Pyridoxamine-Pyruvate Transaminase. II. Characteristics of the Enzyme*

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Improved procedures are described for the purification and determination of pyridoxamine-pyruvate transaminase, which catalyzes the reversible reaction:

$$\text{pyridoxamine} + \text{pyruvate} \underset{b}{\overset{a}{\rightleftharpoons}} \text{pyridoxal} + \text{L-alanine} \qquad (1)$$

The specific activity of the crystalline enzyme (40) is substantially higher than previously reported.[1] In addition to pyridoxamine, 5-deoxypyridoxamine and ω-methylpyridoxamine are excellent substrates in reaction (1a); pyridoxamine-5-P undergoes slow reaction at high concentrations. Of nineteen amino acids tested, only α-aminobutyrate replaced alanine as substrate in reaction (1b). Pyridoxylalanine is a potent and specific inhibitor of the reaction; its affinity for the enzyme ($K_I = 1.8 \times 10^{-7}$ M) is far higher than that for either pyridoxal ($K_M = 1.5 \times 10^{-5}$ M) or L-alanine ($K_M = 2 \times 10^{-3}$ M). Pyridoxine and certain related compounds are much less effective inhibitors.

One mole of pyridoxal, 5-deoxypyridoxal, or pyridoxal 5-phosphate binds per 60,000 g of enzyme. Reduction of the enzyme with sodium borohydride in the absence of pyridoxal does not inactivate it; in the presence of pyridoxal inactivation is proportional to the amount of pyridoxal bound and to the increase in absorbancy at 325 mμ (*p*H 7.0). This absorption maximum is accounted for quantitatively by the formation of ϵ-pyridoxyllysine residues in the reduced protein. The findings show that a lysine residue is part of the active site of the enzyme. The crystalline enzyme contains no metal ions; its amino acid analysis shows it to be unusually rich in proline.

Pyridoxamine-pyruvate transaminase was isolated and crystallized by Wada and Snell (1962a) from a *Pseudomonad* growing upon pyridoxamine (or pyridoxine) as a sole carbon source. This enzyme is unique among transaminases so far studied in that pyridoxal and pyridoxamine serve as substrates, and no coenzyme is required. Glutamate-oxaloacetate *apo*transaminase catalyzes a similar reaction between pyridoxamine and α-ketoglutarate or oxaloacetate, but very much less efficiently (Wada and Snell, 1962b).

Because of the comparative simplicity of the reaction catalyzed and the lack of a coenzyme, pyridoxamine-pyruvate transaminase should be a useful enzyme for study of the mechanism of transamination. Accordingly, further studies have been undertaken to improve methods for assay and isolation of this enzyme, and to characterize it more fully.

EXPERIMENTAL

Assay Procedures for Pyridoxamine-Pyruvate Transaminase.—Two procedures for following reaction (1),

$$\text{pyridoxamine} + \text{pyruvate} \underset{b}{\overset{a}{\rightleftharpoons}} \text{pyridoxal} + \text{L-alanine} \qquad (1)$$

catalyzed by the transaminase, have been used. In *procedure 1*, used chiefly in following reaction (1a), the pyridoxal produced is determined by spectrophotometric measurement of the complex formed between pyridoxal and phenylhydrazine by the procedure of Wada and Snell (1962a) modified by omission of the potassium phosphate buffer, and by stopping the reaction at the time when the yellow color became visible rather than after a fixed time. The high and variable blanks occasionally noted in the original assay were eliminated by using 0.5 ml of 9 M H_2SO_4 (6 M HCl or 6 M H_3PO_4 also are satisfactory) in place of trichloroacetic acid to stop the reaction. Under these conditions, pyridoxal formation was linear for at least 1 hour when 2 μg or less of the pure transaminase was employed. *Procedure 2*, used for most of the work with the pure enzyme, involves spectrophotometric estimation of pyridoxal at 400 mμ, and can be used for following reaction (1) in either direction. It permits the region of linearity at low substrate concentrations to be readily seen. The absorbancy of pyridoxal limits the maximum concentration that can be used to 1 mM. Details of individual reaction mixtures are given with the tables and figures.

Preparation of Samples for Amino Acid and Metal Ion Analysis.—Twenty mg of enzyme (specific activity 38.4) was dialyzed sequentially over a 6-day period against four changes (200 volumes each) of 0.02 M potassium phosphate, four changes (400 volumes each) of distilled water, and finally four changes (400 volumes each) of quartz-distilled H_2O. The solution was then lyophilized and the protein was dried to constant weight over P_2O_5 under reduced pressure. For the amino acid analyses, six samples (3.5 ± 0.4 mg each) of protein were hydrolyzed in duplicate in 1 ml of twice-distilled constant boiling HCl for 24, 48, and 72 hours. The protein-HCl mixtures were frozen at −70°, put under reduced pressure at 0.04 mm Hg, and allowed to thaw slowly to remove dissolved oxygen. The mixture was then refrozen, sealed, and hydrolyzed. Dried protein without hydrolysis was supplied for the metal analyses. All glassware for these experiments was freed of metal contamination by washing successively in soap, alcoholic KOH, aqua regia, and finally by autoclaving for 30 minutes at 121° in quartz-distilled water.

We are indebted to Dr. Bert Vallee, Harvard Medical School, for spectrographic metal analyses.

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Improved Preparation of ε-Pyridoxyllysine.— α-Acetyl-ε-pyridoxyllysine was prepared by the method of Dempsey and Christensen (1962). This compound (258 mg) was hydrolyzed by autoclaving for 40 minutes at 121° in 5 ml of 6 N HCl. After drying under reduced pressure, the residue was dissolved in water, adjusted to *p*H 7.0 with aqueous ammonia, and again dried under reduced pressure. The residue was dissolved in a little water and applied to a 0.9 × 30-cm column of XE-64 resin which had been previously equilibrated with 1 M ammonium formate, *p*H 4.0, and washed with 250 ml of water. The column was washed with 850 ml of water followed by 70 ml of 0.72 M acetic acid to remove unhydrolyzed material. The ε-pyridoxyllysine was then eluted with 0.81 M acetic acid. When spotted on paper, ε-pyridoxyllysine fluoresces blue-white in ultraviolet light, and gives a positive reaction (blue-purple) with ninhydrin and with 2,6-dichloroquinone chlorimide reagent. Fractions of the eluate which showed these reactions appeared 35 ml after the eluant was applied. All such fractions were combined and evaporated under reduced pressure at 60°. The residual oil was dissolved in absolute ethanol and the sample was redried by the same procedure. The oil was again dissolved in ethanol and held overnight at room temperature. Pure ε-pyridoxyllysine crystallized out and was collected by filtration and dried under reduced pressure. The yield was 110 mg of pale yellow crystals, mp 214–214.5°.

Anal. Calcd. for $C_{14}H_{23}O_4N_3$ (297.4): C, 56.50; H, 7.80; N, 14.15. Found: C, 56.42; H, 7.63; N, 13.85.

Miscellaneous.—Saturated ammonium sulfate was neutralized directly with aqueous ammonia solution. The *p*H of all solutions containing enzyme was kept between 7.0 and 7.5 by the addition of aqueous ammonia.

All buffers were prepared and their *p*H was measured at the temperature in use. Dialysis tubing was boiled for 30 minutes in distilled water before use.

Protein determinations were made by the method of Lowry *et al.* (1951); bovine serum albumin was the standard. Purified enzyme, dried to constant weight after analysis by this method, was found to be within 5% of the amount of protein calculated. 4-Deoxypyridoxine, 5-deoxypyridoxamine, ω-methylpyridoxine, pyridoxyl-L-tyrosine, pyridoxyl-DL-valine, pyridoxyl-DL-isoleucine, and pyridoxyl-DL-alanine were gifts from Merck, Sharp and Dohme, and were supplied through the courtesy of Dr. Karl Folkers. The preparation of ω-methylpyridoxal and ω-methylpyridoxamine from ω-methylpyridoxine has been described (Ikawa and Snell, 1954). Other chemicals were from commercial sources.

Results

Improved Preparative Procedure for the Transaminase.—The purification of pyridoxamine-pyruvate transaminase from *Pseudomonas* MA-1 involves precipitation of nucleic acids with protamine, heat inactivation of contaminating proteins, and ammonium sulfate fractionation, followed by acetone fractionation to yield the crystalline transaminase (Wada and Snell, 1962a). We describe herein a greatly simplified batch procedure for this acetone fractionation.

The protein fraction soluble in 45% and insoluble in 60% saturated ammonium sulfate (Wada and Snell, 1962a) was dialyzed for 12 hours at 5° against 100 volumes of 0.02 M potassium phosphate, *p*H 7.0. Acetone was then added in four sequential steps, each employing a volume equal to one-half that of the *initial* dialyzed ammonium sulfate fraction (previous paragraph). No other measurements of volumes were made. The dialyzed enzyme solution was placed in a −20° bath, and acetone at −20° was added in 1 minute with stirring. The mixture was stirred for an additional 2 minutes. Precipitates were removed by centrifugation at −15° for 10 minutes at 15,000 × *g*, suspended immediately at 0° in 0.02 M potassium phosphate, *p*H 7.0, and dialyzed overnight at 5° against this same buffer made 0.001 M in pyridoxal. Supernatant solutions were treated with more acetone as described above. Under these conditions the enzyme always precipitated during the third addition of acetone. The precipitate containing the enzyme could be identified by its large bulk and bright yellow color. After dialysis the enzyme crystallized at 5° within 12 hours after the addition of saturated ammonium sulfate solution to incipient turbidity. It was then recrystallized to constant specific activity (5–7 recrystallizations were required) from 0.02 M potassium phosphate buffer 0.001 M in pyridoxal, at *p*H 7.0, by addition of saturated ammonium sulfate, as described for the initial crystallization. A protocol of a typical purification by this procedure is shown in Table I. Purified enzyme fractions (specific activity > 33) were largely denatured during recrystallization whenever their solution was attempted in buffer not containing pyridoxal. The final preparations obtained by this procedure are entirely free of the insoluble protein noted in the earlier preparations (Wada and Snell, 1962a). The enzyme (30 mg/ml) was stored in crystalline form in 60% saturated ammonium sulfate, *p*H 7.2, 0.001 M in pyridoxal, and was stable at least 6 months at 5° under these conditions. For assay, this suspension of crystalline enzyme was diluted with 10^4 parts of 0.02 M potassium phosphate, *p*H 7.0. Lyophilized crystals stored at room temperature for one month retained 60% of the original activity.

TABLE I
ACETONE FRACTIONATION OF PYRIDOXAMINE-PYRUVATE TRANSAMINASE[a]

Fraction	Description	Specific Activity	Wt. of Pure Enzyme in Fraction (mg)
a	Starting fraction	6.37	34.3
b	Acetone precipitate 1	1.17	
c	2	2.00	
d	3	23.1	33.8
e	4	1.75	
f	Fraction d, recrystallized 7 times	40.0	

[a] The amount of enzyme (column 4) is a calculated value based upon a final specific activity of 40. Specific activity is expressed as μmoles of pyridoxal formed per minute at 37° per mg of protein. The assay was by procedure 1; the assay mixture contained 300 μmoles of Tris, 10 μmoles of pyruvate, 10 μmoles of pyridoxamine, and 10 μmoles of EDTA in 3 ml. The *p*H at 37° was 8.50.

Specific Activity of the Crystalline Transaminase.—The specific activity of the crystalline transaminase (Table I) obtained by this procedure is 5- to 6-fold higher than previously reported.[1] Since the previous prepara-

[1] In accordance with recommendations of the International Commission on Enzyme Nomenclature, specific activity as used in this paper represents μmoles of pyridoxal formed per *minute* per mg of protein (Table I). In the preceding paper (Wada and Snell, 1962a) specific activity of this enzyme was expressed in terms of μmoles of pyridoxal formed per *hour* per mg of protein.

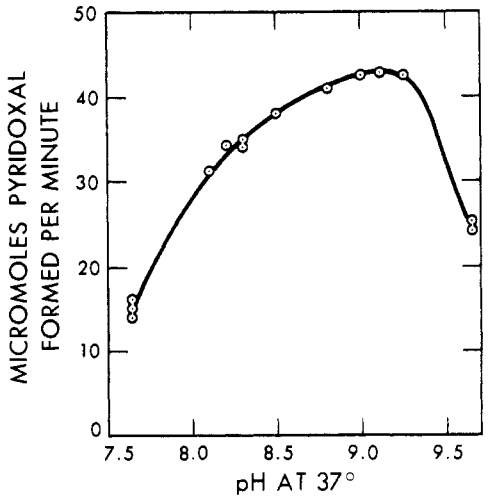

FIG. 1.—*p*H optimum of transamination reaction between pyridoxamine and pyruvate at 37°. Measurements were made in reaction mixture containing per 3 ml: 1.0 μg of crystalline enzyme (specific activity 36.7) 300 μmoles of Tris, 10 μmoles of sodium pyruvate 10 μmoles of pyridoxamine, and 10 μmoles of EDTA. The *p*H values were measured at 37°. Pyridoxal formation was measured by procedure 1.

tion, like the present one, was a homogeneous crystalline protein which interacted stoichiometrically with pyridoxal, some explanation of the markedly different specific activities of the two preparations was sought. Some of this difference results from the presence of the previously noted insoluble material in purified preparations obtained by the older procedure. A larger portion of the difference appears to result from the fact that pyridoxine, which is added to stabilize the enzyme during the purification, and also pyridoxal interfere with the determination of protein by the Lowry method sufficiently to result in serious overestimation of the protein present and consequent underestimation of the specific activities.[2] The higher final specific activity of the crystalline enzyme prepared by the method described here results chiefly from removal of excess residual vitamin B_6 by dialysis after the acetone precipitation.[3]

To ascertain the reason for its yellow color, several preparations of the enzyme were made without the addition of pyridoxal. The yield in these cases was poor owing to extensive denaturation. Two of these preparations were recrystallized seven times (specific activity, 40), then precipitated in 60% saturated ammonium sulfate, *p*H 4.0. The supernatant solutions were then assayed for total vitamin B_6 with *Saccharomyces carlsbergensis* (Rabinowitz and Snell, 1947).[4] One mole of the vitamin was found per 150,000 g of enzyme. From its absorbance maximum at 380 mμ (*p*H 6.8), this was identified as pyridoxal. This amount of bound pyridoxal, which could not be detected in the normal assay procedures by either the Lowry method for protein or the phenylhydrazine assay for pyridoxal, lends the yellow color to the crystalline protein.

*p*H *Optimum and Substrate Specificity.*—The *p*H optimum of the pure enzyme (Fig. 1) is somewhat higher than that previously reported. The transaminase is specific for pyruvate as the keto acid substrate (Wada and Snell, 1962a). K_M values for each substrate,

TABLE II

SUBSTRATE AFFINITIES AND MAXIMUM VELOCITIES OF THE PYRIDOXAMINE-PYRUVATE TRANSAMINASE[a]

	K_M	V_{max} (20°, *p*H 9.1) μmoles/min/mg
Pyridoxamine	6×10^{-5} M	13.8
Pyruvate	3×10^{-4} M	
Pyridoxal	1.5×10^{-5} M	7.2
L-Alanine	2×10^{-3} M	

[a] K_M values and maximum velocities were determined by double reciprocal plots of reaction velocity against substrate concentration. Each reaction vessel contained, per ml, 100 μmoles of potassium carbonate, 3 μmoles of EDTA, and the two substrates for the reaction to be studied. For measuring the K_M value for L-alanine, 0.8 μmole of pyridoxal was used; 3.3 μmoles of each of the other substrates whose concentrations were not being varied was added. The *p*H of each mixture was adjusted to 9.1 at 20°. Initial velocities were measured at 20° by following the rate of change of pyridoxal concentration at 400 mμ (procedure 2). Reaction was allowed to proceed no further than 10% toward the equilibrium position.

TABLE III

REACTIVITY OF PYRIDOXAMINE ANALOGS AS SUBSTRATES FOR THE ENZYME[a]

	K_M	V_{max} (37°, *p*H 8.5) μmoles/min/mg
Pyridoxamine	3.1×10^{-5} M	43
5-Deoxypyridoxamine	3.0×10^{-5} M	30
ω-Methylpyridoxamine	1.7×10^{-3} M	110
Pyridoxamine 5-phosphate	2×10^{-3} M	13

	K_I
Pyridoxine	5.2×10^{-5} M
4-Deoxypyridoxine	1.3×10^{-4} M
Pyridoxyl-L-alanine	1.8×10^{-7} M
Pyridoxyl-L-isoleucine	1.2×10^{-4} M
Pyridoxyl-L-tyrosine	3.6×10^{-5} M
Pyridoxyl-L-valine	1.0×10^{-5} M

[a] Values were calculated from double reciprocal plots of rate of aldehyde formation vs. concentration of the corresponding amine. Each reaction vessel contained, per ml, 100 μmoles of Tris, 10 μmoles of EDTA, 3 μmoles of Na-pyruvate, and 0.03–3 μmoles of amine. The *p*H was adjusted to 8.50 at 37° with HCl, and the reaction was started at 37° by introduction of 1 μg of enzyme. Aldehyde formation was determined with appropriate standards by procedure 1. Inhibitors were tested with pyridoxamine as substrate. Differences between values for V_{max} given here and in Table II arise from differences in assay conditions.

[2] For example, an aliquot of the supernatant solution from the heat step (Wada and Snell, 1962a), initially 0.01 M in pyridoxine and giving a color value equivalent to 40 μg of serum albumin, may contain as little as 6 μg of protein as indicated by the value obtained after extensive dialysis. The true specific activity of the protein in that fraction is thus substantially greater than that previously reported.

[3] An additional factor partially responsible for the low specific activity found previously is the pronounced effect of dilution and temperature on the *p*H of Tris buffers. In the previous work the *p*H was measured at room temperature in the stock buffer solution prior to dilution with other components of the enzymatic reaction mixture, whereas the enzymatic reaction was carried out at 37°. Both factors result in overestimation of the final *p*H, so that the actual assay was run at a *p*H somewhat below the optimum for the enzyme.

[4] This assay was kindly performed by Dr. B. M. Guirard.

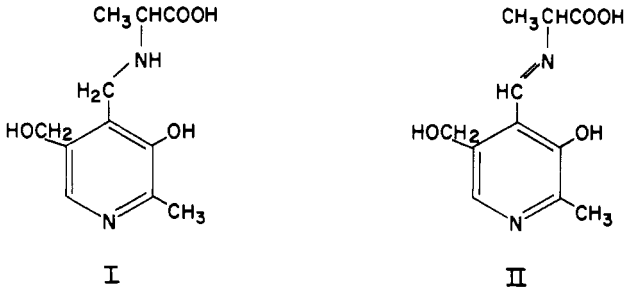

FIG. 2.—Structures of pyridoxylalanine (I) and pyridoxylidenealanine (II).

and the initial maximal velocity of reaction (1) in each direction at 20° and *p*H 9.1, are shown in Table II.

The specificity of pyridoxamine as substrate was examined at 37° and *p*H 8.5 (Table III). 5-Deoxypyridoxamine is equally as effective as pyridoxamine as a substrate. The affinity of the enzyme for ω-methylpyridoxamine is markedly lower than that for pyridoxamine, but the maximum velocity of reaction (1a) is higher with this compound as substrate than with pyridoxamine itself. The enzyme was very strongly inhibited by 10^{-6} M pyridoxylalanine; several other pyridoxylamino acids were very much less effective as inhibitors. This specificity in the inhibitory action of pyridoxylalanine, which differs from pyridoxylidenealanine only in the absence of a double bond (Fig. 2), supports current concepts of the transamination reaction (Snell, 1960, 1963) according to which the aldimine, pyridoxylidenealanine, should be an enzyme-bound intermediate in the reaction catalyzed by this enzyme. Pyridoxine inhibits the reaction and has an affinity for the enzyme similar to that of pyridoxamine and pyridoxal. Each of the inhibitors listed (Table III) was competitive with pyridoxamine as judged from Lineweaver-Burk plots.

At high concentrations, pyridoxamine phosphate was a relatively poor substrate for the enzyme (Table III). The assay employed limited the concentration of aldehyde that could be tested to 1 mM; at this concentration of pyridoxal-5-P the reverse reaction was not found. The possibility that pyridoxal-5-P might serve as a coenzyme for transamination between alanine and a keto acid was investigated by incubating 0.1 mg of the enzyme with 1 μmole of pyridoxal-5-P in 0.2 ml of 0.02 M phosphate, *p*H 7.5, for 10 minutes at 25° and testing this enzyme for ability to catalyze pyruvate formation at *p*H 9.1 and 25° in a system containing in 1 ml potassium carbonate, 100 μmoles; L-alanine, 10 μmoles; α-ketoglutarate or oxalacetate, 3 μmoles; DPNH, 140 μg; and excess lactic dehydrogenase. No pyruvate was formed, as measured by DPNH oxidation, unless pyridoxal was added.

The specificity of the enzyme for alanine was verified by replacing L-alanine as substrate in reaction (1b) by D-alanine, L-serine, L-threonine, glycine, L-leucine, L-glutamate, L-aspartate, β-alanine, DL-α-*n*-valeric acid, DL-β-amino-*n*-butyric acid, DL-α-amino-*n*-butyric acid, L-isoleucine, L-valine, L-methionine, L-tryptophan, L-phenylalanine, L-lysine, L-arginine, or L-histidine, each at a final concentration of 0.1 mg/ml. Of these, only α-aminobutyrate replaced alanine, and the observed rate of the reaction was less than 5% of that found for L-alanine at the same concentration. DL-Alanine ethyl ester and DL-alanine amide showed similarly low activity.

Nature and Number of Binding Sites for Pyridoxal.—It was reported previously without rigorous data that each mole of crystalline enzyme (s_{20} = 7 S; mol wt *ca.* 140,000) bound 2 moles of pyridoxal, apparently in aldimine linkage (Wada and Snell, 1962a). This was confirmed and the nature of the binding sites was clarified in the following way. The pure enzyme (1.6 mg/ml) was freed of as much pyridoxal as possible by dialysis at 5°, first for 48 hours against three changes of 100 volumes each of 0.01 M Tris (*p*H 8.0) containing 0.01 M L-alanine, then for 24 hours against 2 changes (100 volumes each) of 0.01 M Tris–0.01 M potassium phosphate buffer, *p*H 8.0, and finally for 48 hours against three changes (100 volumes each) of 0.02 M potassium phosphate, *p*H 7.0. Even after this time the ultraviolet spectrum of the enzyme indicated that some pyridoxal (< 0.1 mole/mole enzyme) remained

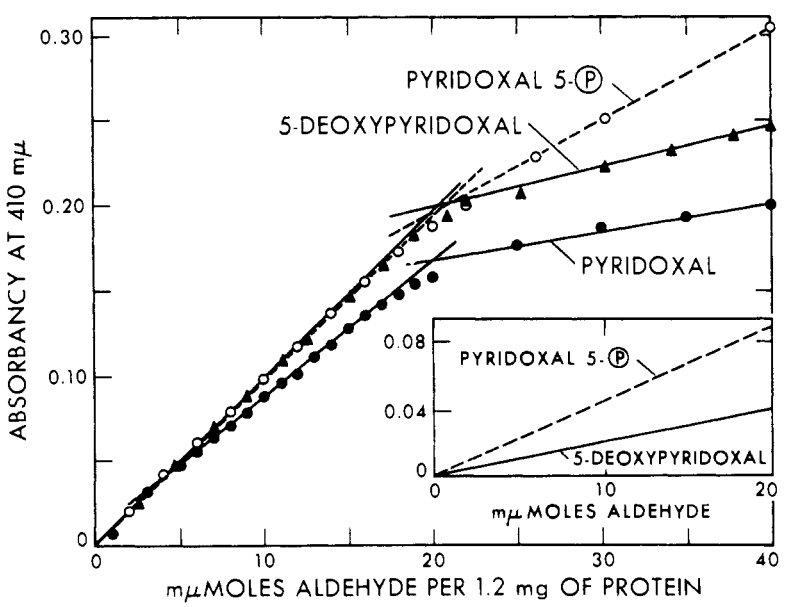

FIG. 3.—Titration of the crystalline transaminase with various aldehydes. The spectra were measured in 1-cm silica cells at *p*H 7.0 and 20° immediately after addition of the aldehyde to a solution of enzyme which was initially free of aldehyde. The variation in the absorbance at 410 mμ with concentration of the free aldehydes (in the absence of protein) is indicated in the inset.

TABLE IV
Properties of Enzyme Protein after Borohydride Reduction in the Presence of Different Aldehydes[a]

	Absorbancy Maximum (mμ)	Absorbancy (at 330 mμ)	Specific Activity
Enzyme alone	330	0.016	25.40
Enzyme plus one equivalent of			
Pyridoxal	329	0.166	0.25
5-Deoxypyridoxal	327	0.152	0.63
Pyridoxal 5-phosphate	325	0.086	8.30
ω-Methylpyridoxal	329	0.134	3.60

[a] Absorbancies at 330 mμ are for 0.1% solutions of protein. $NaBH_4$ in amount equimolar with the aldehyde was added to mixtures containing 1 mole of aldehyde per 60,000 g protein 10 minutes after addition of the aldehyde. Each mixture initially contained 600 μg of enzyme in 1 ml of 0.02 M potassium phosphate, *p*H 7.0. Experiments with pyridoxal and ω-methylpyridoxal were also performed with 1200 μg of protein in 1 ml of 0.5 M potassium phosphate, *p*H 6.5, with identical results. Enzyme activities were measured by procedure 1 after dialysis of the reaction mixtures.

bound. Similar dialysis entirely at *p*H 7.0 was much less effective in removing bound pyridoxal.

Pyridoxal at *p*H 7.0 was then added in measured increments to 1 ml of the dialyzed enzyme solution (1.2 mg of protein per ml), and the absorbancy at 410 mμ was measured immediately after each addition (Fig. 3). The increase in this value was initially linear with the addition of pyridoxal. The point at which the sharp change in slope occurred to an absorbancy characteristic of pyridoxal alone was taken as the end point of the titration and gives a value of 1 mole of pyridoxal bound per 60,000 g of enzyme. Similar titrations with 5-deoxypyridoxal and pyridoxal-5-P give values of 1 mole of aldehyde bound per 59,000 and 61,000 g of enzyme, respectively (Fig. 3). These values confirm previous findings that 2 moles of pyridoxal bind per mole of crystalline enzyme and give a more accurate estimate of the particle weight of the enzyme as 120,000 ± 4000.

Similar titrations were attempted with ω-methylpyridoxal, which also serves as a substrate for this enzyme and hence binds at the active site. However, use of this pyridoxal analog does not lead to enhanced absorbancy at 410 mμ. Instead, the spectrum observed has maxima identical with those of the free aldehyde (253, 318 mμ).

Since the maximum at 410 mμ displayed by the pyridoxal-enzyme complex is characteristic of hydrogen-bonded aldimines of pyridoxal (Metzler, 1957), reduction with sodium borohydride should fix the pyridoxal at the active site in a form stable to hydrolysis (Fischer *et al.*, 1957; Hughes *et al.*, 1962; Dempsey and Christensen, 1962). Accordingly, sodium borohydride equimolar with aldehyde was added to mixtures containing 1 mole of the various aldehydes per 60,000 g of enzyme. Reactions were carried out at 20° in 0.02 M potassium phosphate, *p*H 7.0, and a protein concentration of 0.06%. Immediate disappearance of the absorption maximum at 410 mμ and appearance of a maximum at 320–330 mμ, representing the reduced product, was observed with mixtures containing either pyridoxal or 5-deoxypyridoxal. The spectrum of the mixture of pyridoxal-5-P with enzyme changed much more slowly; even on addition of a 3:1 ratio of borohydride to aldehyde, the changes occurred over a 15-minute

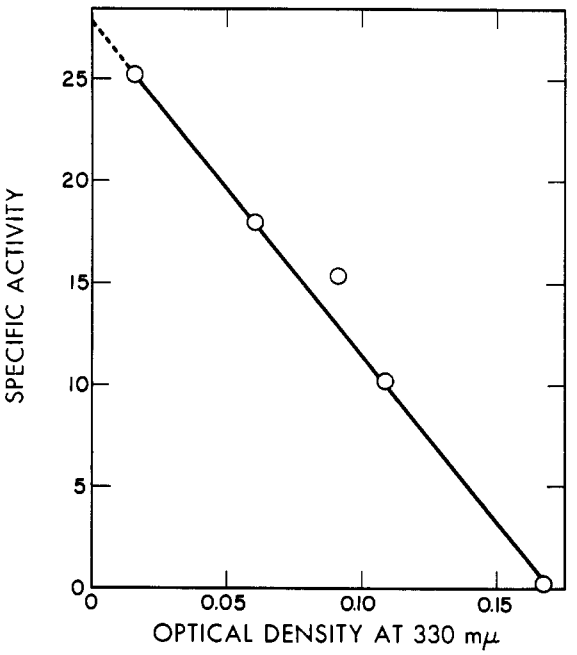

FIG. 4.—Relation between transaminase activity and absorbancy at 330 mμ after sodium borohydride treatment in the presence of increasing amounts of pyridoxal. Purified enzyme (specific activity 30) was largely freed of pyridoxal by exhaustive dialysis as described in the text. Aliquots of this solution containing 1.2 mg of protein per ml were treated with increasing amounts of pyridoxal (0 to 20 mμmoles from left to right) followed by 25 mμmoles of $NaBH_4$. The net absorbancy at 330 mμ and the enzyme activity were measured after dialysis.

period. Although showing no spectral evidence of interaction with the enzyme, a mixture of ω-methylpyridoxal with enzyme was converted instantaneously to a product absorbing at 325 mμ by the addition of equimolar sodium borohydride.

Each protein solution, together with a control solution of enzyme which had been treated with sodium borohydride in the absence of any added aldehyde, was dialyzed for 24 hours at 5° against two changes (2000 volumes each) of 0.02 M potassium phosphate, *p*H 7.0. The solutions were clarified by centrifugation and the ultraviolet spectrum and enzymatic activity of each solution was measured (Table IV). The disappearance of enzymatic activity coincident with appearance of the new absorption band indicates that the aldehydes were, indeed, attached at or near the active site of the enzyme.

These experiments were repeated to determine whether inactivation of enzyme by borohydride was proportional to the amount of pyridoxal added below the equivalance point. The absorbancy at 330 mμ, a measure of the amount of vitamin B_6 attached to the protein after reduction, was directly related to the decrease in specific activity of the enzyme (Fig. 4).

These experiments demonstrate that pyridoxal (or its analogs) is attached at the active site of the enzyme in a form which is reduced by borohydride to yield a stable complex with a spectrum characteristic of pyridoxylamino acids or similar compounds. To determine the mode of binding, the pyridoxal-treated reduced proteins were hydrolyzed for 24 hours at 105° in 6 N HCl. The hydrolysates were dried in vacuum and chromatographed on Whatman No. 1 paper in water-methanol-ethanol-benzene-pyridine-dioxane (25:25:10:10:10:10, Dempsey and Christensen, 1962), in butanol–acetic acid–water (4:1:1), and in 77% ethanol.

TABLE V
AMINO ACID COMPOSITION OF THE PURIFIED ENZYME[a]

Amino Acid	μmole per mg of Protein
Aspartic acid	0.72
Glutamic acid	0.53
Alanine	1.26
Half-cystine	0.04
Glycine	0.73
Isoleucine	0.44
Leucine	0.76
Methionine (+ sulfoxides)	0.24
Phenylalanine	0.15
Proline	0.55
Serine	0.36
Threonine	0.41
Tyrosine	0.29
Valine	0.59
Arginine	0.37
Histidine	0.19
Lysine	0.38
Ammonia	0.55

[a] Analyses were performed by the Oxford Laboratories, Redwood City, California. Preparation of protein hydrolysates followed standard procedures (see text). The values shown are the mean of five analyses performed on 24-, 48-, and 72-hour hydrolysates. No significant differences were observed between values at the three times except for valine and isoleucine, for which the 72-hour values are given. Cysteic acid was not found.

Each such hydrolysate showed the presence in each solvent system of a fluorescent material corresponding in R_F value in the three solvents (0.49, 0.36, 0.02, respectively) to synthetic ε-pyridoxyllysine. The fluorescent area from each sample was eluted with water for spectrophotometry. The eluted material accounted for at least 85% of the absorbancy at 323 mμ observed in the protein solution before and after hydrolysis. The eluted material was then rechromatographed in two additional solvents; in each case it again corresponded to ε-pyridoxyllysine. It also corresponded to authentic ε-pyridoxyllysine in its reactivity to ninhydrin and to 2,4-dichloroquinonechlorimide, and in its fluorescence, which is reversibly quenched by NH_3 fumes. The pyridoxyl derivatives of the other amino acids do not give positive reactions to all three tests. Thus, all the pyridoxal that is bound specifically by the enzyme gives rise on reduction to ε-pyridoxyllysine, and a hydrogen-bonded aldimine of pyridoxal with a lysyl residue appears to be responsible for the absorbancy of the complex at 410 mμ and the consequent yellow color of the enzyme. Such hydrogen bonding of the aldimine appears not to be required for its participation in transamination, however, since ω-methylpyridoxal, which shows no evidence of such bonding, does form an aldimine with the enzyme (as evidenced by its reduction to an inactive complex of the proper absorbancy by borohydride) and does undergo transamination. Conversely, pyridoxal phosphate, which binds as a hydrogen bonded aldimine, does not undergo transamination.

The finding that the ε-amino group of a lysyl residue interacts with pyridoxal in this transaminase as it does with pyridoxal phosphate in glutamic aspartic transaminase (Fischer and Krebs, 1959) indicates that this group is part of the active site of both enzymes. Reasons for believing that such a group may contribute to the catalytic action of the transaminases have been summarized elsewhere (Snell, 1963).

No significant quantities of trace metals were found on spectrographic analysis of the purified enzyme preparations. The amino acid analysis of the enzyme (Table V) reveals it to be unusually rich in proline. In addition to the amino acids listed, small amounts of an unidentified ninhydrin-reactive peak appeared in the lysine region.

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