Computer Analysis of Spectra of Enzyme-Substrate and Enzyme-Inhibitor Complexes Involving Aspartate Aminotransferase*

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SUMMARY

Dicarboxylic acid inhibitors and the amino and keto acid substrates interact with aspartate aminotransferase (EC 2.6.1.1) to form spectrally distinct enzyme-inhibitor and enzyme-substrate complexes. The spectra of solutions of the enzyme and varying concentrations of inhibitor or substrate are analyzed by computer methods to obtain the spectra of the enzyme-inhibitor and enzyme-substrate complexes. Programs have been written which permit the computation of the $pK_a$ values and the dissociation constants of the complexes with the use of the spectral data at many wave lengths. The complete spectra of the individual ionic species of the complexes are drawn. Comparison plots of the experimental spectral data and the calculated spectra can also be obtained. These computer methods are applied to four enzyme-inhibitor complexes and to complexes of the enzyme with its substrates and two pseudosubstrates. Spectra of apoaspartate aminotransferase containing bound pyridoxal phosphate analogues have also been analyzed.

Spectrophotometry is often used to evaluate dissociation constants of enzyme-inhibitor complexes and has been exploited for determination of dissociation constants of both enzyme-inhibitor (1, 2) and enzyme-substrate complexes (3-8) of soluble aspartate aminotransferase. The methods generally used involve construction of linear plots relating the concentrations of inhibitor or of substrate to the changes in absorbance at one or two wave lengths. Extinction coefficients for the complexes at these wave lengths are obtained as well as the formation or dissociation constants. From measurements at various values of pH, the acid dissociation constants of the complexes can also be determined.

Recently, computer methods have been developed which permit evaluation of both acid dissociation constants and formation constants of complexes using entire spectra measured at various pH values and at various concentrations of ligand (9). With such methods, complete spectra of individual ionic species of the complexes could also be obtained. The present work represents an extension of this method to a pyridoxal 5'-phosphate-dependent enzyme, aspartate aminotransferase (EC 2.6.1.1). The extensive previous work of Jenkins et al. (1, 2, 4-6) and of Fasella, Giartosio, and Hammes (7) on the spectral changes undergone by the enzyme upon addition of substrates, pseudosubstrates, and dicarboxylic acid inhibitors makes aspartate aminotransferase an ideal system for testing the computer method. Models describing the various possible complexes between the enzyme and its substrates have been postulated. When the substrate pairs, glutamate-α-ketoglutarate or aspartate-oxaloacetate, are added to the enzyme, a number of enzyme species are found. These include the pyridoxal and pyridoxamine forms of the enzyme, binary intermediate enzyme-substrate complexes, an abortive complex between the pyridoxal form of the enzyme and the keto acid, and possibly an abortive complex between the pyridoxamine enzyme and the amino acid. The computer method provides a means of determining the contribution of the various enzyme forms to the overall spectra and permits, for the first time, calculation of complete spectra of individual ionic species of the complexes with the use of spectral data at many wave lengths.

EXPERIMENTAL PROCEDURE

Materials—The aspartate aminotransferase was donated by Dr. W. T. Jenkins as the highly purified cytoplasmic α-subform (10). The absorbance ratio $A_{364}/A_{340}$ was 3.6 in 0.02 $M$ acetate buffer, pH 5.4. Enzyme concentrations were obtained from the absorbance at 364 $\mu m$ at pH 8.0, with the use of the molar extinction coefficient of $8.20 \times 10^5$ (5, 11). One mole is that amount of enzyme containing 1 mole of bound pyridoxal-P.

Aspartic and glutamic acids were purchased from Nutritional Biochemicals. Oxalacetic and α-ketoglutaric acids were obtained from Calbiochem, and succinic acid was a product of Mallinckrodt. Pyridoxal-P and glutaric and α-methyl-DL-aspartic acids were purchased from Sigma. erythrol-α-Hydroxy-α-aspartic acid was prepared and donated by Dr. W. T. Jenkins. Of the pyridoxal-P analogues, Compounds I (12) and IV were C. Iwata, manuscript in preparation.
prepared in this laboratory and Compounds II and III were supplied by Dr. Walter Korytnyk.

Buffers were prepared from triethanolamine (J. T. Baker Chemical Company), crystallized as the hydrochloride salt, and sodium hydroxide. Tris hydrochloride was prepared from Tris (Fisher) and hydrochloric acid. Stock solutions of substrates and inhibitors were prepared in the appropriate buffer. Solutions of oxaloacetate were prepared immediately before use.

Test solutions—The test solutions were made up in semimicro cuvettes, and each contained approximately $5 \times 10^{-4}$ M enzyme. The concentration of the substrates or inhibitors was varied. In the case of the inhibitors, aliquots of the inhibitor were added to 1 ml of a solution of enzyme, buffered with 0.15 M acetate, 0.1 M triethanolamine, or 0.1 M Tris buffer. Eleven spectra of the glutarate complex were obtained from pH 5 to 9.45; 14 of the complex with succinate at pH 5.15 to 8.77; 18 of the $\alpha$-keto glutarate complex at pH 5.75 to 8.55. To obtain spectra of the enzyme-substrate complexes, either aliquots of keto acid were added to a solution of enzyme and amino acid, or aliquots of amino acid were added to the enzyme with and without keto acid present. Spectra of the enzyme-substrate complexes were obtained in 0.1 M triethanolamine.HCl buffers. Eighteen spectra of the $\alpha$-keto glutarate-glutamate complex were obtained at pH 7.12 to 8.8; 12 spectra of the oxaloacetate-aspartate complex at pH 8.16 to 8.25; 12 spectra of the complex with $\alpha$-methyl aspartate at pH 7.57 to 8.80; and five of the $\beta$-hydroxy aspartate complex at pH 8.25. Spectra of each solution were obtained against a reference containing buffer and keto or amino acid (or both) at the same concentration as the test solution.

pH Measurements—pH measurements were made with a Corning model 12 research pH meter and a Sargent 30070-10 combination electrode, and with a Radiometer PHM 4d pH meter. Standard buffer solutions from Mallinckrodt and Fisher were used for calibration. The pH of each test solution was obtained after the spectra were recorded.

Spectra—Absorption spectra were measured at 25° with Cary model 15 and Cary model 1501 recording spectrophotometers equipped with a Cary-Datex digital output system and an IBM card punch. The absorbances were punched out at 2 nm or at 0.2 kK intervals (1 kK = 10$^3$ cm$^{-1}$). Spectra were corrected for base line errors and for very small amounts of turbidity when necessary. Spectra were obtained of solutions of apoaaspartate aminotransferase and apoglutamate decarboxylase (prepared according to the procedure of Huntley and Metzler (13)) with low amounts of turbidity increasing slowly with time. The difference spectra of the protein with and without turbidity were used to obtain standard plots. It was found that the absorbance due to turbidity increased linearly with wave number over the range 18.8 to 31.3 kK (532 to 320 nm). The parameters, intercept and slope, for the linear correction were calculated from the standard plots and the absorbance at 532 nm (18.8 kK) of the sample protein solutions. Results obtained with this method of turbidity correction were very similar to those obtained with a plot of log A against log wave length (14). The maximum correction at 320 nm necessary in this study was 0.026 for a solution having an A of 0.545.

Spectra Required for Computations—To determine the pK$\alpha$, dissociation constant, and spectra of the ionic forms of the enzyme-inhibitor complexes, the following spectra are necessary: (a) spectral measurements on solutions containing high concentrations of inhibitor at several pH values to obtain a pK; (b) spectra of the enzyme titrated with lower concentrations of inhibitor to obtain the inhibitor dissociation constant. In the case of the enzyme-substrate complexes, spectra at several con-

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Fig. 1. Contour map obtained in determining the inhibitor dissociation constant and the acid dissociation constant for the complex between glutarate and aspartate aminotransferase. The numbers represent the sum of the squares of the deviations $\times 10^4$.

Fig. 2. Absorption spectra of the two ionic forms of the $\alpha$-keto glutarate-aspartate aminotransferase complex (---), and of the two ionic forms of the enzyme-succinate complex (---).
centrations of both the keto acid and the amino acid must be obtained.

**Computer Programs—**The programs used were written in FORTRAN IV language for use with the IBM 360/65 computer. The automatic graphing was done by the Cal-Comp Digital Incremental Plotter.

**Enzyme-bound Pyridoxal-P Analogue—**The apoenzyme of aspartate aminotransferase was prepared according to the procedure of Scardi et al. (15) with an adaptation described previously (11). The apoenzyme and an equivalent concentration of the pyridoxal-P analogue (approximately 5 × 10^{-6} M) were incubated at room temperature in 0.01 M triethanolamine hydrochloride buffer, pH 8.3, for 15 min, sufficient time for complete binding. Spectra of each enzyme-bound analogue were obtained at a number of different pH values. The pH was adjusted with an appropriate amount of either 0.5 M triethanolamine hydrochloride buffer or 0.5 M acetate buffer.

**MODELS**

**Enzyme-Inhibitor Complexes—**Scheme I represents the spectral changes observed with the pyridoxal form of aspartate aminotransferase in the presence of certain dicarboxylic acids or the keto acid substrates. P (absorbing at 364 μm or 27.5 kK) and HP (490 μm or 23.3 kK) are the nonprotonated and protonated forms of the pyridoxal form of the enzyme and are related by a pK of 6.27.

![Diagram](image_url)

**FIG. 3.** Calculated absorption spectra of solutions containing 7.0 × 10^{-6} M aspartate aminotransferase and three concentrations of succinate in 0.1 M triethanolamine, pH 8.10, plotted together with experimental points. ○, 6.17 × 10^{-4} M; □, 2.38 × 10^{-3} M; Δ, 5.55 × 10^{-2} M succinate.

**SCHEME I**

\[
\begin{align*}
H_L & \xrightarrow{K_1} HPL \\
HPL & \xrightarrow{K_3} HP \\
PL & \xrightarrow{K_7} L + P
\end{align*}
\]

where \(L, HL,\) and \(H_2L\) are the ionic forms of the dicarboxylic or keto acid. \(PL\) and \(HPL\) are the nonprotonated and protonated forms of the complex between the inhibitor and enzyme. \(K_5, K_6, K_1,\) and \(K_7\) are acid dissociation constants, and \(K_9\) is a dissociation constant, is

\[
K_9 = C_P C_L / C_{PL}
\]

where \(C_P\) is the concentration of \(P\). The total concentration of all forms of the free enzyme can be expressed as the product of the concentration of any one of the individual ionic forms and some function of the \(K_9\) values and the pH. Thus, \(\alpha, \beta,\) and \(\gamma\) are functions used to relate the ionic forms of the free enzyme, the free inhibitor, and the enzyme-inhibitor complex respectively, and are defined by Equations 2 to 4.

\[
\begin{align*}
\alpha &= 1 + a_H / K_2 \\
\beta &= 1 + a_H / K_5 + (a_H)^2 / K_4 K_3 \\
\gamma &= 1 + a_H / K_7
\end{align*}
\]

where \(a_H\) is the apparent hydrogen ion "activity" and is obtained with a pH meter. The concentrations of total enzyme, \(C_{TP}\), and total inhibitor, \(C_{IL}\), are given by Equations 5 and 6.

\[
\begin{align*}
C_{TP} &= \alpha C_P + \gamma C_P C_L / K_3 \\
C_{IL} &= \beta C_L + \gamma C_P C_L / K_9
\end{align*}
\]

\(K_3, K_4, K_6,\) and the spectra of \(P\) and \(HP\) are known (11). The values for \(C_P\) and \(C_L\) are obtained by the computer through successive approximations. The first estimate of \(C_L\) is given by

\[
C_L = C_{IL} / \beta
\]

and successive estimates of \(C_P\) and \(C_L\) are obtained from Equations 5 and 6.

The trial value for \(K_9\) was determined from a plot of the reciprocal of the change in \(A\) at one wave length against the reciprocal of the inhibitor concentration. Data for these plots were obtained from titrations of the enzyme with inhibitor at one pH. Approximate values for \(K_7\) were obtained with Equation 8.

\[
\Delta A_{max}/\Delta A = 1 + K_9 / a_H (1 + 1 / K_5 C_L)
\]

where \(\Delta A_{max}\) is the absorption of \(HPL\) at \(\lambda_{max}\) at low pH, and \(\Delta A\) is the absorption at the same wave length at a pH near the \(pK_9\) of the complex.

The trial values of \(K_7\) and \(K_9\) did not need to be extremely close to the final values computed and were adjusted by the computer with a modification of the grid method described by Nagano and Metzler (9). The molar extinction coefficients of the two ionic species (\(PL\) and \(HPL\)) were obtained by the method of least squares applied consecutively to the data at each experimental spectral point.

An example of the results obtained for different values of \(K_7\) and \(K_9\) in the glutarate-enzyme complex is shown in Fig. 1. In
In this case, the original trial values were pK\(_7\) of 8.81 and pK\(_8\) of 1.39 (marked by X). The numbers on the contour map are values of the sum of the squares of the deviations calculated for the various grid parameters. Standard deviations of the absorbance values were calculated by dividing the values of squares of the deviations by the total number of experimental points minus the number of constants adjusted (degrees of freedom) and taking the appropriate square root.

These results were checked with a program which calculated these parameters by the method of steepest descent. The results calculated with either the grid program or the steepest descent method were very similar. Comparison plots were also computed to show the goodness of fit between computed spectra and experimental results.

**Enzyme-Substrate Complexes**—The spectral changes that occur upon the addition of both amino acid and keto acid substrates may be described as simply as possible by Scheme II.

![Scheme II](image)

In Scheme II \(L\) is the keto acid and \(B\) is the amino acid. \(PL\) and \(HPL\) are the ionic forms of the abortive complex between the pyridoxal form of the enzyme and the keto acid. \(PX\) represents all of the intermediate enzyme-substrate complexes. Jenkins (6, 8) has reported that pH has no effect on the interconversions of the enzyme-substrate complexes and this was also found in the present study; therefore, only one ionic species has been considered in this scheme. \(PM\) is the pyridoxamine form of the enzyme, the spectrum of which does not change over the pH range of 5.0 to 9.0. Abortive complexes between amino acid and the pyridoxamine form of the enzyme do not appear to be formed with aspartate (6) or glutamate (8). The apparent dissociation constants are

\[
K_{12} = C_P C_B/C_{FX} \tag{9}
\]

\[
K_{11} = C_{PM} C_L/C_{FX} \tag{10}
\]

In this model \(\alpha, \beta, \gamma, \) and \(\delta\) are defined by Equations 2 to 4. The function relating the acid dissociation constants of the amino acid and pH is

\[
t = 1 + a_0/K_{11} + (a_0)^2/K_{11} K_{12} \tag{11}
\]

According to this scheme the total concentrations of the three components are given in Equations 12 to 14.

\[
C_{1P} = \alpha C_P + \gamma C_P C_L/K_5 + C_B C_P/K_{12} + K_{13} C_B C_P/K_{13} L \tag{12}
\]

\[
C_{1L} = \beta C_L + \gamma C_P C_L/K_5 - K_{13} C_B C_P/K_{13} C_L \tag{13}
\]

\[
C_{1B} = \delta C_B + C_B C_P/K_{12} + K_{13} C_B C_P/K_{13} C_L \tag{14}
\]

The first estimate of \(C_L\) is given by Equation 7 and that of \(C_B\) by Equation 15.

\[
C_B = C_{1B}/\delta \tag{15}
\]

Successive estimates of \(C_P, C_L, \) and \(C_B\) are obtained from Equations 12 to 14. Trial values for \(K_{13}\) and \(K_{11}\) were determined from double reciprocal plots. Data for these plots were obtained by titration of the enzyme at one pH with the amino acid at a constant concentration of keto acid, or with keto acid at a constant concentration of amino acid. The trial values of \(K_{13}\) and \(K_{11}\) are automatically adjusted as described for Scheme I.

**RESULTS**

Fig. 2 shows the protonated and nonprotonated forms of the complex of aspartate aminotransferase with \(\alpha\)-ketoglutarate (solid lines) and with succinate (dashed lines). These spectra were plotted automatically, converting the absorption in wave length to wave number. The spectra are interpolated between points to obtain a smooth curve. Similar spectra were obtained for the two ionic forms of the enzyme complexes with oxaloacetate and glutarate.

A comparison plot for the enzyme-inhibitor complex involving succinate is presented in Fig. 3. The solid lines were calculated with the results for \(K_7, K_8, \) and the extinction coefficients obtained by the computer. The points drawn in are the experimentally observed spectra. Very good agreement was obtained between the calculated and observed spectra over the wave length range studied for the two dicarboxylic acids, succinate and glutarate. In Fig. 4, the calculated and observed spectra for the abortive complex with oxaloacetate are shown. In this case there is good fit between the two spectra in the region of the 429 mu (23.3 kK) peak, but not in the region of the 364
The parameters obtained for the enzyme-inhibitor and abortive complexes are summarized in Table I. The standard deviation per absorbance point is included to give an idea of the goodness of fit obtained with each inhibitor. The deviation is higher with α-ketoglutarate and oxaloacetate than with glutarate and succinate because of the previously mentioned poor fit in the region of the 364 μm (27.5 kK) absorbing species. With the exception of the complex with oxaloacetate, the protonated forms of the complexes absorb at longer wave lengths than that of the free pyridoxal form of the enzyme. Furthermore, the protonated forms of the enzyme-inhibitor complexes have higher extinction coefficients than that of the holoenzyme. There is no appreciable shift in the absorption maxima of the non-protonated form of the enzyme in the presence of the inhibitors. The inhibitors all cause a large increase in the pKₐ of the enzyme. The keto acids bind more tightly to the nonprotonated form of the enzyme than do the dicarboxylic acids.

The spectra of the enzyme-substrate complexes obtained with the aminotransferase with its normal substrates and some pseudo-substrates are shown in Fig. 5. These spectra are due only to the enzyme-substrate complexes, since the absorption due to abortive complexes and free enzyme have been subtracted from

Fig. 6. Calculated absorption spectra of solutions containing 4.5 × 10⁻¹ M aspartate aminotransferase, L-glutamate, and α-ketoglutarate in 0.1 M triethanolamine at pH 6.25 plotted together with experimental points. ○, 9.53 × 10⁻⁴ M L-glutamate; △, 9.49 × 10⁻⁴ M L-glutamate and 3.56 × 10⁻⁴ M α-ketoglutarate; □, 9.41 × 10⁻⁴ M L-glutamate and 1.18 × 10⁻⁴ M α-ketoglutarate.

### Table I

| Parameters for enzyme-inhibitor complexes |
|------------------------------------------|
|                                         |
| **Holoenzyme**                           |
| λₘₐₓ (μm) & x 10⁻³                          |
| 429 (25.3) & 6.98                         |
|                                         |
| **Glutarate**                            |
| λₘₐₓ (μm) & x 10⁻³                          |
| 437 (22.9) & 7.96                         |
|                                         |
| **Succinate**                            |
| λₘₐₓ (μm) & x 10⁻³                          |
| 438 (22.8) & 8.58                         |
|                                         |
| **α-Ketoglutarate**                      |
| λₘₐₓ (μm) & x 10⁻³                          |
| 438 (22.8) & 7.90                         |
|                                         |
| **Oxaloacetate**                         |
| λₘₐₓ (μm) & x 10⁻³                          |
| 429 (22.9) & 7.80                         |

* Standard deviation per absorbance point.
the experimentally obtained spectra. Comparison spectra for the glutamate-α-ketoglutarate system appear in Fig. 6. The fit with this substrate pair was good. The fit obtained in the comparison plots for the aspartate-oxaloacetate system was somewhat worse, and the value of standard deviation per absorbance point was higher than that obtained with glutamate-α-ketoglutarate. A comparison plot for the data obtained with erythro-β-hydroxyaspartate is presented in Fig. 7. The fit between the experimental and computed spectra is excellent. Excellent agreement was also obtained for the complex with α-methylaspartate at both pH 7.6 and 8.8. This is consistent with the previous observation that there was no effect of pH on the distribution of the enzyme-substrate complexes with α-methylaspartate (7).

The data obtained for the various enzyme-substrate complexes are presented in Table II. As was observed by Jenkins (8), there are at least four spectrally distinct binary complexes. These complexes absorb at approximately 402 μm (20.6 kK), 430 μm (23.2 kK), 365 μm (27.3 kK), and 330 μm (30.3 kK). The complex with β-hydroxyaspartate is predominately in the quinoid type of intermediate (17), but also has appreciable absorption at 330 μm (30.3 kK). It appears that the 330 μm (30.3 kK) absorption with β-hydroxyaspartate is due to an enzyme-substrate complex rather than to the pyridoxamine form of the enzyme or to an abortive complex between the PMP enzyme and the amino acid. According to our results, the dis-

![Figure 7](image1.png)

**Fig. 7.** Calculated spectra of solutions containing aspartate aminotransferase and different concentrations of erythro-β-hydroxy-DL-aspartate in 0.1 M triethanolamine, pH 8.25, plotted together with experimental points. ○, 2.5 × 10⁻⁴ M; △, 4.98 × 10⁻⁴ M; ●, 9.89 × 10⁻⁴ M; □, 2.20 × 10⁻³ M erythro-β-hydroxy-DL-aspartate.

![Figure 8](image2.png)

**Fig. 8.** Structures of pyridoxal-P analogues

| System                        | λ_max (μm) | ε × 10⁻³ | Dissociation constant for amino acid (M) | Dissociation constant for keto acid (M) | S.D. a |
|-------------------------------|------------|---------|------------------------------------------|-----------------------------------------|-------|
| Glutamate-α-ketoglutarate     | ~500 (20.0)| 0.30    | 4.6 × 10⁻⁴                               | 8.2 × 10⁻²                              | 0.0125|
| ~435 (23.0)                   | 0.45       |         |                                          |                                         |       |
| sh. b 366 (27.3)              | 2.50       |         |                                          |                                         |       |
| 331 (30.2)                    | 6.10       |         |                                          |                                         |       |
| Aspartate-oxaloacetate        | 495 (20.2) | 0.45    | 5.94 × 10⁻⁴                              | 7.1 × 10⁻⁶                              | 0.0158|
| 430 (23.2)                    | 2.20       |         |                                          |                                         |       |
| 337 (30.3)                    | 5.94       |         |                                          |                                         |       |
| α-DL-Methylaspartate          | 430 (23.2) | 3.88    | 4.68 × 10⁻³                              | 7.1 × 10⁻⁶                              | 0.0104|
| 364 (27.5)                    | 5.33       |         |                                          |                                         |       |
| erythro-β-Hydroxy-DL-aspartate| 492 (20.3) | 21.90   | 2.93 × 10⁻⁴                              |                                         | 0.0058|
| sh. 333 (30.0)                | 4.80       |         |                                          |                                         |       |

a Standard deviation per absorbance point.

b Sh., shoulder.
which has since been shown to interact with the enzyme-sub-
zyme, it was thought that a similar shift in pKₐ would be ob-
served with the enzyme-bound pyridoxal-P analogues. The
spectral pKₐ values for the enzyme-pyridoxal-P analogues in the
presence and absence of glutarate are presented in Table III.
The pKₐ values for E-I and E-II were determined according to
the method of Nagano and Metzler (9). Glutarate does cause
a shift in the pKₐ values of E-I and E-II of a magnitude similar
to that observed with the native enzyme. However, E-III and
E-IV exhibited absorption maxima at approximately 370 με (27.0 kK) at pH 8.3 and 5.0, and had no absorption at 429 με (23.3 kK) at pH 5.0. It was of interest to determine whether
the latter two enzyme-bound analogues could be protonated at
all. Since glutarate greatly increases the pKₐ of the native en-
zeine, it was thought that a similar shift in pKₐ would be ob-
served with the enzyme-bound pyridoxal-P analogues. The
spectral pKₐ values for the enzyme-pyridoxal-P analogues in the
presence and absence of glutarate are presented in Table III.
The pKₐ values for E-I and E-II were determined according to
the method of Nagano and Metzler (9). Glutarate does cause
a shift in the pKₐ values of E-I and E-II of a magnitude similar
to that observed with the pyridoxal-P enzyme. When glutarate
was added to either E-III or E-IV at pH 5.0, there was an ap-
preciable increase in absorption at 429 με (23.3 kK). It was
estimated that the pKₐ of the E-III glutarate complex was
approximately 4.8 and the pKₐ of the E-IV glutarate complex
was somewhat less then 4.8.

### DISCUSSION

The computer methods described here enable the calculation
of pKₐ values, dissociation constants, and spectra of enzyme-
inhibitor and enzyme-substrate complexes. The results ob-
tained when these methods were applied to aspartate ami-
transferase agree well with most previously reported data. The
pKₐ values and the dissociation constants for the complexes
with glutarate and succinate are very similar to those reported
by Jenkins (1, 2) using spectrophotometric titrations. The
dissociation constants for the pseudosubstrates, β-hydroxyas-
partate and α-methylaspartate, obtained in this study are com-
parable to that of 3.8 × 10⁻⁴ M reported for β-hydroxyas-
partate (5) and 4.76 × 10⁻³ M for dl-α-methylaspartate (7). How-
ever, the dissociation constants obtained for glutamate,
aspartate, α-ketoglutarate, and oxaloacetate are all lower than
those reported previously (6, 8, 18–20). These differences may
due to differences in the reaction conditions used. Previous
spectral studies had been conducted in pyrophosphate buffer,
which has since been shown to interact with the enzyme-sub-
strate complexes.³ Kinetic studies (19) indicated that no abor-
tive complex was formed with oxaloacetate at concentrations
of 15 mM keto acid. However, these kinetic studies were conducted
with a mixture of subforms of the enzyme in sodium arsenate
buffer at 37°C.

The discrepancies obtained in the spectral changes upon
binding keto acid to aspartate aminotransferase to form an
abortive complex appear to be due to the buffer used. The
results obtained in Tris buffer are fully consistent with the
model used to describe the formation of abortive complexes
(Scheme I) and would indicate that the model is correct. How-
ever, in triethanolamine buffer, the keto acids appear to be
binding differently, and the simple model is not consistent with
the data obtained in this buffer. The results obtained by
analyzing the entire spectra indicate the importance of careful
consideration of the reaction conditions. It appears that the
dissociation constants and the spectral behavior of the abortive
complexes may vary with certain conditions, such as buffer used.
The unusual spectral behavior obtained with the keto acids in
triethanolamine buffer would not have been observed with the
one-wave length approach used by other investigators to deter-
mine dissociation constants. Since the formation constants for
the abortive complexes are low compared with those of the en-
zeine-substrate complexes, it was felt that these discrepancies
would have little effect on the final spectra computed for the
enzyme-substrate complexes.

These methods of computer analysis have several advantages
over previously described methods of analyzing spectrophoto-
metric titrations of enzymes. The computer method utilizes
data at many different wave lengths rather than just at one
wave length. It takes into consideration the spectral pKₐ
values for both the pyridoxal form of the enzyme and the en-
zeine-inhibitor complexes. The absorption spectra of the
individual complexes can be computed. Fewer experimental
spectra are needed to obtain all parameters required in a given
model. Comparison plots can be generated, thus providing a
means of evaluating the model used.

The comparison plots and the low values of standard deviation
per absorbance point obtained for the enzyme-inhibitor com-
plexes would indicate that the spectra of the ionic forms of these
complexes are reliable. In each case, the spectrum of the HPL
form is probably much more accurate than that of the PL form of
the complexes.

Computer analyses can be extended to other studies of the
aspartate aminotransferase system. For instance, some en-
zeine-inhibitor complexes of the enzyme in which pyridoxal-P
has been replaced with a pyridoxal-P analogue have been studied.
Glutarate and α-ketoglutarate do form complexes with the
enzyme-bound analogues. These complexes, like those with the
native pyridoxal form of the enzyme, absorb at longer wave
lengths and have a higher pKₐ than does the free enzyme-bound
analogue. The computer method can also be used to study the
complexes of substrates and inhibitors with other enzymes.

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