Transamination in Pyridoxal Phosphate Systems. Kinetic Studies of the Influence of the Side-chains of Several Amino-acids on 1,3-Prototropic shift in Zinc(II) Aldimine Complexes

By D. Hopgood, Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

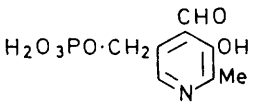
The rates of transamination of 15 amino-acids have been measured in the presence of zinc(II) and pyridoxal-5'-phosphate. On mixing the reactants, zinc(II) aldimine complexes are rapidly (*ca.* 5 min.) formed and these species subsequently transaminate in a slow, second step. Trends in the transamination rates are interpreted as resulting from various properties of the amino-acid side-chains. Side-chains of non-acid–base type appear to influence the rates by inductive and steric effects. The following order of reactivity was observed: alanine > methionine > phenylalanine > α-amino-n-butyric acid > glycine > serine > isoleucine > threonine > valine. The data indicate that electron donation by the side-chain to the α-carbon atom is rate enhancing and that bulky side-chains act to decrease the rates. The latter effect is probably due to steric hindrance either of base abstracting the α-proton or of acid donating a proton to the γ-carbon atom. Side-chains of acid–base type apparently do not effect intramolecular acid–base catalysis of the prototropic shift.

THE role of pyridoxal-5-phosphate (1) (PLP) as cofactor in a large number of enzymic reactions concerned with amino-acid metabolism continues to be a subject of intensive investigation.[1] Model systems which contain an amino-acid, PLP (or an homologue), and in some cases a metal ion, duplicate many of the enzymic reactions, albeit at much lower rates.[2]

[1] E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky, ed., 'Pyridoxal Catalysis: Enzymes and Model Systems,' Interscience, New York, N.Y., 1968.

[2] D. E. Metzler, M. Ikawa, and E. E. Snell, *J. Amer. Chem. Soc.*, 1954, **76**, 648 and references cited therein.

Schiff base (or possibly carbinolamine) condensation products appear to be necessary intermediates in reactions such as transamination, decarboxylation, and



(1)

racemization which occur in both the enzymic and model systems.[2] In the ternary systems containing metal ion, various Schiff base complexes are formed in solution [3-5] and these are postulated [2,6] to be the reactive species rather than uncomplexed aldimine present.

Kinetic studies designed to elucidate mechanistic features of PLP-mediated reactions have been reported for both binary [7] and ternary [6,8] (metal-ion containing) systems. This work was recently reviewed.[9]

The present study reports transamination kinetics of ternary systems containing zinc(II), PLP, and several amino-acids in aqueous solution. Comprehensive equilibrium data for the zinc(II), PLP, glycine, and alanine systems were recently reported by Felty, Ekstrom, and Leussing.[10] Their data are used to aid elucidation of the kinetic data described herein.

Unfortunately, detailed equilibrium data are not available for most of the systems we investigated. Nevertheless, extensive spectrophotometric data have been reported and these are used in the characterization of the reactions. Spectrophotometric measurements demonstrate that Schiff base formation occurs in solutions containing PLP and many amino-acids [4,5]. Addition of metal ions to such solutions results in spectral changes which indicate that stable metal–aldimine complexes are formed.[4,5]

We are principally interested in the influence of the amino-acid side-chains on transamination rates. Such factors as the inductive, steric, and acid-base properties of the side-chains are examined.

EXPERIMENTAL

PLP, pyridoxamine-5′-phosphate (PAP), and the amino-acids (either DL- or L-) were used as supplied by Sigma Chemical Co.

Standard solutions of zinc(II) chloride were prepared and standardized using accepted procedures. In some cases the ionic strengths of reaction solutions were adjusted to $\mu = 0{\cdot}5$M with potassium chloride.

Reaction pH values were attained by the addition of standard sodium hydroxide solution and were measured by a Radiometer pH meter 26. The pH of each reaction solution was self-buffered under the conditions used. The solutions were maintained at 25·0° under an atmosphere of nitrogen in the absence of light. The reactions were monitored by withdrawing aliquots at suitable time intervals and recording their spectra in a 1-cm path-length cell between 200 and 450 nm on a Cary 14 spectrometer.

Two series of kinetic runs were performed. In the first series the transamination rates of DL-alanine were measured over a pH range of 5·6 to 7·5 under the conditions $[Zn^{II}] = 0{\cdot}01$M, [alanine] = 0·03M, $[PLP] = 1{\cdot}0 \times 10^{-4}$M, and $\mu = 0{\cdot}5$M. After mixing, the reactants rapidly (<5 min.) yielded Schiff-base species as shown by the u.v. spectra of the solutions.[4] Published stability constants were used to calculate the species distributions in the solutions.[10] The calculations were done on high-speed digital computers using standard numerical procedures to solve the mass balance equations. The reaction conditions were chosen so that greater than 98% of the PLP present would initially react to form Zn^{II} aldimine species (3)—(7). The pH range was restricted because this condition was not satisfied below pH 5·6 under the conditions used. Above pH 7·5 precipitation of zinc complexes occurred.

The second series of runs was done under similar conditions to the first. The transamination rates of 15 amino-acids were measured at pH 5·6 and/or pH 7·0 under the conditions $[Zn^{II}] = 0{\cdot}01$M, [amino acid] = 0·03M, $[PLP] = 1{\cdot}0 \times 10^{-4}$M.

Most of the reactions were monitored until equilibrium was reached. However in some cases mould growth in the solutions precluded this. Pseudo-first-order rate constants were obtained in these systems by using the Guggenheim method.

RESULTS

The rates of transamination of the system Zn^{II} (0·01M)–Alanine (0·03M)–PLP ($1{\cdot}0 \times 10^{-4}$M) were measured over the pH range of 5·6—7·5. After the reactant solutions were mixed the PLP rapidly reacted (less than 5 min.) to give spectra with bands at 378 and 269 nm. Much slower reaction then occurred; the bands at 378 and 269 nm decreased with the concomitant increase of a band at 323 nm. Spectra from a typical run are depicted in Figure 1. All the runs gave spectra which had sharp isosbestic points at *ca.* 340 and 290 nm. The spectral changes indicate that the first fast reaction is condensation of PLP with alanine to give aldimine species [4] and that the second slower reaction is transamination of the aldimine species to give ketimine species or free pyridoxamine-5′-phosphate (2) (PAP). The final spectra of the reaction solutions were identical to spectra observed when PAP was added to the solutions. Decarboxylation of alanine did not occur (no CO_2 evolution was observed).

The distributions of the aldimine species formed after the completion of the initial fast reaction were calculated from

[3] G. L. Eichhorn and J. W. Dawes, *J. Amer. Chem. Soc.*, 1954, **76**, 5663; P. Fasella, H. Lis, N. Siliprandi, and G. Bagliani, *J. Inorg. Nuclear Chem.*, 1959, **8**, 620; L. Davis, F. Roddy, and D. E. Metzler, *J. Amer. Chem. Soc.*, 1961, **83**, 127; D. L. Leussing and N. Huq, *Analyt. Chem.*, 1966, **38**, 1388.

[4] Y. Matsuo, *J. Amer. Chem. Soc.*, 1956, **79**, 2011.

[5] R. L. Blakely, *Biochem. J.*, 1955, **61**, 315; J. Cattaneo, J. C. Senez, and P. Beaumont, *Biochim. Biophys. Acta*, 1960, **44**, 543; N. Lucas, M. King, and S. J. Brown, *Biochem. J.*, 1962, **84**, 118; Y. Matsushima and A. E. Martell, *J. Amer. Chem. Soc.*, 1967, **89**, 1322.

[6] J. B. Longenecker and E. E. Snell, *J. Amer. Chem. Soc.*, 1957, **79**, 142.

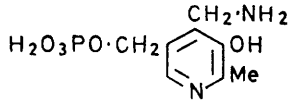
[7] B. E. C. Banks, A. A. Diamantis, and C. A. Vernon, *J. Chem. Soc.*, 1961, 4235; T. C. Bruice and R. M. Topping, *J. Amer. Chem. Soc.*, 1963, **85**, 1480, 1488, 1493.

[8] D. E. Metzler and E. E. Snell, *J. Amer. Chem. Soc.*, 1952, **74**, 979; Y. Matsushima and A. E. Martell, *ibid.*, 1967, **89**, 1331; M. E. Farago and T. Matthews, *J. Chem. Soc.* (*A*), 1969, 609.

[9] T. C. Bruice and S. J. Benkovic, ' Bio-organic Mechanisms,' W. A. Benjamin, New York, 1966, vol. 2, pp. 226—300.

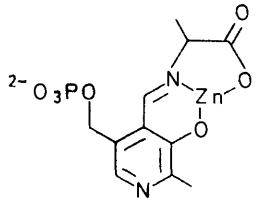
[10] W. L. Felty, C. G. Ekstrom, and D. L. Leussing, *J. Amer. Chem. Soc.*, 1970, **92**, 3006.

reported equilibrium constants.[10] This data shows that greater than 98% of the PLP is present as the zinc–aldimine

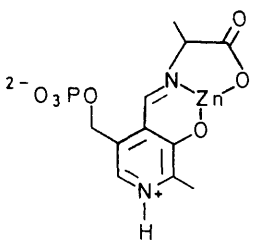


(2)

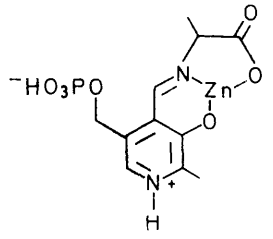
complexes (3)—(7) over the pH range 5·6—7·5. The distribution of these species are depicted in Figure 2.*



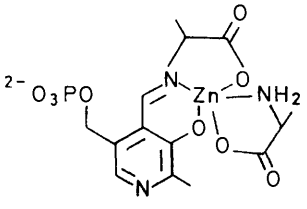
(3) ZnAP



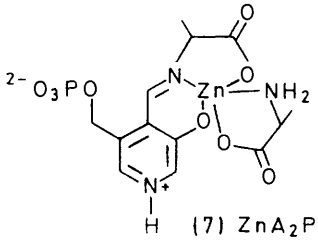
(4) ZnAPH



(5) $ZnAPH_2$

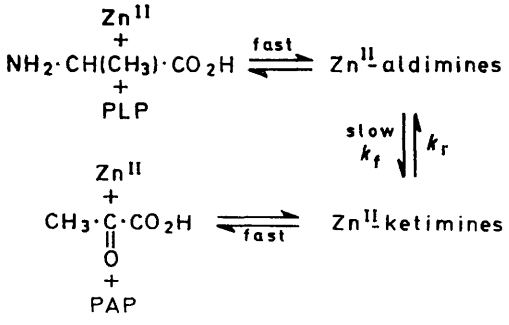


(6) ZnA_2P



(7) ZnA_2PH

We investigated the possibility that uncomplexed aldimine species significantly contribute to the observed transamination rates. Some reactions of alanine–PLP mixtures were measured in the presence of various concentrations of KH_2PO_4 and K_2HPO_4 buffers. Their transamination rates were too slow to account for any significant portion of the rates measured in the zinc ion systems. Thus the overall reaction sequence is:



The concentrations of Zn^{II} and alanine were in large excess of the PLP concentration. Identical pseudo-first-order rate constants (k_{obs}) were obtained either from the rate of decrease of the aldimine band absorbance at 378 nm or

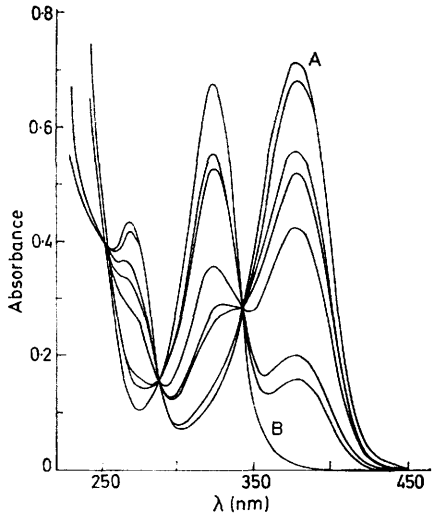


FIGURE 1 Spectra from the Zn^{II} (0·01M)–alanine (0·03M)–PLP ($1·0 \times 10^{-4}$M). System at 25·0°, pH 7·00. The scan times (in min) are: 10(A); 320; 1434; 1805; 2864; 7190; 8623; and 23,245(B)

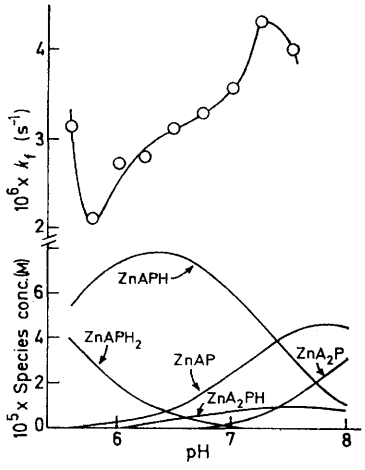


FIGURE 2 Transamination rates and species distribution of the system Zn^{II} (0·01M)–alanine (0·03M)–PLP ($1·0 \times 10^{-4}$M) at 25·0°, μ = 0·5M

from the increase of the PAP band at 323 nm.† These constants are given in Table 1.

The overall rate of transamination, k_f, is related to k_{obs} by the expression $k_{obs} = k_f + k_r$ for a reversible first-order reaction.[11] The final equilibrium values of the absorbances

* For convenience the molecular formulae are written as ZnA_1PH_m where A represents the alanine anion and P the PLP trianion.

† Excellent linear plots of log $(A_\infty - A_t)$ *vs.* time were obtained. (A_t and A_∞ are the absorbances at times t and ∞ respectively.)

[11] A. A. Frost and R. G. Pearson, ' Kinetics and Mechanism,' 2nd edn., Wiley, New York, 1961, p. 186.

of the bands at 378 and 323 nm were used to calculate values of the apparent equilibrium constant for each run.

$$K_{app} = \frac{[Zn^{II}ketimine]_t}{[Zn^{II}aldimine]_t} = \frac{[PAP]_t}{[Zn^{II}aldimine]_t}$$

These values are also given in Table 1. Rates of transamination (k_f) were calculated from $k_f = k_{obs}K_{app}/(1 + K_{app})$ and these are given in Table 1. The pH-rate (k_f) profile is plotted in Figure 2. The profile exhibits a minimum at pH 5·8 and a maximum at pH 7·3 over the pH

TABLE 1

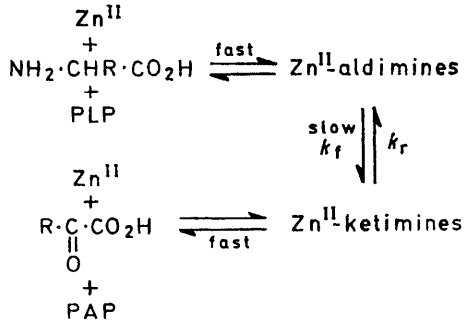
Transamination rates of the system Zn^{II} (0·01M)–alanine (0·03M)–PLP ($1·0 \times 10^{-4}$M) at 25·0° and μ = 0·5M

pH	10^6k_{obs} (s^{-1})	K_{app}	10^6k_f (s^{-1})
5·61	3·25	31·0	3·15
5·80	2·49	5·40	2·10
6·03	3·07	8·0	2·73
6·25	3·15	8·25	2·81
6·50	3·40	11·6	3·13
6·75	3·57	12·6	3·31
7·00	3·80	16·5	3·58
7·25	4·63	13·7	4·32
7·52	4·44	9·5	4·02

range 5·6—7·5. The rates do not vary by more than a factor of 2 under these conditions.

The rates of transamination of 15 α-amino-acids were measured under conditions similar to those of the alanine system except that the ionic strength was not adjusted to μ = 0·5M.

All the systems exhibit spectral changes which are very similar to those of the alanine system (Figure 1). After mixing of the reactants the absorbance of bands due to free PLP rapidly decreased with a concomitant increase in the absorbance of bands at *ca.* 380 and 270 nm. These data indicate that Zn^{II}aldimine species were formed.[4] Slow reaction then proceeded to give a band at 323 nm; the spectra exhibiting isosbestic points at *ca.* 340 and 290 nm. The magnitude of these spectral changes indicate that at least 98% of the PLP in all systems initially reacts to give Zn–aldimine complexes and that transamination occurs in a slow, second step to yield PAP species. The overall reactions are thus very similar to that of the alanine system:



Pseudo-first-order rate constants (k_{obs}) were determined as in the alanine system.* Values of k_{obs} from the systems Zn^{II} (0·01M)–amino acid (0·03M)–PLP ($1·0 \times 10^{-4}$M) at pH 5·6 and/or pH 7·0 are given in Table 2. This Table also contains values of K_{app} and k_f calculated in the same way as described for the alanine system.

Unfortunately no comprehensive equilibrium data have been measured for these systems excepting those containing either alanine or glycine.[10] The equilibrium constants reported for the glycine systems are very similar to those of the alanine system. Hence under the analogous conditions used here the initial Zn^{II}–aldimine species distributions of the alanine and glycine systems are virtually identical at any given pH.

Even though comprehensive equilibrium data are lacking for most of the systems, a large body of data exists which may be used to estimate species distributions. These data include the proton dissociation constants of the amino-acids [12,13], formation constants of Zn^{II}–amino-acid complexes,[12] conditional formation constants of aldimines in PLP–amino-acid solutions,[4] and extensive u.v./visible spectral data of PLP and its derivatives.[4,5]

The systems are conveniently divided into two groups. One group contains the amino-acids with side-chains of Brönsted acid-base type; these are aspartic acid, glutamic acid, ornithine, lysine, asparagine, and glutamine. The other group contains the amino-acids with side-chains of little or no acid–base character. The properties of this latter group indicate that they condense with PLP to form aldimine species analogous to those formed in the alanine system. Various thermodynamic parameters such as proton dissociation constants,[12,13] formation constants with divalent zinc,[12] conditional constants with PLP,[4] are all relatively invariant within this group of amino-acids. These data, taken together with the very similar reaction spectra of the systems under discussion, strongly suggest that at a given pH the initial Zn^{II}–aldimine species distributions in each of the systems are similar to each other. Thus it is likely that any marked trends which occur in the transamination rates are caused by properties associated with the side-chains. In many of the systems k_f values were measured at both pH 5·6 and 7·0. In these cases the values differ by less than a factor of 2. At either pH the values of k_f are in the order alanine > methionine > phenylalanine > α-amino-n-butyric acid (ABA) > glycine > serine > isoleucine > threonine > valine.

The effects of the acid–base side-chains on the initial Zn^{II}–aldimine species distributions are difficult to evaluate. Each of the amino-acids in this group can potentially bind as a tridentate chelate to Zn^{II} and form aldimine species with PLP which can possibly bond as tetradentate ligands. Several authors have investigated aqueous solution equilibria of most of these amino-acids with Cu^{II} [14–16] and Ni^{II}.[14] Most of these amino-acids appear to bind both as bidentate and as tridentate ligands depending on pH, concentrations, and other factors. Another complication is that both ornithine and lysine can potentially condense with PLP using either the α- or the ω-amino-groups. The reaction spectra do not distinguish between these various possibilities but only indicate that Zn^{II}–aldimine species are formed in the

* In some cases A_∞ values were not measurable and the Guggenheim method was used to obtain values of k_{obs}. These values were then used to calculate A_∞ values and hence K_{app}.

[12] *Chem. Soc. Special Publ.*, 1961, No. 17.

[13] G. Kortüm, W. Vogel, and K. Andrussow, 'Dissociation Constants of Organic Acids in Aqueous Solution,' Butterworths, London, 1961.

[14] G. R. Brubaker and D. H. Busch, *Inorg. Chem.*, 1966, **5**, 2110.

[15] E. W. Wilson, jun., M. H. Kasperian, and R. B. Martin, *J. Amer. Chem. Soc.*, 1970, **92**, 5365, and references cited therein.

[16] K. M. Wellman, T. G. Mecca, W. Mungall, and C. R. Hare, *J. Amer. Chem. Soc.*, 1968, **90**, 805.

TABLE 2

Transamination rates of the systems Zn^{II} (0·01M)–amino acid (0·03M)–PLP ($1·0 \times 10^{-4}$M) at 25·0°

		pH 5·6			pH 7·0
Amino acid	R	10^6k_{obs} (s^{-1})	K_{app}	10^6k_f (s^{-1})	$10^6k_{obs} = 10^6k$ (s^{-1})
Glycine	–H	0·71	0·79	0·31	0·24
Alanine	$-CH_3$	2·70	31·7	2·62 *	2·95 *
α-Amino-n-butyric Acid	$-C_2H_5$	0·86	2·54	0·62	1·20
Phenylalanine	$-CH_2{\cdot}Ph$	1·6	1·62	1·0	
Isoleucine	$-i\text{-}C_4H_9$				~0·05
Valine	$-i\text{-}C_3H_7$				~0·02
Aspartic acid	$-CH_2{\cdot}CO_2^-$	10·0	11·5	9·2	2·28
Glutamic acid	$-(CH_2)_2{\cdot}CO_2^-$	14·0	2·25	9·7	
Ornithine	$-(CH_2)_2{\cdot}NH_3^+$				3·5
Lysine	$-(CH_2)_4{\cdot}NH_3^+$				2·04
Serine	$-CH_2OH$	0·44	1·29	0·25	0·11
Threonine	$-CH(OH){\cdot}CH_3$				0·025
Methionine	$-(CH_2)_2{\cdot}S{\cdot}CH_3$				2·02
Asparagine	$-CH_2{\cdot}CO{\cdot}NH_2$				4·0
Glutamine	$-(CH_2)_2{\cdot}CO{\cdot}NH_2$				2·0

* These rates differ from the analogous rates given in Table 1 because of ionic strength effects.

first fast step and that these transaminate in a second much slower step.

DISCUSSION

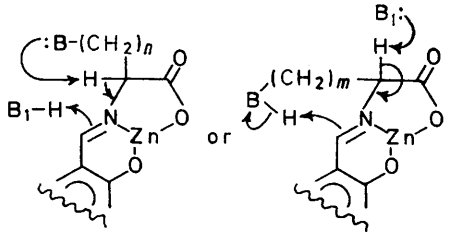
The Zn^{II}–alanine–PLP system (Table 1) contains five Zn^{II}–aldimine species (Figure 2) which exist in appreciable concentrations over the pH range 5·6—7·5. Each of these species may transaminate at rates which significantly contribute to the observed transamination rates of the system (k_f). Apart from reaction paths which may be first-order in Zn^{II}–aldimine species, there may also exist acid–base catalysed paths. Apart from the ubiquitous solvent, the only acid–base species present in appreciable concentration is the alanine zwitterion ($[NH_3^+{\cdot}CH(CH_3){\cdot}CO_2^-] = 2·83 \times 10^{-2}$M and $1·83 \times 10^{-2}$M at pH 5·6 and 7·0 respectively). A least-squares analysis was carried out to test various rate laws of the form:

$$-d[Zn^{II}\text{–aldimines}]/dt = (1 + [NH_3^+{\cdot}CH(CH_3){\cdot}CO_2^-]) \sum_{l=1}^{2}\sum_{m=0}^{2} k_{lm}[ZnA_lPH_m]$$

A minimum of five or six of these paths are necessary to describe adequately the pH-rate profile depicted in Figure 2. Obviously, measurement of a very large number of rates is necessary before a detailed kinetic analysis of the alanine system can be effected.

This study is mainly concerned with the elucidation of the effects that the various amino-acid side-chains have on the transamination rates.

The side-chains of aspartic and glutamic acids [$-(CH_2)_{1,2}{\cdot}CO_2^-$], of ornithine and lysine [$-(CH_2)_{3,4}{\cdot}NH_3^+$], and of asparagine and glutamine [$-(CH_2)_{1,2}{\cdot}CO{\cdot}NH_2$] may conceivably effect intramolecular acid–base catalysis of the prototropic shift (Scheme 4). The other amino-acids have side-chains which are unlikely to influence the transamination rates other than by their inductive and steric properties. The inductive and steric effects may be separated by utilizing Taft σ* parameters.[17] These parameters are a measure of the inductive or polarizing effect of a group and they have been experimentally determined for side-chains of several of the amino-acids under discussion. The following σ* values[17] are used in the Taft plot [$\log (k_f/k_{f_{Ala}}) = \sigma^*\rho^*$] depicted



in Figure 3: $HOCH_2^-$, +0·55; H^-, +0·49; $PhCH_2^-$, +0·22; Me, 0·0; Et, −0·10; Bu^i, −0·12; and Pr^i, −0·20. Of the systems included in Figure 3, the rates of these containing glycine, alanine, and serine are

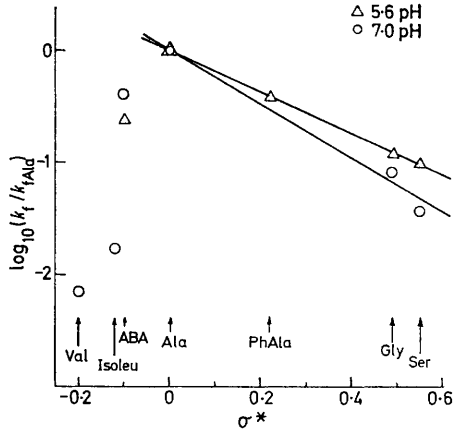
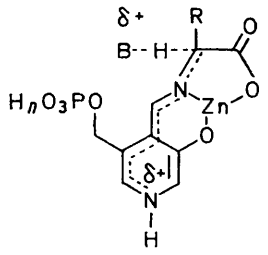


FIGURE 3 Taft plot of transamination rates of some systems Zn^{II} (0·01M)–amino-acid (0·03M)–PLP ($1·0 \times 10^{-4}$M) at 25·0°

expected to be influenced only by the inductive effects of the small side-chains. These systems exhibit linear Taft plots at both pH 5·6 and 7·0. The rate from the phenylalanine system at pH 5·6 also follows the same relationship. The ρ* values are −1·8 and −2·5 at pH

[17] R. W. Taft, *J. Amer. Chem. Soc.*, 1953, **75**, 4231.

5·6 and 7·0 respectively. The large negative ρ^* values indicate that electron donation to the α-carbon markedly enhances the rate of transamination. A widely accepted opinion [2,9] regarding transamination reactions is that electron withdrawal from the α-carbon results in enhanced rates by weakening the C–H bond. The principal evidence supporting this hypothesis is that conjugative electron-withdrawal by the aromatic ring nitrogen atom appears to be an important rate-increasing factor. However, as Pullman [18] pointed out, the α-carbon is not part of the conjugated system except in a



transition state of the type above. Thus any rate enhancement which results from conjugative electron withdrawal is probably due to resonance stabilization of the transition state. On the other hand, the α-carbon atom increases its electron density in attaining partial sp^2 character in the transition state. Thus it is not surprising that inductive electron-donation from α-carbon substituent groups also decreases the activation energy of the reaction. Consequently it appears that both inductive electron-donation and conjugative electron-withdrawal are rate-enhancing factors. On the basis of their inductive properties, the rates of the amino-acids which do not follow the linear relationships depicted in Figure 3 are markedly lower than expected. However the effect of their chains on the rates is in the order Et $\gg$ Bu^i > Pr^i which is an order of increasing bulkiness and branching. These side-chains probably decrease the rates by sterically hindering the approach of either the base abstracting the α-proton or the acid donating a proton to the γ-position.

The influences of the acid–base side-chains on the rates are impossible to determine at this time. However none of the rates of these systems differ from those of the alanine system by more than a factor of 4. Hence we tentatively conclude that intramolecular acid–base catalysis does not appear to be an important influence on the rates of prototropic shift.

I gratefully acknowledge financial support by the Wisconsin Alumini Research Foundation. I am indebted to Professor D. L. Leussing (Department of Chemistry, Ohio State University) for many invaluable discussions and for the use of his laboratories where an initial study was carried out. This preliminary study was supported by the National Science Foundation. I also thank the University of Wisconsin Computer Center for the use of their facilities.

[1/1676 *Received*, 13*th September*, 1971]

[18] B. Pullman, in ' Chemical and Biological Aspects of Pyridoxal Catalysis,' ed., E. E. Snell, P. M. Fasella, A. E. Braunstein, and A. Rossi Fannelli, Macmillan, New York, 1963, p. 103.