The Interaction of Aspartate Aminotransferase with α-Methylaspartic Acid*

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ABSTRACT: The equilibrium constant for the reaction of α-methylaspartate with aspartate aminotransferase has been determined spectrophotometrically over the pH range 5–8 at 25°. The spectrum of the enzyme–amino acid complex has peaks at 362 and 430 mμ and is independent of pH. Optical rotatory dispersion measurements show that the complex has a Cotton effect centered about 362 mμ; the magnitude of the Cotton effect is much smaller than that observed for the free enzyme.

The pH dependence of the binding constant can be ascribed to the fact that the free enzyme has an ionizable group with a pK of 6.3 (the pK value can be determined independently) and only the basic form of the enzyme interacts with α-methylaspartate. These results suggest that the protonated amino acid interacts with the basic form of the enzyme to form at least two distinct enzyme–α-methylaspartate complexes. On the basis of this and other evidence a detailed mechanism for enzymatic transamination is proposed.

An important approach to the study of enzymatic mechanisms is an investigation of the interaction of substrate analogs with the enzyme. If an analog can be found which undergoes only part of the catalytic reaction, information about possible reaction intermediates can sometimes be obtained. This type of investigation has been particularly useful in understanding the mechanism of action of aspartate aminotransferase: detailed studies of the interaction of enzyme with dicarboxylic acids, carbonyl reagents (Bonavita and Scardi, 1958; Jenkins and Sizer, 1959; Polyanovsky and Torchinsky, 1961; Velick and Vavra, 1962; Banks *et al.*, 1963; Hammes and Fasella, 1963; Sizer and Jenkins, 1963; Braunstein, 1964), and β-*erythro*-aspartic acid (Jenkins, 1961, 1964a) have been reported. A substrate analog of particular interest is α-methylaspartic acid since it would be expected to bind to the aldimine form of the enzyme in a manner analogous to the substrate, except that the tautomerization step in the reaction mechanism would not occur because of the methyl group in the α position. Previous workers have shown that α-methylaspartate is an inhibitor of the enzyme at pH 6.3 (Jenkins *et al.*, 1959b) and that its addition to the enzyme causes marked changes in the absorption spectrum (Jenkins *et al.*, 1959b) and rotatory dispersion of the enzyme (Torchinsky and Koreneva, 1964). These results have been interpreted in terms of Schiff base formation between α-methylaspartate and enzyme-bound pyridoxal phosphate; confirmatory evidence has been obtained by reduction of the enzyme–amino acid complex and isolation of the pyridoxal α-methylaspartate derivative (Braunstein, 1964).

We present here a quantitative study of the absorption spectrum of aspartate aminotransferase in the presence of α-methylaspartate as a function of pH and amino acid concentration; some rotatory dispersion measurements were also made. The characteristic binding constant of the enzyme–amino acid interaction has been determined at various pH values. The implications of the results obtained for the over-all transamination reaction are discussed.

Experimental Section

The α form of supernatant aspartate aminotransferase was prepared from pig hearts as previously described (Martinez-Carrion *et al.*, 1965). DL-α-Methylaspartic acid was obtained from Sigma Chemical Co. All other chemicals were standard reagent grade.

Absorption spectra were measured with a Beckman DK 2 spectrophotometer whose cell compartment was thermostated at 25.0°. Solutions used for the spectrophotometric measurements were approximately 10^{-4} M (with respect to coenzyme concentration) in enzyme. Rotatory dispersion curves were obtained with a Cary 60 spectropolarimeter at 27° (±1). Solutions were approximately 5×10^{-5} M for measurements made in the wavelength region 300–600 mμ and approximately 5×10^{-6} M for measurements in the 220–300 mμ wavelength region. All pH measurements were made with a Radiometer pH meter.

Enzyme concentrations were determined spectrophotometrically: a molar extinction coefficient (per mole of coenzyme) of 8.2×10^3 M^{-1} cm^{-1} at 362 mμ and pH 8.5 was used (Martinez-Carrion *et al.*, 1965). Spectra of the enzyme in the presence of increasing

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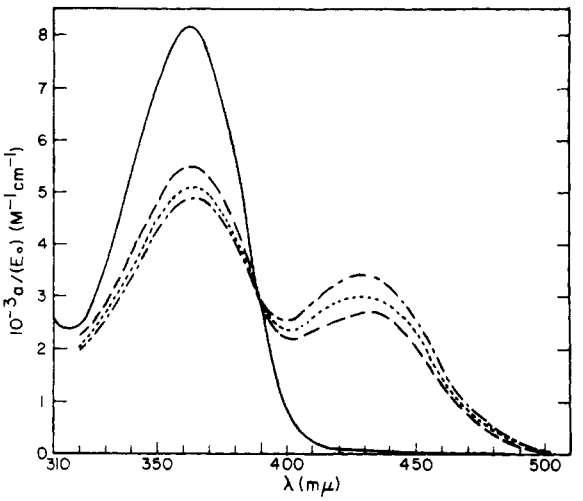

FIGURE 1: Absorption spectra of aspartate aminotransferase in the presence of increasing concentrations of α-methylaspartic acid at pH 8.0; L-α-methylaspartate concentration: 0 M ———, 1.41×10^{-2} M – – –, 2.78×10^{-2} M . . ., 6.67 and 9.10×10^{-2} M – · – ·. Experimental details are given in the text.

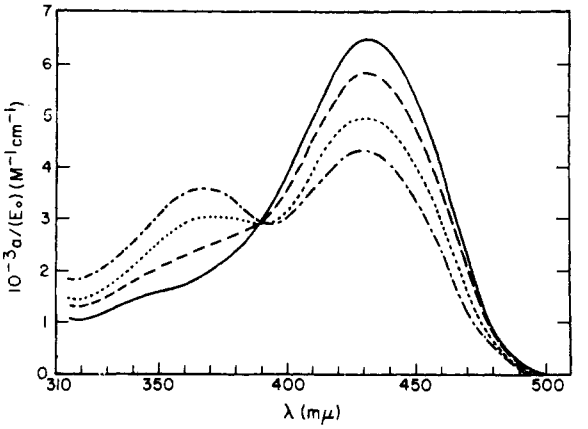

FIGURE 2: Absorption spectra of aspartate aminotransferase in the presence of increasing concentrations of α-methylaspartic acid at pH 5.0; L-α-methylaspartate concentration: 0 M ———; 3.36×10^{-2} M – – –, 0.107 M . . .; 0.214 M – · – ·. Experimental details are given in the text.

concentrations of α-methylaspartate were recorded over the wavelength region 315–500 mμ at pH 5.0 (0.2 M sodium acetate), 6.0 (0.14 M potassium phosphate), 7.0 (0.14 M potassium phosphate), and 8.0 (0.14 M tris(hydroxymethyl)aminomethane). Spectra were recorded at different times after preparing the solutions; the spectra were unchanged over a time period extending between 15 sec and 3 hr after mixing the reactants. Thus the reaction between enzyme and α-methylaspartate is quite rapid. The amino acid does not have an absorption spectrum of significant magnitude over the wavelength region investigated.

Rotatory dispersion measurements of the enzyme were made over the wavelength region 200–600 mμ in the absence of α-methylaspartate and in the presence of 0.1 M DL-amino acid at pH 8.0.

Results and Analysis of Data

Spectra of the enzyme in the presence of increasing concentrations of α-methylaspartate at pH 5 and 8 are shown in Figures 1 and 2. The concentrations of α-methylaspartate are reported as one-half of the total amino acid concentration since it is assumed that only the L isomer reacts with the enzyme. The binding of D-amino acids to this particular transaminase has never been detected. Large concentrations of α-methylaspartate tend to produce the same features in the spectra of the enzyme at all pH values, namely, two peaks of about equal height appear at 430 and 362 mμ, with a slight asymmetry in the latter peak at higher wavelengths. Below pH 6, the addition of amino acid causes a drop in the absorbancy at 430 mμ and an increase at 362 mμ, while at high pH values the absorbancy decreases at 362 mμ and increases at 430 mμ. At pH 8.0, the spectrum of the enzyme changes very little when the concentration of DL-α-methylaspartate is greater than 0.14 M, which indicates the enzyme is essentially saturated. At lower pH values, saturation of the enzyme was not observed, even at the highest amino acid concentrations employed.

The binding constant and extinction coefficient of the complex at a given pH value can be determined according to the following analysis. The binding constant is written as

$$K = \frac{(E\alpha A)}{(E)(\alpha A)} \tag{1}$$

where EαA designates the complex, E the enzyme, and αA the amino acid. Since the concentration of α-methylaspartate is always much greater than that of the enzyme, the concentrations can be written as

$$(\alpha A) = (\alpha A_T) \tag{2}$$

$$(E\alpha A) = (a - a_0)/(\epsilon_{E\alpha A} - \epsilon_E) \tag{3}$$

$$(E) = a_0/\epsilon_E - (a - a_0)/(\epsilon_{E\alpha A} - \epsilon_E) \tag{4}$$

where (αA_T) is the total concentration of α-methylaspartate, a_0 is the absorbancy of the solution before addition of amino acid, a is the absorbancy of the solution at a given concentration of amino acid, and ϵ designates the extinction coefficients. Substitution of eq 2–4 into eq 1 gives

TABLE I: Molar Extinction Coefficients of the Transaminase–L-α-Methylaspartate Complex at Various pH Values and 25°.[a]

pH	$10^{-3}\epsilon$ (M^{-1} cm^{-1})	
	362 mμ	430 mμ
5.0	4.07 ± 0.4	3.70 ± 0.4
6.0		4.24 ± 0.4
7.0	4.95 ± 0.4	3.82 ± 0.4
8.0	4.61 ± 0.2	3.64 ± 0.2
Weighted average[b]	4.57 ± 0.2	3.76 ± 0.2

[a] Molarity is expressed in terms of the coenzyme concentration. [b] The weighting factor used was the reciprocal of the square of the estimated experimental errors given above.

$$\frac{(E_0)}{a - a_0} = \frac{1}{(\alpha A_T)}\left[\frac{(E_0)}{Ka_0(1 - \epsilon_{E\alpha A}/\epsilon_E)}\right] - \frac{(E_0)}{a_0(1 - \epsilon_{E\alpha A}/\epsilon_E)} \quad (5)$$

Both sides of eq 5 have been multiplied by the total enzyme concentration, E_0, so that eq 5 is written in terms of apparent molar extinction coefficients, $a/(E_0)$. A plot of $(E_0)/(a - a_0)$ *vs.* $1/(\alpha A_T)$ permits evaluation of K and $\epsilon_{\alpha A}$ (since ϵ_E is known). Some typical plots of the data according to eq 5 are shown in Figure 3. The extinction coefficients of the complex at 362 and 430 mμ at various pH values are given in Table I. The binding constants at various pH values are reported in Table II.

TABLE II: Association Constants for the Formation of the α-Methylaspartate–Transaminase Complex at 25°.

pH	K (M^{-1})	$10^{-2}K°$ (M^{-1})[c]
5.0[a]	22	4.6 ± 0.4
5.0[b]	21	4.4 ± 0.4
6.0[a]	150	4.5 ± 0.4
7.0[a]	330	4.0 ± 0.4
7.0[b]	280	3.3 ± 0.4
8.0[a]	420	4.3 ± 0.2
8.0[b]	420	4.3 ± 0.2
	Weighted average[d]	4.2 ± 0.2

[a] Absorbancy changes at 430 mμ used for the determination of K. [b] Absorbancy changes at 362 mμ used for the determination of K. [c] pH-independent association constant. [d] The weighting factor used was the reciprocal of the square of the estimated experimental errors given above.

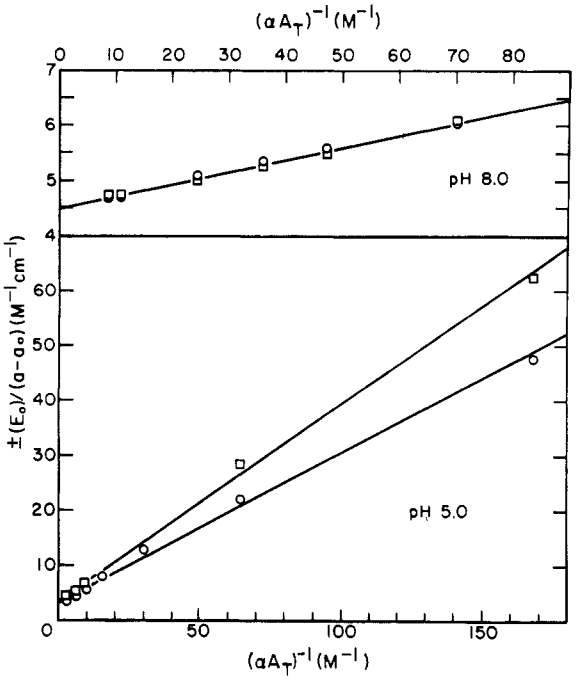

FIGURE 3: Some typical plots of the total enzyme concentration divided by $\pm(a - a_0)$ *vs.* the reciprocal of the total L-α-methylaspartate concentration, $(\alpha A_T)^{-1}$, at pH 5.0 and 8.0 at 362 (□) and 430 (○) mμ.

The rotatory dispersion curves of the enzyme and the enzyme–α-methylaspartate complex in the 300–600 mμ range are shown in Figure 4. At wavelengths shorter than 300 mμ, the two dispersion curves coincide within experimental error and are identical with those previously reported for aspartate aminotransferase in the presence and absence of substrates (Fasella and Hammes, 1965). The value of the rotation at the minimum of the protein Cotton effect (231 mμ) was 6350 ±150°.

Discussion

The presence of two distinct peaks in the spectrum of the enzyme–α-methylaspartate complex suggests the existence of at least two isomeric forms of the complex. Alternatively a single species may be present with two spectral peaks; however, in the native enzyme spectral peaks at similar wavelengths are associated with *different* forms of the coenzyme. The asymmetry of the 362 mμ peak at long wavelengths may indicate the presence of still a third isomer; however, the asymmetry is not great enough to justify such a conclusion. The spectrum of the complex is independent of pH (*cf.* Table I); this indicates that if isomeric equilibria exist, they probably do not involve side chain groups of the enzyme whose ionization states change in the pH range 4–9.

Since the ionization state of the amino acid does not change in this pH range, the variation of the binding

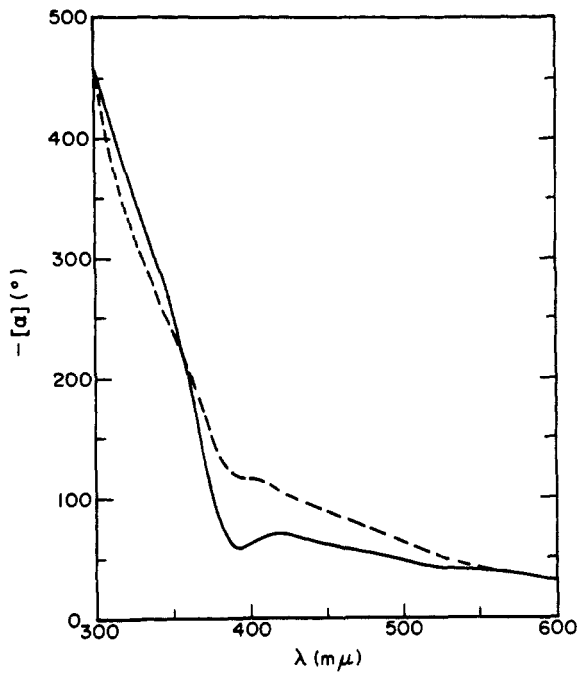

FIGURE 4: Specific rotation of aspartate aminotransferase alone (———) and in the presence of 0.1 M DL-α-methylaspartic acid (- - -), pH 8.05.

constant with pH is probably due to a change in the ionization state of the enzyme. An ionizable group with a pK of 6.3 previously has been implicated as being at the active site (Jenkins, 1961; Velick and Vavra, 1962; Hammes and Fasella, 1963). If only the basic form of the enzyme is assumed to bind α-methylaspartate, the measured binding constant can be written as

$$K = \frac{K^\circ}{1 + (H^+)/K_A} \qquad (6)$$

where K° is a pH-independent binding constant and K_A is the acid ionization constant. Values of K° calculated at various pH values by use of eq 6 are given in Table II. It can be seen that K° is, as assumed, independent of pH. If the amino acid also binds to the protonated enzyme, the characteristic binding constant must be at least a factor of 100 smaller than K°. This finding lends further support to the hypothesis that in the enzymatic reaction an amino acid with a protonated amino group reacts with a nonprotonated site of the enzyme. Since neutral hydroxylamine binds more strongly to the protonated enzyme site (Velick and Vavra, 1962; Hammes and Fasella, 1963), the optimal condition for binding apparently is the presence of only one shared proton in the complex formed. A behavior similar to that of hydroxylamine has been observed for the reaction of transaminase with other carbonyl reagents (Jenkins *et al.*, 1959a,b; Jenkins and Sizer, 1963).

The rotatory dispersion curve does not have a Cotton effect centered around 430 mμ in agreement with the results of previous workers (Torchinsky and Koreneva, 1964). This is in marked contrast to the results obtained with the form of the free enzyme having an absorption maximum at 430 mμ and to the carboxylic acid–enzyme complexes (Fasella and Hammes, 1964; Torchinsky and Koreneva, 1964). Since previous studies (Fasella and Hammes, 1964, 1965; Torchinsky and Koreneva, 1964) have shown that the coenzyme Cotton effect is either abolished or inverted by carbonyl reagents and the natural substrates, the simplest explanation for this behavior is that a complex is formed between enzyme and α-methylaspartate which destroys the aldimine linkage between enzyme and coenzyme, *i.e.*, a Schiff base is formed. The form of the complex with a spectral peak at 362 mμ has a Cotton effect associated with it which is much smaller than that found with the native enzyme. Apparently Cotton effects for enzyme–substrate complexes are either absent (Fasella and Hammes, 1965) or much smaller in magnitude than found for the enzyme alone. This again must be due to destruction of the enzyme–coenzyme aldimine linkage which causes the coenzyme to be held much less rigidly in the complex than in the free enzyme. As previously observed (Fasella and Hammes, 1965), the protein Cotton effect, which has a minimum at 231 mμ, is insensitive to events occurring at the active site.

The results presented here strongly suggest the existence of enzyme–substrate complexes which have absorption maxima at 430 and 360 mμ. Previous studies have suggested the presence of complexes with spectral maxima at 360 and 330 mμ (Hammes and Fasella, 1962). Saturation of transaminase with aspartate produces spectral peaks at 330, 430, and 490 mμ (Jenkins, 1964b). Finally β-*erythro*-aspartate bound to the enzyme also produces a spectral peak at 490 mμ (Jenkins, 1961; Czerlinski and Malkewitz, 1964). Therefore a minimal mechanism for enzymatic transamination should include intermediates with characteristic absorption peaks at 330, 360, 430, and 490 mμ. A speculative mechanism of transamination for half of the over-all reaction is shown in Figure 5. This mechanism assumes all of the observed spectra belong to substances lying on the main enzymatic pathway and that each spectral peak is associated with a different reaction intermediate. Presently these assumptions cannot be proved or disproved. This mechanism is also consistent with the many kinetic studies which have been performed on this enzyme. The first step in the mechanism is transfer of a proton from the amino acid to the aldimine; this is necessary before the Schiff base between coenzyme and substrate can be formed. This initial complex then forms a protonated Schiff base; both of these complexes would be expected to have an absorption peak around 430 mμ. Transfer of a proton to the ε-amino group (which previously was involved in the aldimine linkage) yields an intermediate with an expected absorption maximum around 360 mμ. The quinoid structure, with an absorption maximum around 490 mμ, has been previously suggested as an inter-

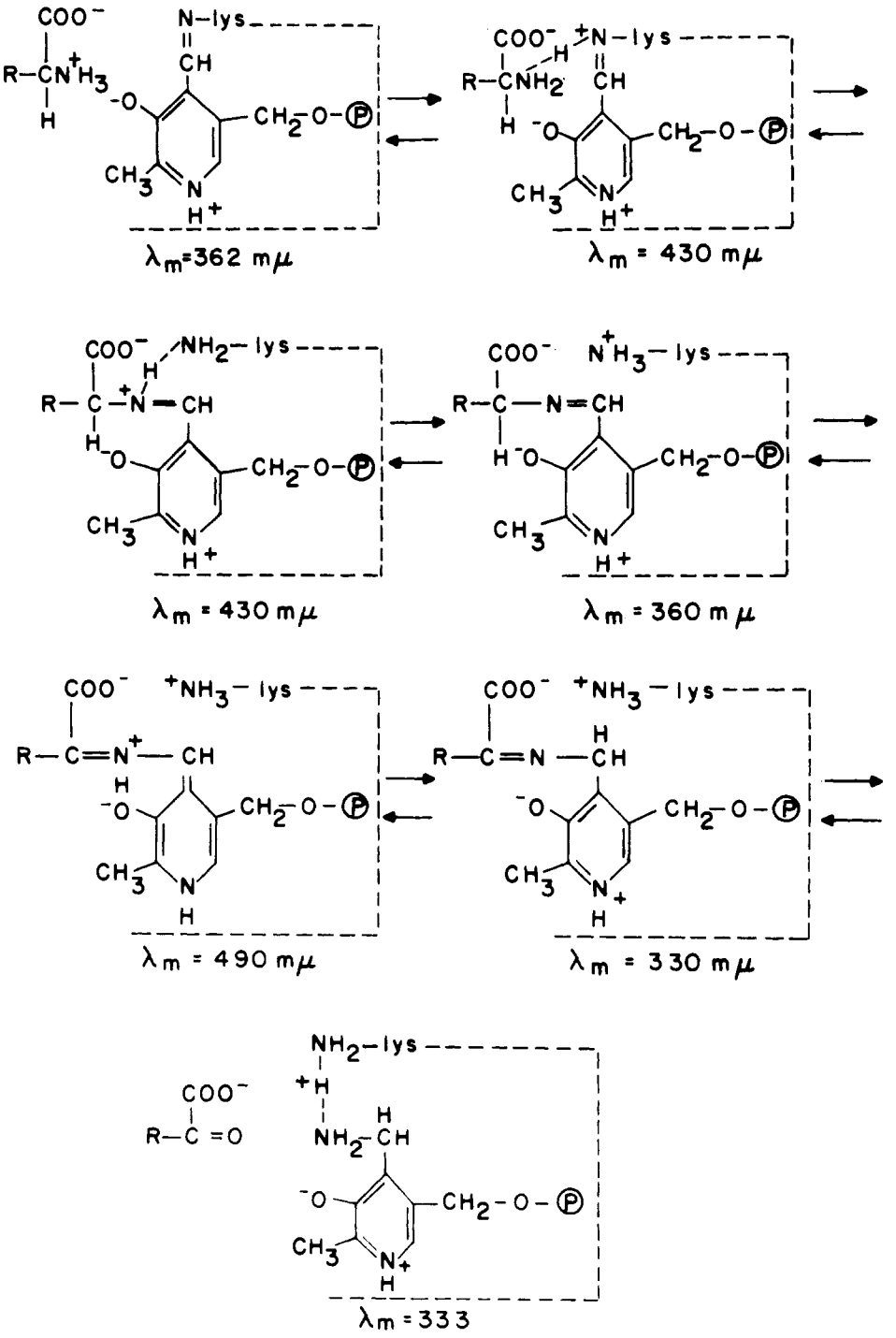

FIGURE 5: A possible mechanism for the "half-reaction" of enzymatic transamination. (The protonated Schiff base of the two intermediates with λ_{max} 430 mμ should also have the charged proton close to the phenolic oxygen; this was not possible to show in two dimensions and still illustrate the movement of the proton in the mechanism.)

mediate (Snell, 1962; Jenkins, 1961, 1964a), and the conversion of this intermediate to the new Schiff base with a spectral maximum at 330 mμ is probably the slowest intramolecular step in the mechanism (Velick and Vavra, 1962; Hammes and Fasella, 1962; Czerlinski and Malkewitz, 1964). The amino group of the enzyme and of the pyridoxamine ordinarily would be expected to be protonated in the pH range under consideration, but in this case the equilibrium constant of the half-reaction would be pH dependent; instead, the equilibrium constant of the half-reaction is independent of pH in the pH range 5–9 if the previously mentioned

ionization of the enzyme group with a pK of 6.3 is taken into account.[1] To resolve this dilemma, the pyridoxamine form of the enzyme in Figure 5 is shown with a proton being shared between the two amino groups. Further evidence for such a structure has been obtained by Turano and Giartosio (1964), who found that in the aminic form of the enzyme both the pyridoxamine amino group and the amino group on the protein lysine residue which forms an aldimine bond with the coenzyme in the aldehydic form of transaminase are not readily acetylated. The suggested mechanism is quite logical in a chemical sense and is a direct extension of that previously proposed by Snell (Snell, 1962). A rather disturbing feature of the available results is that the pH dependence of equilibrium and kinetic parameters seems to indicate that no amino acid side chains of the enzyme are directly involved in the enzymatic process other than those necessary for binding of the coenzyme. (Of course, several ionizable groups might be involved which compensate in such a manner that no pH dependence is observed.) On the other hand, model transaminase systems, which generally involve acid–base catalysis, are considerably less efficient than the enzyme (Cordes and Jencks, 1962; Bruice and Topping, 1963; French and Bruice, 1964, 1965). This suggests the protein does play an important, but unknown, role in the catalysis. Kinetic studies of the α-methylaspartate–transaminase system are currently in progress and may reveal further details of the general mechanism of transamination.

References

Banks, E. E., Lawrence, A. J., Vernon, C. A., and Wooton, J. F. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. F., Fasella, P., Braunstein, A., and Rossi-Fanelli, A., eds., Pergamon Press, Oxford, p. 197.

Bonavita, V., and Scardi, V. (1958), *Experientia 14*, 78.

Braunstein, A. (1964), *Vitamins Hormones 22*, 453.

Bruice, T. C., and Topping, R. M. (1963), *J. Am. Chem. Soc. 85*, 1480, 1488, 1493.

Cordes, E. H., and Jencks, W. P. (1962), *Biochemistry 1*, 773.

Czerlinski, G., and Malkewitz, J. (1964), *Biochemistry 4*, 1127.

Fasella, P., and Hammes, G. G. (1964), *Biochemistry 3*, 530.

Fasella, P., and Hammes, G. G. (1965), *Biochemistry 4*, 801.

French, T. C., and Bruice, T. C. (1964), *Biochemistry 3*, 1589.

French, T. C., and Bruice, T. C. (1965), *Biochemistry 4*, 77.

Hammes, G. G., and Fasella, P. (1962), *J. Am. Chem. Soc. 84*, 4644.

Hammes, G. G., and Fasella, P. (1963), *J. Am. Chem. Soc. 85*, 3939.

Jenkins, W. T. (1961), *J. Biol. Chem. 236*, 1121.

Jenkins, W. T. (1964a), *J. Biol. Chem. 239*, 1742.

Jenkins, W. T. (1964b), Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., p. 172.

Jenkins, W. T., Orlowsky, S., and Sizer, I. W. (1959a), *J. Biol. Chem. 234*, 2657.

Jenkins, W. T., and Sizer, I. W. (1959), *J. Biol. Chem. 234*, 1179.

Jenkins, W. T., Yphantis, D. A., and Sizer, I. W. (1959b), *J. Biol. Chem. 234*, 51.

Martinez-Carrion, M., Turano, C., Riva, F., and Fasella, P. (1965), *Biochem. Biophys. Res. Commun. 20*, 206.

Polyanovsky, O. L., and Torchinsky, Yu. (1961), *Dokl. Acad. Nauk SSSR, 141*, 488.

Sizer, I. W., and Jenkins, W. T. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., Fasella, P., Braunstein, A., and Rossi-Fanelli, A., eds., Pergamon Press, Oxford, p. 123.

Snell, E. E. (1962), *Brookhaven Symp. Biol. 15*, 32.

Torchinsky, Y., and Koreneva, L. G. (1964), *Biochim. Biophys. Acta 79*, 426.

Turano, C., and Giartosio, A. (1964), Abstracts, Sixth International Congress of Biochemistry, Vol. IV, New York, N. Y., 178.

Velick, S. F., and Vavra, J. (1962), *J. Biol. Chem. 237*, 2109.

[1] C. A. Vernon, E. E. Banks, and A. J. Lawrence, personal communication.