The Enzymatic Oxidation of Pyridoxine and Pyridoxamine Phosphates

HIROSHI WADA AND ESMOND E. SNELL

From the Department of Biochemistry, University of California, Berkeley, California

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Extracts of rabbit liver and brain are known (1, 2) to oxidize pyridoxine 5-phosphate to pyridoxal 5-phosphate much faster than they oxidize free pyridoxine to pyridoxal. Comparison of the two activities during limited purification indicated that different enzymes catalyzed the two reactions (1). Pogell (3) had shown previously that oxidation of pyridoxamine 5-phosphate was catalyzed by an enzyme present in rabbit liver. This enzyme was purified approximately 15-fold and was shown to be distinct from both monoamine oxidase and diamine oxidase.

Further investigations of the purification and properties of pyridoxine 5-phosphate oxidase and of pyridoxamine 5-phosphate oxidase, reported herein, lead to the conclusion that the two enzymes are probably identical. In the course of these investigations, a simple photometric method has been developed for the determination of pyridoxal or pyridoxal 5-phosphate in enzymatic reaction mixtures.

EXPERIMENTAL PROCEDURE

Materials—Deoxypyridoxine phosphate (deoxypyridoxine-P) and isoriboflavin were kindly provided by Dr. Karl Folkers of Merck and Company, Inc. Pyridoxine 5-phosphate (pyridoxine-P) (4), w-methylpyridoxamine phosphate (w-methylpyridoxamine-P) (5), pyridoxal oxime (6), and pyridoxal 5-phosphate oxime (pyridoxal-P oxime) (4) were synthesized by the procedures described in the references cited. Isopyridoxal was isolated from the culture medium of Pseudomonas sp. 1A grown on pyridoxine (7). 4-Pyridoxic acid phosphate (4-pyridoxic acid-P) was synthesized by shaking a 1% aqueous solution of pyridoxal 5-phosphate (pyridoxal-P) in the dark for 24 hours at 30° with an excess of freshly prepared silver oxide. The mixture was saturated with H₂S and filtered. The product crystallized on concentrating the solution in a vacuum and analyzed correctly.

Alumina Cy was prepared according to Willstätter and Kraut (8). DEAE-cellulose (Brown Company) was recyrled through acidic, aqueous solutions. Phenylhydrazine was the most satisfactory hydrazine tested, and yields with pyridoxal-P a stable, intense yellow color (λ<sub>max</sub> = 410 μm) at room temperature or below (Figs. 1 and 2). Pyridoxal reacts more slowly but gives the same high extinction on heating at 60° (Fig. 1). This difference in rate of color development can be increased by decreasing the temperature and by increasing the acidity of the reaction mixture, and permits determination of pyridoxal-P in the presence of a moderate excess of pyridoxal.

2. Phenylhydrazine Reagent—Phenylhydrazine hydrochloride (2 g) is dissolved in 100 ml of 18 N H₂SO₄. The reagent can be kept in the refrigerator for several weeks but should be renewed when any brown color appears.

3. Determination of Pyridoxal or Pyridoxal-P—To 3.8 ml of deproteinized sample containing 0.01 to 0.1 μmole of pyridoxal or pyridoxal-P is added 0.2 ml of phenylhydrazine reagent. When pyridoxal-P is to be determined in the absence of pyridoxal, the samples are allowed to stand 10 minutes at room temperature, and read at 410 μm in a Bausch and Lomb Spectronic-20 colorimeter. For determination of pyridoxal alone, or of pyridoxal plus pyridoxal phosphate, the solutions are heated at 60° for 20 minutes, and allowed to stand at room temperature for 10 minutes before reading at 410 μm. The absorbancy at this wave length follows Beer's law in the concentration range 0 to 0.025 mm pyridoxal or pyridoxal-P, and is equal for the two compounds. The rates and extent of color development under various conditions are shown in Fig. 1.

4. Determination of Pyridoxal-P in Presence of Excess Pyridoxal—For this purpose, color development with pyridoxal is inhibited by decreasing the temperature to 0° (Fig. 1) and increasing the acidity by addition of 0.5 ml of 18 N H₂SO₄. Phenylhydrazine reagent (0.2 ml) is added and after 30 minutes at 0° the absorbancy is measured at 410 μm. Under these conditions, amounts of pyridoxal up to 1.0 μmole did not contribute to the color produced.

5. Specificity of Procedure—α-Ketoglutarate, pyruvate, and similar α-keto acids do not interfere. Of several other naturally occurring carbonyl compounds tested, only glyoxylate interfered; its color yield after heating was only about 0.5% that of equimolar amounts of pyridoxal. 5-Deoxypyridoxal and p-nitro-

* The slower color development with pyridoxal reflects the fact that this compound exists primarily as the hemiacetal in acidic solutions (9); with 5 deoxypyridoxal, color develops at the same rate as with pyridoxal phosphate. At pH values between 4.0 and 7.0, pyridoxal reacts much more rapidly with phenylhydrazine.
salicylaldehyde give color similar to pyridoxal, whereas iso-
pyridoxal, salicylaldehyde, and benzaldehyde do not.

The method is very suitable for determination of pyridoxal-P or pyridoxal in enzymatic reaction mixtures. When applied to crude materials such as urine or liver extract, the absorbancy at 410 mμ is somewhat greater than can be accounted for by the expected pyridoxal content of the sample. Difficulty from this source was experienced only in the initial phases of this investigation, before purification of the enzyme was effected. In these instances, an appropriate blank containing the enzyme extract but no substrate was used to correct the values for pyridoxal-P production. As shown by data of Fig. 2, the phenylhydrazine procedure is substantially more sensitive than either the ethanolamine procedure used by Metzler and Snell (12) for estimation of pyridoxal in nonenzymatic reaction mixtures or the direct spectrophotometric estimation of pyridoxal-P in 0.1 N NaOH. It is more convenient, but less sensitive and less specific than the apotryptophanase method (13) used in earlier investigations. Although less sensitive than a recently described procedure based on fluorimetry of the cyanohydrins of pyridoxal or of pyridoxal-P (14), the phenylhydrazine procedure has the advantage of being insensitive to presence of an excess of other vitamin B6 derivat-
ives. The method of choice among these various procedures obviously is dictated by the sensitivity required and by other experimental considerations.

**Determination of Enzyme Activity**—Stopped 50-ml Erlen-
meyer flasks containing enzyme, substrate (1 μmole of pyri-
doxine-P or of pyridoxamine-P), and Tris buffer (pH 8.0, final
concentration 0.2 m) in a volume of 3.5 ml were shaken at 37° for 30 minutes. The reaction was stopped by addition of 0.3 ml of 100% (weight per volume) trichloroacetic acid. If a precipi-
tate formed, the mixture was centrifuged and 3 ml of the superna-
tant were taken for determination of pyridoxal-P. With purified
enzyme preparations, no precipitate appeared, and the entire
sample was used for this determination. Specific activities of
oxidase preparations are given in terms of the mμmoles of
pyridoxal phosphate formed per mg of protein per hour under
these conditions. Variations from these conditions are given
with the tables and figures.

Protein was determined by the method of Lowry et al. (15) or
by measurement of optical density at 280 mμ against crystalline
bovine serum albumin as standard (16). Ammonia was deter-
mined colorimetrically with ninhydrin (17) after its separation
from reaction mixtures by the microdiffusion technique of Con-
way (18).

**RESULTS**

**Source Material**—Rabbit liver was a more active source of
pyridoxine-P oxidase than either rat or beef liver, and was there-
fore used as a source material. The livers of freshly slaughtered
animals were held at -10° to -15° until used. Brain and kid-
ney were much less active than liver. All preparative steps
except those specified were carried out at 0–5°.

**Purification of Oxidase**

1. **Extraction and Acid Treatment**—Frozen rabbit liver (150 g)
was thawed, diced, homogenized for 5 minutes in a Waring
Blendor with 600 ml of 0.02 M potassium phosphate buffer, pH
7.0, and centrifuged for 30 minutes at 18,000 × g. The superna-
tant were taken for determination of pyridoxal-P. With purified
enzyme preparations, no precipitate appeared, and the entire
sample was used for this determination. Specific activities of
oxidase preparations are given in terms of the mμmoles of
pyridoxal phosphate formed per mg of protein per hour under
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from reaction mixtures by the microdiffusion technique of Con-
way (18).

**Table I**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Ratio* PNP oxidase PMP oxidase</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>610</td>
<td>33.0</td>
<td>6.5</td>
<td>100</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>520</td>
<td>16.4</td>
<td>13.0</td>
<td>83</td>
<td>1.16</td>
</tr>
<tr>
<td>3</td>
<td>485</td>
<td>10.8</td>
<td>18.0</td>
<td>70</td>
<td>1.14</td>
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<tr>
<td>4</td>
<td>69</td>
<td>32.5</td>
<td>36.4</td>
<td>59</td>
<td>1.08</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>11.0</td>
<td>166.0</td>
<td>27</td>
<td>1.05</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>431.5</td>
<td></td>
<td>1.10</td>
</tr>
</tbody>
</table>

* PNP = pyridoxine phosphate, PMP = pyridoxamine phosphate.
After 60 minutes, the active precipitate was collected by centrifugation and dissolved in approximately the initial volume of water. The clear solution was dialyzed against distilled water overnight and centrifuged to remove precipitated inactive protein.

4. Alumina C\textsubscript{17} Gel Treatment—The dialyzed solution (Fraction 4) was adjusted to pH 6.0 with 1.0 M acetic acid and centrifuged. To each 10-ml portion of the supernatant solution was added 2.0 ml of alumina C\textsubscript{17} gel (7.0 mg of alumina). After 20 minutes of constant stirring, the suspension was centrifuged and the supernatant solution was treated a second time with the same amount of alumina gel. This procedure was repeated four more times. For elution, each gel precipitate was suspended separately in 10 ml of 0.5 M potassium phosphate buffer, pH 8.0, stirred occasionally for 20 minutes, and centrifuged.

Most of the oxidase activity appeared in the eluates from the third and fourth gel treatments. These were combined (Fraction 5) and used for most of the experiments.

5. Fractionation on DEAE-Cellulose—Fraction 5 (100 mg of protein) was dialyzed against distilled water overnight, poured over a 1.7 x 12-cm column of DEAE-cellulose, and eluted in a linear gradient established between 0.01 M potassium phosphate buffer (pH 8.0) and buffer plus 0.2 M NaCl. The elution pattern (Fig. 3) was quite reproducible. The most active fraction (Fraction 6) contained protein of specific activity 432, purified approximately 66-fold over the protein of the initial extract.

Throughout the purification procedure, oxidase activity toward pyridoxine-P exactly paralleled that toward pyridoxamine-P (Table I and Fig. 3), thus providing strong evidence that both catalytic activities are due to a single protein.

Properties of Partially Purified Enzyme—Properties of the enzyme were determined with 0.1 ml of Fraction 5 (Table I) per assay flask as the enzyme source in all instances.

1. Effect of pH—Both pyridoxine-P and pyridoxamine-P were oxidized optimally between pH 9.0 and 10 (Fig. 4). At pH 9.7 both substrates are oxidized to almost the same extent; below pH 8.0, pyridoxine-P is by far the better substrate. Because the aminomethyl group of pyridoxamine-P (pK = 10.9 (19)) is largely protonated below pH 9.0, the unprotonated group is probably attacked preferentially by the enzyme.

2. Inhibition of Oxidase by Pyridoxal-P and Its Alleration by Amines—The pronounced difference in the pH activity pattern with Tris and phosphate buffers (Fig. 4) suggested that the reaction product, pyridoxal-P, inhibited the oxidation and that Tris partially overcame this inhibition through formation of a less inhibitory Schiff's base. In support of this idea, Fig. 5A demonstrates that the initial rate of pyridoxal-P formation is the same in both buffers, but decreases much more rapidly in phosphate than in Tris buffer. Other amino compounds that form derivatives with pyridoxal-P exert an activating effect similar to that of Tris (Fig. 5B); cysteine, which readily forms a...
labile thiazolidine derivative with pyridoxal-P (20), was more effective than Tris. Finally, addition of pyridoxal-P decreases production of pyridoxal-P by the oxidase from both substrates

Penicillamine was even more effective than cysteine in this respect, as was also semicarbazide; phenylhydrazine was inhibitory. These compounds, unlike Tris and the less effective amino acids, partially inhibit formation of the colored reaction product between pyridoxal phosphate and phenylhydrazine. This was corrected by heating the deproteinized sample with the phenylhydrazine reagent at 60°C for 90 minutes, as in the determination of pyridoxal, and with the use of a standard curve derived from known amounts of pyridoxal phosphate in the presence of concentrations of carbonyl reagent identical to those used in the enzymatic reaction mixtures.

The effect is more pronounced when pyridoxamine-P is the substrate, and is much reduced by addition of Tris.

An initially puzzling observation was the failure of pyridoxal-P formation to increase linearly with enzyme concentration when pyridoxamine-P was the substrate under the standard assay conditions, even though 90% or more of the initial substrate remained at the end of the assay period. Pogell (3) noted the same phenomenon without further comment. It results in an apparent decrease in specific activity of the enzyme as its concentration in the assay mixture is increased, and appears to be caused by the accumulation of sufficient pyridoxal-P to inhibit further oxidation of pyridoxamine-P regardless of enzyme concentration. Because inhibition by pyridoxal-P is competitive with substrate, the phenomenon can be prevented by increasing the initial concentration of pyridoxamine-P. It is also observed when pyridoxine-P is the substrate if that compound is used at an initial concentration one-third of that normally employed.

3. Comparative Affinities of Oxidase for Pyridoxine-P and Pyridoxamine-P—Michaelis constants calculated from the substrate concentration curves (Curves 1 and 2, Fig. 7) are 3.1 \times 10^{-4} \text{ M} and 1.4 \times 10^{-4} \text{ M} for pyridoxine-P and pyridoxamine-P, respectively. Extrapolated maximal velocities for the two substrates are identical. The higher affinity of the enzyme for pyridoxine-P correlates with the fact that pyridoxal-P inhibits oxidation of this substrate to a smaller extent than it does that of pyridoxamine-P. Further strong evidence that a single enzyme oxidizes both substrates is the fact that pyridoxal-P formation is not additive in the presence of both substrates (Fig. 7A) and that the substrate with the higher affinity, pyridoxine-P, strongly inhibits pyridoxal-P production from pyridoxamine-P, as measured either directly or by release of ammonia (Fig. 7B).

4. Resolution of Oxidase and Reactivation by FMN—Data of Pogell (3) and of Morise et al. (2) indicated that the prostatic group of the oxidase should be either FAD or FMN. The enzyme was resolved by the following modification of the method of Warburg and Christian (21). Saturated ammonium sulfate solution was adjusted to pH 2.8 with HCl and added dropwise with stirring to an equal volume of the oxidase preparation (Fraction 5, Table I). After 5 minutes at 0°C, the precipitate was collected by centrifugation, resuspended in cold ammonium sulfate solution (35% saturated, pH 2.8), again collected by centrifugation, and finally dissolved in a volume of 0.2 M phosphate buffer, pH 8.0, equal to the original volume of the enzyme preparation. Some insoluble matter present was centrifuged and discarded. This preparation was essentially inactive in the absence of added flavin coenzymes; it was fully reactivated for oxidation of both pyridoxine-P and pyridoxamine-P by low concentrations of FMN (Fig. 8). The Michaelis constant for FMN was 3.1 \times 10^{-4} \text{ M}. FAD was about 0.1% as active as FMN; an FMN-free sample of FAD prepared by chromatography (22) showed this same low activity, but it is not known

Those results were obtained in 0.2 M Tris buffer and were unchanged by increasing its concentration. It thus becomes apparent that although the products of interaction (Tris and other amino compounds) with pyridoxal-P are less inhibitory than free pyridoxal-P, they retain reduced inhibitory activities. This is evident also from the fact that different amines, at their maximally effective concentrations, activate to a different extent (Fig. 5B).

The abbreviations used are: FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetate.

We are indebted to Dr. F. M. Huennekens for this preparation.
whether this activity is due to FAD per se, or whether traces of FMN are formed from FAD by spontaneous breakdown or by contaminating enzymes in the oxidase preparation. It is clear, however, that FMN must serve as the prosthetic group in vivo. Synthetic lysoflavin 5'-phosphate (23) was active at a concentration similar to FAD; free riboflavin showed slight activity at high concentrations, whereas isoriboflavin and cyclic FMN phosphate (24) were wholly inactive.

5. Rates of Heat Inactivation of Oxidase Activities—Oxidase preparations (Fraction 5) were held at pH 5.0 or pH 7.0 and at various temperatures for 10 minutes. Their activities for oxidation of pyridoxamine-P and pyridoxine-P (Fig. 9) disappeared at the same rate, again indicating that a single enzyme system catalyzes both reactions. Separate experiments showed that neither salts nor substrates conferred added heat stability on the enzyme.

6. Effects of Chelating Agents, Metal Ions, and Vitamin B₆ Analogues on Oxidase Activity—Oxidase activity was not decreased by 1 mM 8-hydroxyquinoline, o-phenanthroline, α,α-dipyridyl, or EDTA. This was observed both for the holoenzyme tested in the customary assay, and in experiments in which each of these chelating agents was incubated with the resolved apo-oxidase for 10 minutes before addition of FMN and substrate. Mg⁺⁺, Mn⁺⁺, Fe⁺⁺, Fe⁺⁺⁺, or Zn⁺⁺ at concentrations of 1 mM did not affect activity of the oxidase; 1 mM Hg⁺⁺ or Cu⁺⁺, and 0.1 mM p-chloromercuribenzoate inhibited to the extent of 90 to 95%.

Unphosphorylated vitamin B₆ derivatives were essentially without inhibitory activity (Table II). Each of the phosphorylated analogues tested, however, was a rather effective inhibitor of the oxidase. The inhibitors are uniformly less effective when pyridoxine-P serves as substrate in place of pyridoxamine-P, a clear indication that they act by competing with the normal substrates. Pyridoxal-P oxime, the most effective of the inhibitors found, appears to have a somewhat higher affinity for the oxidase than either of its normal substrates. When ω-methylpyridoxine-P was tested as substrate, a compound indistinguishable from pyridoxal-P by the phenylhydrazine test was formed. This was assumed to be ω-methylpyridoxal-P. The rate of its formation equaled that of pyridoxal-P formation from pyridoxamine-P under the same conditions.

7. Oxidation of Pyridoxamine—In agreement with the report of Pogell (3), purified preparations of the enzyme were found to oxidize pyridoxamine to pyridoxal when the reaction was carried out near pH 10.0 (Fig. 10); pyridoxine was not oxidized under these conditions.

Fragmentary data indicate that pyridoxamine is oxidized by the pyridoxine (pyridoxamine) phosphate oxidase. Prepara

### Table II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Pyridoxine-P</th>
<th>Pyridoxamine-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine</td>
<td>10⁻³</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>10⁻³</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>10⁻³</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>Isopyridoxal</td>
<td>10⁻³</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>4-Deoxypyridoxine</td>
<td>10⁻³</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4-Deoxyriboflavin-P</td>
<td>10⁻³</td>
<td>42.0</td>
<td>70.2</td>
</tr>
<tr>
<td>4-Deoxyriboflavin-P</td>
<td>2 x 10⁻⁴</td>
<td>34.0</td>
<td>54.0</td>
</tr>
<tr>
<td>4-Pyridoxide acid</td>
<td>10⁻³</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>4-Pyridoxide acid-P</td>
<td>10⁻³</td>
<td>33.5</td>
<td>62.0</td>
</tr>
<tr>
<td>4-Pyridoxide acid-P</td>
<td>2 x 10⁻⁴</td>
<td>23.5</td>
<td>52.0</td>
</tr>
<tr>
<td>Pyridoxal oxime</td>
<td>10⁻³</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Pyridoxal-P oxime</td>
<td>2 x 10⁻⁴</td>
<td>80.0</td>
<td>83.6</td>
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<td>10⁻⁶</td>
<td>57.0</td>
<td>67.5</td>
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<tr>
<td>Pyridoxal-P oxime</td>
<td>10⁻⁴</td>
<td>17.0</td>
<td>31.0</td>
</tr>
</tbody>
</table>

* Assay conditions as specified in text.
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It is probable, therefore, as postulated previously (1, 25), that the main pathway of pyridoxal-P formation from pyridoxine is via pyridoxine-P. A similar situation exists with pyridoxamine. This compound is readily phosphorylated to pyridoxamine-P (13, 26), which in turn is readily oxidized enzymatically to pyridoxal-P. Although enzymatic oxidization of free pyridoxamine to pyridoxal does occur, data cited herein indicate that it becomes important only at substantially higher concentrations of pyridoxamine. An alternate route from free pyridoxamine to pyridoxal is by way of transamination with oxaloacetate, but this route, too, becomes important only at higher concentrations of pyridoxamine. Because all the evidence indicates that the oxidase studied here is responsible for the oxidation of both pyridoxine-P and pyridoxamine-P to pyridoxal-P, this enzyme, together with the pyridoxal kinase, assumes a central role in the conversion of both pyridoxine and pyridoxamine to pyridoxal-P. This role is consistent with, and goes to explain, the otherwise puzzling observation that pyridoxine (and, to a lesser extent, pyridoxamine) is markedly superior to pyridoxal in overcoming the inhibitory action of 4-deoxypyridoxine in yeast (33) and in chick embryos (34).

**DISCUSSION**

The enzymatic transformations of vitamin B₆ that are known to occur in mammalian tissues are summarized in Fig. 11. Pyridoxine, pyridoxal, and pyridoxamine are converted with approximately equal efficiency to the corresponding 5-phosphates by the pyridoxal kinase of animal tissues (13) and of yeast (25, 26). Oxidation of pyridoxine to pyridoxal has been detected in mammalian livers (27), but relatively high concentrations are required and the reaction is much slower than the oxidation of pyridoxine-P to pyridoxal-P (1). It is probable, therefore, as postulated previously (1, 25), that the main pathway of pyridoxal-P formation from pyridoxine is via pyridoxine-P. A similar situation exists with pyridoxamine. This compound is readily phosphorylated to pyridoxamine-P (13, 26), which in turn is readily oxidized enzymatically to pyridoxal-P. Although enzymatic oxidization of free pyridoxamine to pyridoxal does occur, data cited herein indicate that it becomes important only at substantially higher concentrations of pyridoxamine. An alternate route from free pyridoxamine to pyridoxal is by way of transamination with oxaloacetate, but this route, too, becomes important only at higher concentrations of pyridoxamine. Because all the evidence indicates that the oxidase studied here is responsible for the oxidation of both pyridoxine-P and pyridoxamine-P to pyridoxal-P, this enzyme, together with the pyridoxal kinase, assumes a central role in the conversion of both pyridoxine and pyridoxamine to pyridoxal-P. This role is consistent with, and goes to explain, the otherwise puzzling observation that pyridoxine (and, to a lesser extent, pyridoxamine) is markedly superior to pyridoxal in overcoming the inhibitory action of 4-deoxypyridoxine in yeast (33) and in chick embryos (34).

**SUMMARY**

A simple photometric procedure involving reaction of pyridoxal and pyridoxal phosphate with phenylhydrazine has been developed for the estimation of these compounds in enzymatic reaction mixtures. With the aid of this method, an enzyme that oxidizes pyridoxine phosphate to pyridoxal phosphate has been purified approximately 65-fold from rabbit liver. The purified enzyme, like the crude extract, also converts pyridoxamine phosphate to pyridoxal phosphate.

Comparative studies of purification, pH optima, and rates of heat inactivation all indicate that the two enzymatic activities are properties of a single enzyme. Oxidation of both substrates is strongly inhibited by the product of the reaction, pyridoxal phosphate. This inhibition is markedly decreased by Tris buffer, cysteine, and other compounds that form Schiff's bases or thialdine derivatives of pyridoxal phosphate. The enzyme is a flavoprotein, and can be resolved from its prosthetic group by precipitation with ammonium sulfate under acid conditions. Riboflavin 5'-phosphate fully reactivates the apoenzyme for oxidation of both substrates; flavin adenine dinucleotide reactivates only at concentrations 1000 times higher.

The oxidase is inhibited by relatively high concentrations of Cu⁺, Hg⁺², and p-chloromercuribenzoate. Unphosphorylated analogues of vitamin B₆ are not effective inhibitors, but some phosphorylated analogues of the vitamin (4-deoxypyridoxine phosphate, 4-pyridoxic acid phosphate, and especially pyridoxal phosphate oxime) are potent inhibitors with affinities for the oxidase approaching or surpassing those of its substrates.

The transformations that vitamin B₆ undergoes in vivo are discussed briefly. It is concluded that the primary pathway of pyridoxal phosphate formation from pyridoxine or pyridoxamine in animal tissues is phosphorylation by the pyridoxal kinase followed by action of the oxidase studied here.

**REFERENCES**


2. **MORISUE, T., MORINO, Y., SAKAMOTO, Y., AND ICHIHARA, K., J. Biochem. (Japan), 46, 16 (1960).**


6. **H. Wada, and E. E. Snell, to be published.**
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