamine-P oxidase. Attempts to separate them from each other were not successful. In microorganisms, the evidences for the identity of two enzymatic activities were also presented.

METHODS AND MATERIALS

Culture.

Alcaligenes faecalis was grown and harvested as previously reported.¹⁾

Activity measurement.

Twenty five-ml Erlenmeyer flasks containing enzyme, substrate (500 μ moles of pyridoxine-P or of pyridoxamine-P), and Tris·HCl buffer (pH 8.6, final concentration 0.2M) in a total volume of 2.5 ml were shaken at 30°C for 30 minutes. The reaction was stopped by the addition of 0.5 ml of 50% (weight per volume) trichloroacetic acid.

Specific activities of the pyridoxine-P and pyridoxamine-P oxidase are expressed as units per mg of protein where one unit is defined as the amount of protein that forms one μ mole of pyridoxal-P in one hour at 30°C.

Analytical methods.

Pyridoxal-P was determined as noted in previous

The following abbreviations were used; pyridoxine-P, pyridoxine 5'-phosphate, pyridoxamine-P, pyridoxamine 5'-phosphate and Tris, Tris-(hydroxymethyl)-aminomethane.

1) S. Yamamoto, T. Tochikura and K. Ogata, This Journal, **29**, 200 (1965).

2) H. Wada and E. E. Snell, *J. Biol. Chem.*, **236**, 2089 (1961).

paper.¹⁾ Ammonia was determined colorimetrically³⁾ after its separation from the reaction mixtures by the technique of Conway.⁴⁾ The other analytical methods were described in previous paper.¹⁾

Chemicals.

Pyridoxine-P and pyridoxamine-P were prepared from pyridoxamine by the phosphorylation with the mixture of phosphorous pentoxide and phosphoric acid.⁵⁾ Cyclic flavin mononucleotide phosphate was prepared by the method of Kilgour and Huenenkenes.⁶⁾ DEAE-Cellulose was the product of Brown, Germany. Hydroxylapatite was prepared by the method of Tiselius et al.⁷⁾ The other chemicals were commercial products.

RESULTS

Purification of Pyridoxine-P and Pyridoxamine-P Oxidase from *Alcaligenes faecalis*.

All operations were performed at 0~5°C.

(1) **Extraction.** *Alcaligenes faecalis* cells were suspended in 0.02 M potassium phosphate buffer, pH 7.0 containing 0.01% 2-mercaptoethanol. The cell suspension was disrupted for 20 minutes with Kaijo-Denki 19 Kc/s ultrasonic oscillator, then centrifuged at 10,000×g for 30 minutes.

(2) **Protamine sulfate treatment.** To the supernatant solution (Fraction I) was added freshly prepared 1.5% solution of protamine sulfate in 0.1 M potassium phosphate buffer, pH 7.0, sufficient to supply 15 mg of protamine sulfate for each 100 mg of extracted protein. The mixture was stirred for 15 minutes, centrifuged, and residue discarded.

(3) **Ammonium sulfate fractionation.** The supernatant solution (Fraction II) was fractionated by an addition of solid ammonium sulfate to yield the fractions insoluble between 0 to 30%, 30 to 50%, 50 to 70% and over 70% levels. During the ammonium sulfate fractionation, the pH was kept to 7.0 with dilut-

ed ammonium hydroxide. The mixture was stirred for 30 minutes, then the precipitated protein was centrifuged out. In each case, the precipitate was dissolved in 0.05 M potassium phosphate buffer (pH 7.0). The major portion of activity was present in the 30 to 50% saturation. The enzyme solution was dialyzed for 30 hours against four changes of 500 volumes each of 0.05 M potassium phosphate buffer, pH 7.0, with stirring (Fraction III).

(4) **DEAE-Cellulose column chromatography.** The dialyzed Fraction III was poured over a 30×300-mm column of DEAE-cellulose equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. After the Fraction III was passed through, the column washed with the same buffer. The protein was eluted stepwisely with 0.05 M, 0.1 M and 0.2 M potassium phosphate buffer at pH 7.0. The elution pattern was quite reproducible. The active fractions eluted with 0.1 M potassium phosphate buffer (Fraction IV) were collected and the active protein concentrated by the addition of solid ammonium sulfate according to the method described in the ammonium sulfate fractionation. The precipitates obtained by centrifugation were dissolved in a small amount of 0.001 M potassium phosphate buffer at pH 7.0, and dialyzed for 30 hours against four changes of 500 volumes each of 0.001 M potassium phosphate buffer, pH 7.0 with stirring (Fraction V).

(5) **Hydroxylapatite column chromatography.** The dialyzed Fraction V was subjected to hydroxylapatite column chromatography. The hydroxylapatite was equilibrated with 0.001 M potassium phosphate buffer, pH 7.0. The Fraction V was passed through the column, then the protein was stepwisely eluted with 0.001 M, 0.005 M, and 0.01 M potassium phosphate buffer, pH 7.0. The active fractions eluted with 0.005 M potassium phosphate buffer at pH 7.0 were pooled and concentrated by the lyophilization (Fraction VI).

3) S. Akamatsu, *J. Biochem. (Japan)*, **39**, 203 (1952).

4) E. J. Conway, "Microdiffusion analysis and volumetric error," D. Van Nostrand Company, New York, 1950.

5) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **76**, 169 (1954).

6) G. L. Kilgour and F. M. Huenenkenes, *J. Am. Chem. Soc.*, **79**, 2256 (1957).

7) A. Tiselius, S. Hjertén, Ö. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

The above procedure resulted in a purification of 490-fold over Fraction I with the overall yield of about 9% (Table I).

TABLE I. SUMMARY OF PURIFICATION ON PYRIDOXINE-P AND PYRIDOXAMINE-P OXIDASE

Fraction		Total Protein mg	Total units	Specific activity	NP/MP*
I	Cell-free extract	106260	4092000	38.5	1.58
II	Protamin treatment	68260	3960000	58.5	1.60
III	Ammonia sulfate fractionation	19034	3720000	195	1.61
IV	DEAE-Cellulose fractionation	612	1776000	2902	1.61
V	Ammonium sulfate fractionation	304	1097000	3611	1.60
VI	Hrdoxylapatite fractionation	21	377000	17952	1.60

* NP/MP; Ratio of pyridoxine-P oxidase/pyridoxamine-P oxidase.

With the purified enzyme preparation of *Alcaligenes faecalis*, the enzyme activities as functions of incubation time and enzyme concentration were investigated. Pyridoxal-P formation was a linear function of incubation time for at least 30 minutes but deviated slightly by 1 hour. The enzyme activities were directly proportional to enzyme concentration. Pyridoxal-P formation is not additive in the presence of both substrates (Fig. 1).

Properties of Purified *Alcaligenes faecalis* Pyridoxine-P and Pyridoxamine-P Oxidase.

FIG. 1. Enzyme Activity as a Function of Incubation Time and Enzyme Concentration.

The enzyme activity was assayed under the standard assay conditions. In Fig. 1A, the incubation was carried out for 30 minutes. In Fig. 1B, the enzyme preparation of 10 μ g of protein was used.

—○— Pyridoxine-P as substrate
—●— Pyridoxamine-P as substrate
—x— Both substrates

Stoichiometry. Table II shows that the decrease in pyridoxine-P associated with the formation of a stoichiometrical amount of pyridoxal-P. On the other hand, a stoichiometrical amount of pyridoxal-P and of ammonia were formed from pyridoxamine-P. A separate experiment performed in Warburg vessels showed that the loss of one mole of pyridoxine-P or pyridoxamine-P was associated with the decrease of half mole of oxygen by the action

TABLE II. STOICHIOMETRY OF PYRIDOXINE-P AND PYRIDOXAMINE-P OXIDASE

Assay	Enzyme Preparation	Substrate	Oxygen Uptake μ moles	Pyridoxine-P decreased μ moles	Pyridoxamine-P decreased μ moles	Pyridoxal-P formed μ moles	Ammonia formed μ moles
A	Fraction IV	{ Pyridoxine-P Pyridoxamine-P		1.094	0.902	0.925 0.867	0.000 0.870
B	Fraction IV	{ Pyridoxine-P Pyridoxamine-P	1.3 1.2	2.748	2.414	2.600 2.400	
A	Fraction VI	{ Pyridoxine-P Pyridoxamine-P		1.418	0.972	1.374 0.948	0.000 0.916
B	Fraction VI	{ Pyridoxine-P Pyridoxamine-P	2.6 1.9	5.216	3.896	5.180 3.808	

The enzyme preparation of 3.64 mg (Fraction IV) or 1.45 mg (Fraction VI), and each substrates of 5 μ moles were used. A) Reaction was carried out in the Conway microdiffusion vessel at 30°C for 1 hour. Saturated potassium carbonate of 1.0 ml was added to stop the enzyme activity and to diffuse ammonia. Diffusion was proceeded at 37°C for more 3 hours-incubation. B) The assay mixture in Warburg vessel was incubated at 30°C for 1 hour. Oxygen uptake was measured manometrically.

of pyridoxine-P and pyridoxamine-P oxidase either in the presence of catalase (Fraction IV) or in the absence of catalase (Fraction VI).

In the supernatant of the reaction mixture, no other vitamin B₆ than pyridoxal-P, and pyridoxine-P or pyridoxamine-P as substrate was detected by the method of paper chromatography.

Effect of pH on activities. The optimal pH of pyridoxine-P and pyridoxamine-P oxidase was found at about 8.6 under the standard assay conditions though pyridoxine-P and pyridoxamine-P oxidase was more active in Tris·HCl buffer than in potassium phosphate buffer (Fig. 2). The pronounced difference in

FIG. 2. Pyridoxal-P Formation by the Pyridoxine-P and Pyridoxamine-P Oxidase as a Function of pH and Buffer Composition.

—○— Pyridoxine-P as substrate
—●— Pyridoxamine-P as substrate

the pH activity pattern with Tris·HCl and potassium phosphate buffer suggested that the reaction product, pyridoxal-P, inhibited the

oxidation and that Tris·HCl buffer partially overcame this inhibition through the formation of a less inhibitory Schiff's base as pointed out by Wada and Snell.²⁾ The formation of a less inhibitory Schiff's base will be present in the following of this series.

Effect of incubation temperature. The rates of both oxidation reaction were measured at different temperatures. From the slope of the line obtained by plotting the log of the velocity against the reciprocal of the absolute temperature, the activation energy was calculated from Arrhenius equation⁸⁾ (Fig. 3).

FIG. 3. Arrhenius Plots for the Reaction of Pyridoxine-P and Pyridoxamine-P Oxidase.

The enzyme preparation, 31 μg of protein was used. The enzyme activity was assayed under the standard assay conditions except at various temperatures as indicated.

—○— Pyridoxine-P as substrate
—●— Pyridoxamine-P as substrate

A discontinuity occurred in the plot at a temperature of 29°C. Enzyme has an activa-

8) M. Dixon and E.C. Webb, "Enzymes," Longmans, Green and Co., Ltd., London, 1964, p. 150.

tion energy of 26600 cal below 29°C and 9200 cal above 29°C for pyridoxine-P oxidation, and 24600 cal and 9200 cal for pyridoxamine-P oxidative deamination, respectively. It is not clear why such change could occur, but similar discontinuities have been observed in lipase,⁹⁾ urease,¹⁰⁾ fumarase¹¹⁾ and choline-esterase¹²⁾ except for the temperature at which they occur.

Substrate specificity.

By using the enzyme preparation of various stages, the reactivity of a variety of the derivatives of vitamin B₆ was examined and the results are summarized in Table III. Pyridoxine-P and pyridoxamine-P were the efficient

TABLE III. SUBSTRATE SPECIFICITY OF PYRIDOXINE-P AND PYRIDOXAMINE-P OXIDASE

Enzyme Preparation	Pyridoxal-P or Pyridoxal formed, $\mu\text{moles/mg/hr}$			
	Fraction I	Fraction III	Fraction IV	Fraction VI
Pyridoxine	1.6	0	0	0
Pyridoxine-P	38.5	195	3611	17952
Pyridoxamine	5.4	8	2	0
Pyridoxamine-P	24.4	121	2243	11150

The enzyme activity was assayed under the standard assay conditions except the derivatives of vitamin B₆ were added at the concentration of $1 \times 10^{-2}\text{M}$.

substrates and substitution of pyridoxine-P and pyridoxamine-P by the free vitamin B₆ derivatives resulted in no activity by the purified enzyme preparation. By the cell-free extract, however, pyridoxamine was oxidized to pyridoxal in agreement with the reports of Pogell,¹³⁾ and Wada and Snell.²⁾

Effect of substrate concentration.

The rates of the pyridoxine-P and pyridoxamine-P oxidation as a function of the concentration of each of the substrates were plotted according to the method of Lineweaver-Burk.¹⁴⁾

9) I. W. Sizer and E. S. Josephson, *Food Res.*, **7**, 201 (1942).

10) I. W. Sizer, *J. Gen. Physiol.*, **22**, 719 (1939).

11) V. Massay, *Biochem. J.*, **53**, 72 (1953); *Biochem. J.*, **55**, 172 (1953).

12) W. M. Fitch, *Biochemistry*, **2**, 1221 (1963).

13) B. M. Pogell, *J. Biol. Chem.*, **232**, 761 (1958).

14) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

FIG. 4. Effect of Pyridoxine-P Concentration on Reaction Rate.

The enzyme preparation, 2 μg , was used. The enzyme activity was assayed under standard assay conditions except at various concentration of pyridoxine-P as indicated. The insert represents the Lineweaver-Burk plot.

FIG. 5. Effect of Pyridoxamine-P Concentration on Reaction Rate.

The enzyme preparation, 5 μg , was used. The enzyme activity was assayed under standard assay conditions except at various concentration of pyridoxamine-P as indicated. The insert represents the Lineweaver-Burk plot.

FIG. 6. Stability of Pyridoxine-P and Pyridoxamine-P Oxidase against Heating and Ultraviolet Light Irradiation.

The enzyme preparation 10 μ g of protein was dissolved in 1.0 ml of Tris-HCl buffer. The enzyme solution was heated for 10 minutes at various temperatures as indicated and irradiated for various minutes as indicated at pH 8.0. The remaining enzyme activity was assayed under the standard assay conditions.

○ — Pyridoxine-P as substrate
● — Pyridoxamine-P as substrate

Michaelis constants for pyridoxine-P and pyridoxamine-P were found to be 3.6×10^{-5} M and 1.5×10^{-4} M, respectively (Fig. 4 and 5).

Rates of inactivation by heat and ultraviolet light.

Oxidase preparations were held at pH 6.0, 7.0 or 8.0 and at various temperatures for 10 minutes. Their activities for the oxidation of pyridoxine-P and pyridoxamine-P were stable up to 40°C, but unstable above 45°C. Their activities disappeared at the same rate (Fig. 6A).

In attempt to obtain a coenzyme-free preparation, the oxidase preparation was irradiated by the mercury lamp (10W, 5 cm high from enzyme) with constant stirring to prevent heating at 2~3°C. The lamp has peak transmission at 265 μ . As shown in Fig.

6B, their activities for the oxidation of both substrates, pyridoxine-P and pyridoxamine-P disappeared at the same rate. All attempts to reverse the inactivation were unsuccessful.

Cofactor requirement.

The bound coenzyme of the oxidase has been identified as flavin mononucleotide by two quite different procedures, i.e., (a) by the extraction by the method of Yagi and Ishiguro,¹⁵⁾ (b) by the resolution by the acid ammonium sulfate treatment.

First, the prosthetic group was removed from enzyme of 5.2 mg by heating at 80°C for 10 minutes. The paper chromatography with three different solvents and paper electrophoresis of the extracted solution, reveal-

15) K. Yagi and I. Ishiguro, *Medicine and Biology*, 17, 105 (1950).

ed one spot corresponding to flavin mononucleotide. The corresponding flavin spot was extracted with distilled water. The spectra in acid, alkali and neutral solutions are identical with those of authentic specimen treated with same way. Fluorescence spectra at pH 7.0 were also identical.

Secondly, the enzyme was resolved by following modification of the method of Warburg and Christian¹⁶⁾ in order to demonstrate the cofactor requirement of the enzyme. The following procedures were performed at 0~5°C. Saturated ammonium sulfate solution was adjusted to pH 4.0 with 1N acetic acid and added dropwise with stirring to an equal volume of the enzyme solution. After the mixture had been stirred for ten minutes, the ammonium sulfate concentration was increased to 90% saturation by an addition of solid ammonium sulfate. The precipitate formed was collected by centrifugation after twenty minutes, washed with a saturated ammonium sulfate solution of pH 4.0, then dissolved in 0.01M potassium phosphate buffer, pH 7.0. Some insoluble matter present was centrifuged and discarded. The enzyme resolved by the treatment of acid ammonium sulfate was essentially inactive in absence of flavin coenzyme. It was reactivated for the oxidation of both pyridoxine-P and pyridoxamine-P by flavin mononucleotide. A flavin mononucleotide-free flavin adenine dinucleotide prepared by Dowex 1×4 column chromatography,¹⁷⁾ followed by paper chromatography with butanol-acetic acid-water solvent (4:1:5)¹⁸⁾ under dark showed no activity at a concentration of 1×10^{-4} M. Free riboflavin and cyclic flavin mononucleotide phosphate were wholly inactive (Table IV). With both pyridoxine-P and pyridoxamine-P as substrate, the Michaelis constant for flavin mononucleotide as calculated from the Lineweaver-Burk

TABLE IV. ACTIVATION OF RESOLVED ENZYME BY DERIVATIVES OF FLAVIN

Substrate	Pyridoxal-P formed, $m\mu$ moles	
	Pyridoxine-P	Pyridoxamine-P
None	0	0
Riboflavin	0	0
FMN*	129	71
FMN**	128	71
Cyclic FMN	0	0
Phosphate	0	0
FAD*	68	42
FAD**	0	0

* Commercial product.

** Purified by column chromatography, followed by paper chromatography (See Text).

The enzyme activity was assayed under the standard conditions. The resolved enzyme of 102 μ g of protein was preincubated for 20 minutes with the derivatives of flavin before an addition of substrates. The derivatives of flavin were added at the concentration of 1×10^{-4} M.

FIG. 7. Effect of Flavine Mononucleotide Concentration for Activation of Resolved Enzyme.

The enzyme activity was assayed under the standard assay conditions. The resolved enzyme 102 μ g of protein was preincubated with flavine mononucleotide for ten minutes before the addition of substrates. The insert represents the Lineweaver-Burk plot.

—○— Pyridoxine-P as substrate
—●— Pyridoxamine-P as substrate

16) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).

17) K. Yagi, J. Okuda and Y. Matsuoka, *Nature*, **175**, 555 (1955).

18) H. Katagiri, H. Yamada and K. Imai, *J. Vitaminol.*, **5**, 307 (1959).

plot¹²⁾ was 1.8×10^{-8} M (Fig. 7).

Effect of metal ions.

Mg^{++} , Mn^{++} , Fe^{++} , Fe^{+++} , Co^{++} , Zn^{++} , Ni^{++} , Ca^{++} , Li^{+} , Al^{+++} , or MoO_4^{--} at a concentration of 1×10^{-3} M did not affect both activities of the oxidase either by direct addition or by preincubation of metal with enzyme at 10 hours at 4°C.

Cupric ion accelerated the enzyme activity of pyridoxine-P oxidation at a concentration of 1×10^{-3} M, to about 30% of the rate without the cupric ion, but exerted no effect on the enzyme activity of pyridoxamine-P oxidation at the same concentration of cupric ion (Fig. 8).

FIG. 8. Effect of $HgCl_2$ and $CuSO_4$ Concentration for Pyridoxine-P and Pyridoxamine-P Oxidase.

The enzyme activity was assayed under the standard assay conditions. The enzyme preparation of 7.2 μ g of protein was preincubated with $CuSO_4$ and $HgCl_2$ for 20 minutes before the addition of substrates.

○ $CuSO_4$ as indicated concentration
● $HgCl_2$ as indicated concentration

Mercuric ion accelerated both enzyme activities at a concentration of 1×10^{-3} M, to about 30% the rate without the mercuric ion.

Inhibitor.

p-Chloromercuribenzoate did not show the effect on the both enzyme activities at a concentration of 1×10^{-3} M. The metal-binding agents, including α, α' -bipyridyl, *o*-phenanthroline, sodium azide, sodium pyrophosphate, ethylenediaminetetraacetic acid, thio urea, 8-hydroxyquinoline sulfate, and sodium citrate at a concentration of 1×10^{-3} M showed no effect on the both enzymatic activities either by direct addition or by the preincubation of inhibitor with enzyme at 4°C for 10 hours.

DISCUSSION

Although pyridoxine, pyridoxamine and pyridoxal independently can serve as the source of vitamin B₆ for mammals and for many microorganisms, each must be converted to pyridoxal-P, the active coenzymatic form of vitamin B₆ in amino acid and amine metabolisms. With the exception of transamination, in which under the certain condition pyridoxamine-P also is active, pyridoxal-P specifically requires in a large number of reaction in amino acid and amine metabolisms. But, knowledge of the interconversion of vitamin B₆ in microorganisms has not yet been thoroughly investigated and is fragmentary investigated as reviewed by Snell.¹⁹⁾

In earlier work, pyridoxal phosphokinase was found to occur widely in microorganisms,²⁰⁾ and it was assumed to participate in the formation of pyridoxal-P in microorganisms. Pogell¹³⁾ demonstrated the presence of the enzyme which catalyzed pyridoxamine-P oxidation in rabbit liver, and reported the new pathway of the formation of pyridoxal-P. In separate approach, Morisue et al.²¹⁾ reported the occurrence of a flavoprotein that catalyzed pyridoxine-P oxidation in rabbit liver. Recently, Wada and Snell²⁾ concluded

19) E. E. Snell, "Proceedings of the Vth International Congress of Biochemistry", Vol. IV, "Molecular Basis of Enzyme Action and Inhibition", p. 268, Pergamon Press, London, 1963.

20) J. Hurwitz, *J. Biol. Chem.*, **205**, 935 (1953).

21) T. Morisue, Y. Morino, Y. Sakamoto and K. Ichihara, *J. Biochem. (Japan)*, **48**, 28 (1960).

that a single enzyme, for which pyridoxine-P was the preferred substrate, possessed both activities in rabbit liver. In the course of investigations of the vitamin B₆ metabolism in microorganisms, we have found that the pyridoxine-P and pyridoxamine-P oxidase occurred generally in aerobic bacteria. The purpose of the present work is to characterize the formation of pyridoxal-P from pyridoxine-P and pyridoxamine-P in microorganisms as well as the nature of the enzyme. The highly purified enzyme prepared from the cell-free extract of *Alcaligenes faecalis* was used throughout the present work.

The properties of the pyridoxine-P and pyridoxamine-P oxidase obtained from *Alcaligenes faecalis* resemble those reported of Wada and Snell²¹ for the oxidase obtained from the extract of rabbit liver. The formation of pyridoxal-P proceeded stoichiometrically in parallel with the disappearance of pyridoxine-P or pyridoxamine-P. The enzyme has pH optimum at alkaline, and is inhibited by the reaction product, i.e., pyridoxal-P.

On the cofactor requirement, previous workers^{2,8,19} reported the requirement of flavin mononucleotide or flavin adenine dinucleotide, but it was not known whether two activities is due to flavin adenine dinucleotide *per se*, or whether trace of flavin mononucleotide is formed from flavin adenine dinucleotide by spontaneous breakdown or by contaminating enzymes in the oxidase preparation. Clear evidence of the requirement of only flavin mononucleotide for the enzyme activities was obtained by the present work using the acid ammonium sulfate-treated enzyme preparation. The result of Lineweaver-Burk plot reveals the relatively high affinity of flavin mononucleotide to the enzyme protein.

No sensitivity of the enzyme to SH-inhibitors such as *p*-chloromercuribenzoate, mercuric chloride and cupric sulfate indicated that the enzyme contains no functional SH-group. Previous workers, reported these chemicals as

the inhibitors for the pyridoxine-P and pyridoxamine-P oxidase of rabbit liver. However, these SH-inhibitors inhibit to some extent of 10 to 40% in the oxidase of the other microorganisms such as *Pseudomonas fragi*, *Azotobacter agilis* and *Micrococcus ureae** It is not known whether *Alcaligenes* oxidase contained the functional SH-group effectively masked, or not contained the functional SH-group.

Several lines of evidence indicated that both pyridoxine-P oxidation and pyridoxamine-P oxidative deamination were catalyzed with a single enzyme. First, the ratios of the two activities did not affect the purity of the protein. Secondly, when both substrates were presented to the enzyme, each at concentrations saturating the enzyme, there was no summation in the rate of pyridoxal-P formation. Thirdly, the catalytic activities on both substrates were destroyed at identical rate during heat treatment at three different pH and during ultraviolet light irradiation of the enzyme. Fourthly, flavin mononucleotide is specifically the required prosthetic group for both activities.

Studies on the conversion of pyridoxine and pyridoxamine have provided the evidence for existence of two pathways, one of them via pyridoxal and another via pyridoxine-P or pyridoxamine-P. But, the oxidation of pyridoxine-P and pyridoxamine-P proceeded more readily than the oxidation of pyridoxine and pyridoxamine. The higher apparent affinity of pyridoxine-P and pyridoxamine-P oxidase for its substrate fortifies the proposition that pyridoxine-P and pyridoxamine-P oxidase is a key-enzyme in the formation of pyridoxal-P. Pyridoxine-P and pyridoxamine-P oxidase, together with pyridoxal phosphokinase²² and phosphotransferase,^{23,24} represents the pathways for the conversion of pyridoxine

* S. Yamamoto, T. Tochikura and K. Ogata, Unpublished observation.

22) D. B. McCormick, M. E. Gregory and E. E. Snell, *J. Biol. Chem.*, **236**, 2076 (1961).

23) S. A. Kumar and C. S. Vaidyanathan, *Biochim. Biophys. Acta*, **73**, 98 (1963).

24) K. Ogata, Y. Tani, S. Yamamoto and T. Tochikura, *This Journal*, **28**, 809 (1964).

and pyridoxamine to pyridoxal-P in microorganisms. Moreover, these pathways has been supported by three reports that pyridoxine-P²⁵⁾ and pyridoxamine-P^{26,27)} were the predominant

ing forms of vitamin B₆ group present in cells.

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26) J. C. Rabinowitz and E. E. Snell, *J. Biol. Chem.*, **169**, 643 (1947).

27) W. S. McNutt and E. E. Snell, *J. Biol. Chem.*, **173**, 801 (1948).