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The Stereochemistry of Enzymatic Transamination[*]

H. C. Dunathan, L. Davis, P. Gilmer Kury, and M. Kaplan

ABSTRACT: An approach to the determination of the complete stereochemistry of enzymatic transamination is described. Stereospecificity in the enzymatic labilization of one of the 4-methylene protons of pyridoxamine has been demonstrated in the transamination of pyridoxamine catalyzed by apoglutamate–oxaloacetate transaminase.

Both enantiomers of the 4-($CHD-NH_2$) pyridoxamine have been prepared. These compounds show the expected kinetic isotope effects in the enzymatic transamination. This effect provides a convenient way to compare the symmetries of monodeuteriopyridoxamine samples derived from different enzymes. It is suggested that the symmetry of the hydrogen labilized at the pyridoxamine 4-methylene group may be related to the symmetry of the amino acid substrate. A tentative assignment of the absolute symmetry of the monodeuteriopyridoxamines is made.

The large family of enzymes utilizing pyridoxal phosphate as cofactor catalyze a great variety of transformations of amino acids (Braunstein, 1963).

In all cases the mode of action of the cofactor can be understood in terms of the original mechanism of Braunstein and Schemyakin (1953) and Snell (Metzler *et al.*, 1954).[1] In this formulation all of the enzymatic reactions involve a common intermediate, the cofactor amino acid Schiff base. The properties of reaction, substrate, and stereospecificity are then imposed on this intermediate by the apoenzyme. Given the relative simplicity of this Schiff base intermediate and the limited numbers of conformations it can assume, one may hope to achieve a real understanding of the basis for reaction and stereospecificity in this group of enzymes. In an earlier paper we suggested that reaction specificity in pyridoxal phosphate enzymes must involve enzymatic control of the amino acid C_α–N bond conformation (Dunathan, 1966). In this paper we begin to define the precise stereochemistry of enzymatic transamination.

The fundamental step of transamination is the tautomerism or 1,3-prototropic shift shown in Figure 1. This simple reaction must take place within the confines of only a few stereochemical variables. These can be listed simply: (1) the configuration at the amino acid α-carbon (C_α); (2) the configuration of the proton added to the pyridoxal carbon (C_p); (3) the conformation about the C_α–N single bond; (4) the conformation of the C_p=N double bond; and (5) the stereochemistry of the proton transfer (see Figure 1). In any real enzymatic transamination, the configuration of the amino acid is known, the conformation about the C_p=N is almost certainly "*trans*," and the C_α–N conformation is restricted to one of the two in which the C_α–H bond lies in a plane perpendicular to the plane of the cofactor π system (Dunathan, 1966).

The only real unknowns are the configuration of the proton added to the pyridoxal carbon, the choice of the C_α–N conformation, and the stereochemistry of proton transfer. This transfer will be called *cis* if the CH bond breaking and making both take place on the same side of the π system plane or *trans* if on opposite sides.

These five variables have an "algebraic" relationship to each other such that knowledge of any four of the five will define the fifth. Definition of all five will in turn define a large part of the geometry of the active site and will restrict considerably the mechanistic possibilities for the prototropic shift.

In stating these unknowns we have assumed enzymatic stereospecificity in adding and removing a proton at the pyridoxamine 4-methylene carbon. This is certainly to be expected considering the number of examples of enzymatic discrimination between the protons of a X–CH_2–Y grouping (Rose, 1966).

In this paper we describe proof of this stereospecificity at pyridoxamine and the isolation of the two monodeuteriopyridoxamine enantiomers. We have made tentative assignment of the absolute configuration of these compounds.

[1] The requirement for pyridoxal phosphate in phosphorylase (Fisher *et al.*, 1958) cannot be explained by the Snell–Braunstein mechanism.

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In these experiments we have chosen to work with the deuterium-labeled cofactor since this holds the possibility of the involvement of optical rotatory dispersion–circular dichroism and nuclear magnetic resonance measurements in determining the relative and absolute symmetries of monodeuteriopyridoxamine.

Since these determinations require considerable quantities of the cofactor molecules, it was essential to use an enzyme which transaminates but does not tightly bind the cofactor. Several enzymes are known which transaminate the nonphosphorylated forms of the B_6 cofactors (Wada and Snell, 1962a,b). In these reactions the pyridoxal and pyridoxamine act as freely diffusable substrate molecules. We have used the apoenzyme form of glutamate–oxaloacetate transaminase which has been shown to catalyze reaction 1 (Wada and Snell, 1962a).

pyridoxamine + α-ketoglutarate $\rightleftharpoons$ pyridoxal +

L-glutamate (1)
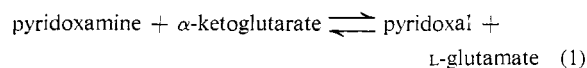
Materials and Methods

Pyridoxal and pyridoxamine were obtained from both Koch-Light Ltd. and from Sigma Chemicals Inc. Deuterium oxide (>99.5%) was purchased from Columbia Organic Chemicals, Inc.

Assays and kinetic measurements were made with both a Baush & Lomb 505 and a Gilford-DU spectrometer. Nuclear magnetic resonance spectra were determined with Varian Models A-60 and HR100.

Hologlutamate–oxaloacetate transaminase was isolated from porcine heart following Martinez-Carrion and coworkers' (1965) modification of Jenkins and coworkers' (1959) procedure. The apoenzyme was prepared from the pyridoxamine phosphate form of the enzyme (Jenkins and D'Ari, 1966) by $(NH_4)_2SO_4$ precipitation (Wada and Snell, 1962a).

The apoenzyme activity was assayed in a 3-ml volume which was 4.8 mM in pyridoxamine and 0.25 mM in 2-ketoglutarate and buffered at pH 8.5 with 0.1 N Tris. Pyridoxal formation was followed at 390 mμ. Under the assay conditions pyridoxal–Schiff base equilibria are rapidly established and the pyridoxal follows Beers' law in the optical density range observed. The yield of apoenzyme activity varied markedly in different preparations. At best the ratio of turnover numbers holo/apo was about 500.

Pyridoxal and pyridoxamine were separated and purified by the method of Peterson and Sober (1954).

Preparation of Deuteriopyridoxamine. Pyridoxamine–pyridoxal (3:1) mixtures in D_2O at pD 4–5 were allowed to stand at room temperature for several days. The mixtures were freeze dried, taken up in pH 7.5 phosphate buffer, and separated on an Amberlite CG 120 column at alkaline pH. Good yields of deuterated pyridoxamine were obtained but the pyridoxal is largely destroyed, apparently by carbon–carbon condensation reactions.

Deuterium content was determined by nuclear magnetic resonance spectroscopy in deuterioacetic acid solvent. The 4-CH_2NH_2 and the 5-CH_2OH resonances saturate at about the same rate. Using the 5-CH_2OH as a standard the deuterium content at the 4-CH_2NH_2 posi-
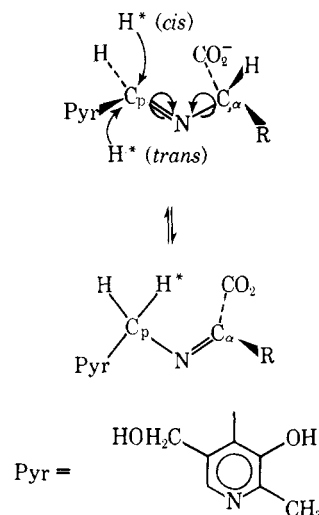

FIGURE 1: Stereochemical variables in transamination. The amino acid C_α configuration is related to the configuration of H* at C_p in pyridoxamine by three variables. These are: the C_α–N and C_p–N bond conformations and the *cis* or *trans* nature of the tautomerization.

tion could be determined on a small sample within a few per cent.[2]

Kinetic Runs. CO_2-free distilled water was used for all solutions and the solutions were protected from atmospheric CO_2. The short exposure necessary during mixing did not seem to affect the results when closed cuvets were used. In all kinetic isotope effect measurements, at least triplicate determinations were made, alternating the hydrogen and deuterium compounds.

Results

Preparation of Deuteriopyridoxamine. Pyridoxamine and pyridoxal undergo relatively rapid nonenzymatic transamination at room temperature and pH values below 7. A detailed study of this transamination will be reported elsewhere. The pH dependency apparently reflects the requirement that the pyridine ring nitrogen be protonated for effective labilization of the 4-methylene protons. Pyridoxamine, 95% deuterated at the 4-methylene group, was isolated from this transamination. Deuterium is also introduced in the 2-methyl group (20–30%) and at the 6-ring position (5%).

Apoglutamate–Oxaloacetate Transaminase. No attempt was made to separate the isozyme mixture which makes up porcine glutamate–oxaloacetate transaminase (Martinez-Carrion *et al.*, 1965). Our purification steps presumably leave us with a mixture of the major supernatant isozymes. Since these isozymes maintain their separate identities when in the apo form, the results reported here for the apo reaction are pertinent to each of the major isozymes.

The apoenzyme is much more labile than the hologlutamate–oxaloacetate transaminase and shows un-
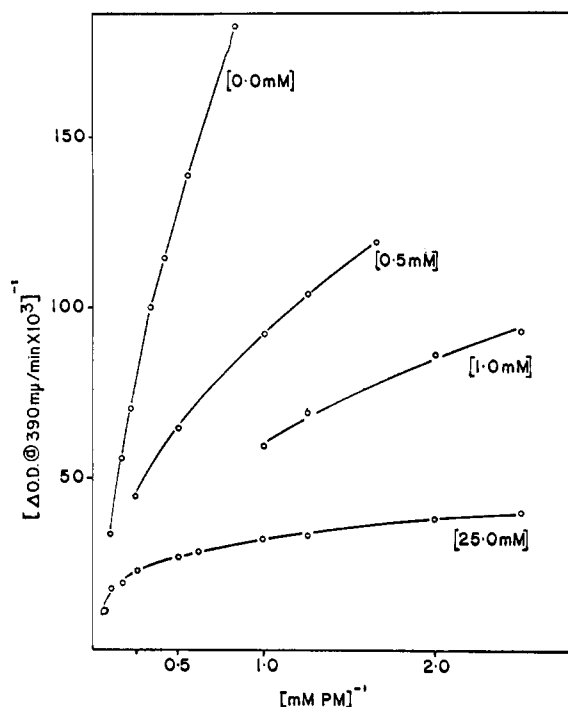

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FIGURE 2: Lineweaver–Burk plots of the rate of pyridoxal formation with pyridoxamine the variable substrate. Initial velocities were measured at 37° in 0.1 M Tris (pH 8.5) which was 0.25 × 10^{-3} M in 2-ketoglutarate. Concentration of added carbonate is shown in brackets.
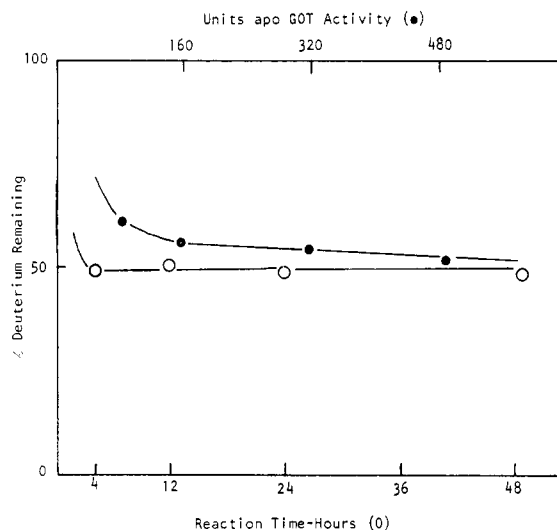

FIGURE 3: Loss of deuterium label from the 4-methylene position of D_2-pyridoxamine in transamination with ketoglutarate catalyzed by apoglutamate–oxaloacetate transaminase. Per cent deuterium is shown as a function of time and of enzyme concentration.

usual kinetic behavior. Figure 2 shows a Lineweaver–Burk plot with pyridoxamine as the variable substrate. There is a marked deviation from linearity. This may represent an isozyme component with a very large K_m or some second-order interaction of pyridoxamine with the apoglutamate–oxaloacetate transaminase. Unfortunately the other substrate, ketoglutarate, acts as an inhibitor above $\sim 10^{-3}$ M precluding examination of its simple binding behavior (Wada and Snell, 1962a). Figure 2 also shows the rather dramatic effect of low carbonate–bicarbonate concentrations on the reaction rate and kinetic constants. It is apparent that carbonate or bicarbonate binds with a K_{eff} of 1 × 10^{-3} M and that it increases both pyridoxamine binding and V_{max} (up to high pyridoxamine concentrations). This effect is not found with formate, acetate, or sulfate.

Enzyme Stereospecificity in Proton Removal from Pyridoxamine. A solution which was 36 mM in dideuteriopyridoxamine, 0.40 mM in α-ketoglutarate, and 26 mM in L-glutamate and buffered at pH 8.5 with 0.1 N carbonate was incubated with 6000 units of apoenzyme.[3] Deuterium loss with time was followed by removing aliquots of the mixture, isolating the pyridoxamine, and analyzing by nuclear magnetic resonance spectroscopy. In a second experiment five aliquots of the same reaction mixture were incubated for 8 hr, each with different amounts of added apoglutamate–oxaloacetate trans-

[3] A unit of apoglutamate–oxaloacetate transaminase activity is defined as that amount of enzyme which gives an initial rate of ΔOD = 0.001/min at 390 mμ under the conditions of the assay.

aminase activity. Figure 3 shows the loss of exactly half of the 4-methylene deuterium in both experiments.

When nondeuterated pyridoxamine was incubated with ketoglutarate, glutamate, and apoenzyme in D_2O solvent, the recovered pyridoxamine contained 53% deuterium at the 4-methylene position. This product must be the enantiomer of the monodeuteriopyridoxamine described above.

For convenience we have adopted the following notation in referring to these labeled components (see Figure 4). Pyridoxamine dideuterated in the 4-methylene position is referred to as D_2-pyridoxamine. The monodeuteriopyridoxamine with a proton in the "activated" configuration is HD-pyridoxamine and its enantiomer with the deuteron in the "active" configuration is DH-pyridoxamine. When it is not obvious, the enzyme used in the preparation is indicated in parenthesis as: DH-pyridoxamine (apoglutamate–oxaloacetate transaminase).

Kinetic Isotope Effects. A kinetic isotope effect is observed in the apoglutamate–oxaloacetate transaminase transamination of deuterated pyridoxamine. Table I

TABLE I: Kinetic Isotope Effects in the Transamination of Pyridoxamine.

$k_{H_2\text{-}PM}/k_{D_2\text{-}PM}$	$= 1.7\text{-}2.0^a$
$k_{H_2\text{-}PM}/k_{HD\text{-}PM(apo\text{-}GOT)}$	$= 1.09 \pm 0.05$
$k_{D_2\text{-}PM}/k_{DHPM\text{-}(apo\text{-}GOT)}$	$= 1.00$

^{a} The isotope effect is very sensitive to traces of carbonate. The value approaches 2.0 at low pyridoxamine concentration when all precautions are taken to avoid exposure to CO_2. H_2-PM, D_2-PM, and HD-PM = H_2-, D_2-, and HD-pyridoxamine; apo-GOT = apoglutamate–oxaloacetate transaminase.

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TABLE II: Effect of Carbonate on the Kinetic Isotope Effect in Pyridoxamine Transamination.

Concn of HCO$_3^-$ + CO$_3^{2-}$ (mM)	$k_{H_2\text{-PM}}/k_{D_2\text{-PM}}$	Rel Rates (H$_2$-PM)
0	1.90	1
3.3	1.15	2.8
6.5	1.09	3.1
16.6	1.07	3.4

FIGURE 4: Deuterium-substituted pyridoxamine species. R$_1$ = R$_2$ = H, H$_2$-pyridoxamine; R$_1$ = H, R$_2$ = D, HD-pyridoxamine or DH-pyridoxamine, the enantiomeric mono-deuteriopyridoxamines; R$_1$ = R$_2$ = D, D$_2$-pyridoxamine, dideuteriopyridoxamine.

shows the results with D$_2$-pyridoxamine, HD-pyridoxamine, and DH-pyridoxamine. The observed effects confirm the enantiomeric nature of the two monodeuterio-pyridoxamines and confirm their high optical purity. Table II shows the effect of carbonate-bicarbonate concentrations on the kinetic isotope effect. The rate of the deuteriopyridoxamine transamination increases more rapidly with carbonate concentration than that of the unlabeled pyridoxamine. This finally eliminates the isotope effect.

Symmetry Comparison. The observed kinetic isotope effect can be used to compare symmetries of mono-deuteriopyridoxamine samples prepared with other enzymes. One such comparison is reported in this journal (Ayling *et al.*, 1968). Optical rotatory dispersion measurements might also provide a convenient basis for comparison; however, so far no reproducible Cotton effect or circular dichroism has been observed.

Discussion

Demonstration of stereospecific proton removal in the apoglutamate–oxaloacetate transaminase catalyzed transamination of pyridoxamine provides another example of enzymatic discrimination between enantiomeric protons. This stereospecificity has now been demonstrated in a second enzyme, pyridoxamine–pyruvate transaminase, raising a question as to the relative configuration of the pyridoxamine proton labilized by the various transaminases. In this case it has been shown that both enzymes remove the same pyridoxamine proton (Ayling *et al.*, 1968). Since both enzymes are L-amino acid specific, one is tempted to predict a direct relationship between the labilized pyridoxamine proton configuration and the configuration of the amino acid substrate. If found, this relationship would imply that the detailed stereochemistry of all enzymatic transaminations is the same. A number of transaminases are available for this comparison, including some specific for D-amino acids (Martinez-Carrion and Jenkins, 1965).

A number of other pyridoxal phosphate dependent enzymes which do not normally catalyze transamination can produce or utilize the pyridoxamine form of the cofactor (Novogrodsky *et al.*, 1963; Schirch and Jenkins, 1964.) The relative stereochemistry of these enzymes can be explored with the asymmetrically labeled pyridoxamine now available.
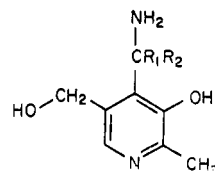
The observation of a small kinetic isotope effect in the apoglutamate–oxaloacetate transaminase catalyzed transamination of D$_2$-pyridoxamine is expected. The magnitude of the true isotope effect for CH bond breaking is certain to be reduced by the reversible formation of the anion of the cofactor substrate Schiff base on the enzyme surface. The isotope effect as measured by the rate of pyridoxal formation depends upon both the isotope effect in the CH bond-breaking step and a complex function of the relative rates of protonation of C$_p$ and C$_\alpha$, the isotope effects in this protonation, and the rate of loss of deuterium from the catalytically active group(s) at the active site. The holoenzyme reaction also exhibits a small isotope effect when α-deuterioamino acid substrates are used (Banks, 1965). Since the apoglutamate–oxaloacetate transaminase reaction does not show straightforward kinetic behavior, it has not been possible to detect any influence of deuterium substitution on K_m for pyridoxamine. The results of Table I and II indicate that if such an effect exists it is small compared with that found with pyridoxamine–pyruvate transaminase (Ayling *et al.*, 1968).

The effect of carbonate–bicarbonate on the apoglutamate–oxaloacetate transaminase transamination is reminiscent of the effects of phosphate on various apotransaminases. Wada has found both inhibition and activation with different apoglutamate–oxaloacetate transaminase isozymes. The mitochondrial apoglutamate–oxaloacetate transaminase is activated by phosphate while our supernatant apoglutamate–oxaloacetate transaminase is inhibited by phosphate (Morino and Wada, 1963).

The most attractive interpretation of the carbonate effect is that carbonate or bicarbonate binding leads to an enzyme conformation which is more effective in the CH bond-breaking step of transamination, thus speeding the reaction while eliminating the primary kinetic isotope effect. Any alternative explanation would require a rather drastic change in the transamination mechanism. It should be pointed out that apoglutamate–oxaloacetate transaminase shows the same stereospecificity in removing a pyridoxamine proton in the presence or absence of carbonate.

The observed kinetic isotope effect is the most convenient way to compare symmetries of asymmetrically monodeuterated pyridoxamine samples. One such comparison has been completed (Ayling *et al.*, 1968) and others are planned. It is less satisfactory when deuterio-pyridoxamine samples of less than 100% optical purity
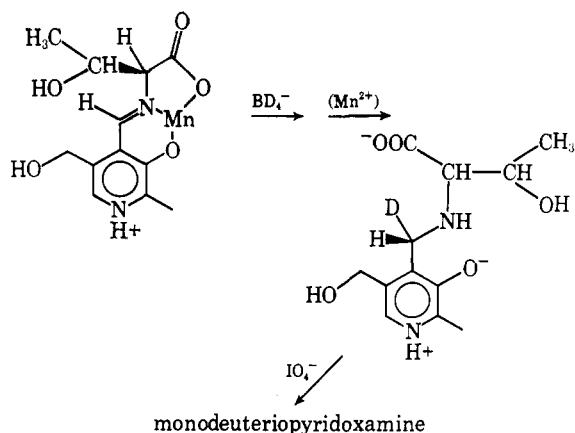
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monodeuteriopyridoxamine

FIGURE 5: An asymmetric synthesis of monodeuteriopyridox-amine. When the L-threonine-PL-Mn complex is reduced, attack from the least hindered side by BD_4^- leads to the S configuration at the new asymmetric carbon.

are being compared. This is because of the inaccuracies in rate measurements due to traces of carbonate.

We have completed an asymmetric synthesis of HD- and DH-pyridoxamine in which the direction of asymmetric induction is predictable. The reaction sequence is outlined in Figure 5. In this synthesis the geometry of the threonine–pyridoxal Schiff base is controlled through metal complex formation. Reduction of this complex with borodeuteride is assumed to take place more rapidly on the less hindered side of the double bond. The monodeuteriopyridoxylthreonine is freed of metal ion and cleaved with periodate. The monodeuterio-pyridoxamine products of this synthesis, using D- and L-threonine, give different primary isotope effects in the apoglutamate–oxaloacetate transaminase catalyzed transamination. Although the degree of asymmetric induction is not large, the results allow tentative assign-ment of the S configuration to DH-pyridoxamine (apoglutamate–oxaloacetate transaminase).[4]

The key unknown in enzymatic transamination is the cis or trans nature of the tautomerization. In recent years a number of important results have been obtained in organic systems which are reasonably good models for enzymatic transamination (Cram and Guthrie, 1966; Ohlsson et al., 1966; Guthrie et al., 1967). In these systems one commonly obtains a high degree of stereospecificity in the prototropic shift. This is often accompanied by a direct transfer of deuterium label from one terminus to the other. The extent of this transfer is a function of the nature of the base, the solvent, etc. These results are interpreted in terms of a cis 1,3 transfer, usually involving a single group acting as proton acceptor and donor. In many cases the inter-mediate is best viewed as an ion pair which retains the asymmetry of the starting material.

A number of factors make this mechanism a probable

one for the enzymatic reaction. The active site repre-sents a relatively low dielectric medium much like the solvents used in these model studies. A cis mechanism would allow maximum binding on one side of the co-factor–substrate complex while the other side is exposed to the catalytically active apoenzyme residue(s). This would provide a clear relationship between binding specificity and stereospecificity in the enzymatic reaction. Finally, molecular orbital calculations (Perault et al., 1961) indicate a high local negative charge on the $C_\alpha \cdots N \cdots C_p$ portion of the intermediate anion or "quininoid form." This charge distribution would be consistent with ion-pair formation.

Experiments in enzymatic systems which test for a transfer of isotopic label from C_α to C_p or the reverse can give information on this point. If no transfer is observed no firm conclusions are possible since rapid exchange with the solvent could compete with transfer. However, if any transfer of label is observed, a cis mechanism is strongly indicated. Recently, deuterium from α-deuterio-L-alanine has been shown to be transfered directly to pyridoxamine in the pyridoxamine–pyruvate trans-aminase catalyzed reaction (Ayling et al., 1968). Al-though the per cent transfer is small, it is consistent with transfer by a single NH_2 group in which a normal iso-tope effect discriminates against deuterium transfer and in which exchange with solvent is about as rapid as the transfer to C_p. We hope to find a similar transfer with the apoglutamate–oxaloacetate transaminase reaction. Establishing the cis mechanism and making firm assign-ment of HD-pyridoxamine absolute symmetry will allow complete definition of the stereochemistry of transamin-ation in these enzymes.

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[4] This asymmetric synthesis is being repeated under a variety of conditions with different amino acids in an attempt to in-crease the degree of asymmetric induction. These results will be reported elsewhere.

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Stereochemistry of Transamination Catalyzed by Pyridoxamine–Pyruvate Transaminase *

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ABSTRACT: In the reaction of pyridoxamine with pyruvate, giving pyridoxal and L-alanine, catalyzed by pyridoxamine–pyruvate transaminase, stereospecific transfer of a single deuterium atom to and from the 4-methylene position of pyridoxamine occurs. By conducting the reaction with unlabeled pyridoxamine in D_2O or with dideuteriopyridoxamine in H_2O, the two enantiomers of monodeuteriopyridoxamine are readily obtained. Direct comparison of products showed that the absolute stereospecificity of transfer in this reaction is the same as that observed earlier in the reaction of pyridoxamine with α-ketoglutarate, giving pyridoxal and L-glutamate, catalyzed by apoglutamate–oxaloacetate transaminase. Deuterium in either the active or inactive position of pyridoxamine causes an increase in the Michaelis constant for pyridoxamine for the transaminase reaction catalyzed by pyridoxamine–pyruvate transaminase. The Michaelis constant of the co-substrate, pyruvate, is also increased slightly by the presence of deuterium in the active position of pyridoxamine. A relatively small kinetic isotope effect also is observed. By conducting the transaminase reaction in D_2O, α-deuterio-L-alanine was prepared. When this compound was used with pyridoxal as the substrate for the transaminase reaction in H_2O solution, significant amounts of deuterium were transferred to the pyridoxamine formed. The result shows that an internal prototropic shift occurs during enzymatic transamination that is consistent only with the cis removal and addition of a proton presumably via a single group of the apoenzyme which functions as a general base–acid catalyst.

Reaction 1, catalyzed by apoglutamate–oxaloacetate transaminase (Wada and Snell, 1962a), serves as a model for a half-reaction of the over-all transamination

$$\text{pyridoxamine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyridoxal} + \text{L-glutamate} \quad (1)$$
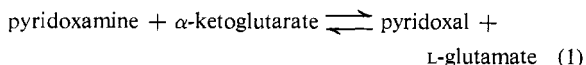
reaction (reaction 2) catalyzed by the corresponding holoenzyme. In reaction 1, pyridoxal and pyridoxamine act as poorly bound analogs of pyridoxal phosphate and pyridoxamine phosphate; the reaction proceeds much more slowly than the over-all reaction 2. Duna-

$$\text{L-glutamate} + \text{oxaloacetate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{L-aspartate} \quad (2)$$
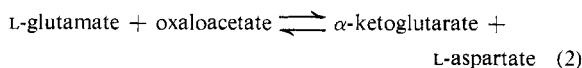
than et al. (1968a,b) showed that only one of the two protons present on the 4-methylene carbon atom of pyridoxamine was labilized during reaction 1, thus demonstrating the stereospecificity of proton transfer in this and, by implication, other transamination reactions.

We have now extended these observations to pyridoxamine–pyruvate transaminase, a pyridoxal phosphate independent enzyme that catalyzes reaction 3 (Wada and Snell, 1962b). This enzyme, which catalyzes an essential

$$\text{pyridoxamine} + \text{pyruvate} \rightleftharpoons \text{pyridoxal} + \text{L-alanine} \quad (3)$$
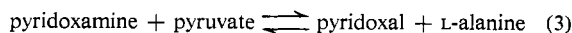
step in the degradation of pyridoxamine by a soil bacterium (Burg et al., 1960), is exceptionally useful for this type of investigation since it shows relatively high affini-

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