# Machine Computation of Equilibrium Constants and Plotting of Spectra of Individual Ionic Species in the Pyridoxal–Alanine System[1]

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**Abstract:** A method is described by which a digital computer with an automatic plotter can draw a smooth curve to fit experimental spectral data points at 5-mμ intervals with supplemental points, where needed, at 2.5-mμ intervals. Programs have been written that compute, from spectral data at up to 75 wavelengths, as many as three successive dissociation constants for a light-absorbing compound. A least-squares method involving successive approximations is used to obtain the best fit of all p$K$ values to 0.01 unit. The spectra of the individual ionic forms (up to four) are drawn, and the extinction coefficients are also punched out on cards. New data for pyridoxal at 50° are reported. Equilibria involving a second nonlight-absorbing component and reversible formation of a compound or complex are also considered. Methods are provided for finding the formation constant, the successive acid dissociation constants, and the absorption spectra of individual ionic forms for the compound or complex. The graphs of the log of the apparent formation constant *vs.* pH and the pH profiles of the concentrations (or percentages) of individual ionic forms are drawn automatically. An additional program provides for the plotting of experimental spectral data, together with calculated spectra, and the plotting of experimental data and calculated curves of absorbance *vs.* pH at any desired wavelength. Two systems involving formation of Schiff's bases are analyzed in detail.

Measurements of absorption spectra are used frequently to evaluate equilibrium and rate constants in complex chemical and biological systems. The availability of precise recording spectrophotometers makes it feasible to collect data over a broad range of wavelengths, but often, to simplify calculations, data for only one or a few selected wavelengths are employed in the computations.

The use of digital computers for the analysis of chemical equilibria has been developed by de Maine and Seawright,[3] Sillén and Ingri,[4] Conrow, *et al.*,[5] Sullivan, *et al.*,[6] and Wiberg.[7] With a modern high-speed computer, it is possible to treat very complex systems in which numerous equilibria of dissociation of protons and formation of complexes are taken into account. It is feasible to use spectral data at many wavelengths in such computations, and to obtain a graphical display of the results with an automatic curve plotter.

This report describes a method by which a digital computer with an automatic plotter fits a smooth curve to experimental points to closely reproduce the complete electronic spectral absorption curve of a substance. This method when used in conjunction with an appropriate computational program permits the evaluation of acid dissociation constants and formation constants for complexes and compounds and the automatic plotting of the spectra of individual ionic species. It also provides a direct comparison between experimentally observed spectra and those predicted from the equilibrium constants and the spectra of the individual ionic species. Dissociation constants and spectra of individual ionic forms have been evaluated for vitamin $B_6$ aldehyde (pyridoxal) at 50° and for 5-deoxypyridoxal at 25°. The system pyridoxal–alanine at 50° has been studied in detail and formation constants and acid dissociation constants for Schiff bases are presented for this system and for the system 5-deoxypyridoxal–leucine at 25°.

The approach described should be of use in studying many different problems of chemical equilibria. Programs will be made available to persons desiring them.

## Recording and Plotting of Spectral Data

Data are transcribed from the tracings obtained with a standard recording spectrophotometer. If spectra change with time as a result of slow chemical reactions (*e.g.*, between pyridoxal and alanine) it is necessary to extrapolate the spectra to zero time to analyze the initial equilibria. This is accomplished by measuring the spectra repeatedly at intervals of 10, 30, 60, 90, 120, and 180 min after mixing. The computer makes a linear extrapolation, taking into account the time of

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(3) P. A. D. de Maine and R. D. Seawright, "Digital Computer Programs for Physical Chemistry," Vol. I, The MacMillan Co., New York, N. Y., 1962.

(4) L. G. Sillén, *Acta Chem. Scand.*, **16**, 159 (1962); N. Ingri and L. G. Sillén, *ibid.*, **16**, 173 (1962); L. G. Sillén, *ibid.*, **18**, 1085 (1964); N. Ingri and L. G. Sillén, *Arkiv Kemi*, **23**, 97 (1965).

(5) K. Conrow, G. D. Johnson, and R. E. Bowen, *J. Am. Chem. Soc.*, **86**, 1025 (1964).

(6) J. C. Sullivan, J. Rydberg, and W. F. Miller, *Acta Chem. Scand.*, **13**, 2023 (1959).

(7) K. B. Wiberg, "Computer Programming for Chemists," W. A. Benjamin, Inc., New York, N. Y., 1965.

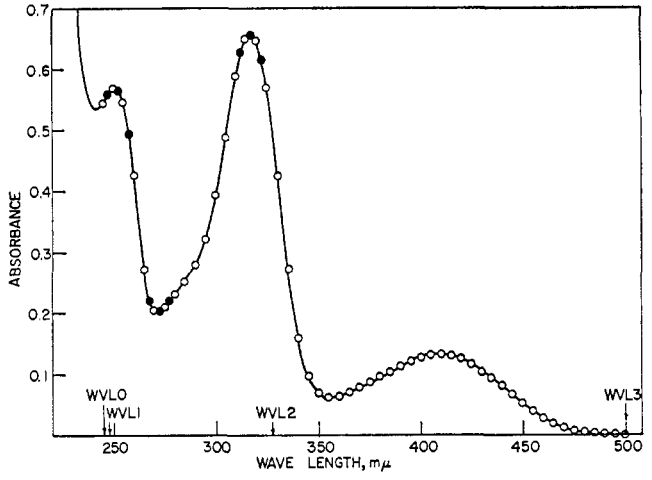



Figure 1. Method of transcription of data from experimentally obtained absorption spectra. The solid line is the absorption spectrum of a solution $1 \times 10^{-4}$ $M$ in pyridoxal and 0.1 $M$ in DL-alanine at pH 7.0 and 50°. This spectrum was selected as typical of those considered in this paper: O, points at 5-mμ intervals between wavelength limits WVL0 and WVL3 whose absorbance values, after any correction for solvent blanks, are transcribed to punched cards; ●, supplemental points added in the wavelength range WVL1–WVL2.

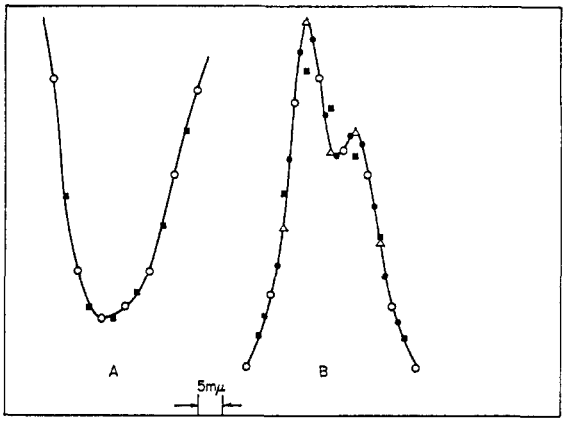



Figure 2. Comparison of interpolation procedure for curves with broad and narrow valleys and peaks. Curves A and B represent assumed experimental curves, ———, with the selected data points at 5-mμ intervals, O; △, supplemental data points, and ■, interpolated points from the first step (see text); ●, interpolated points from the second step.

measurement of every point in the spectrum. According to the spectrum, suitable wavelength limits WVL0, WVL1, WVL2, and WVL3 are chosen (Figure 1). WVL0 and WVL3 are lower and upper limits between which absorbance data are collected at 5.0-mμ intervals. The spectral peaks of pyridoxal and its derivatives are sharper in the lower wavelength region than at longer wavelengths. To accurately portray the spectra in this lower region, supplemental data near the absorption peaks must be provided. These supplemental data are recorded in the region from WVL1 to WVL2, again at 5.0-mμ intervals. Since we define WVL1 = WVL0 + 2.5, the supplemental data fall at wavelengths halfway between those of the principal set of data (Figure 1). In this work WVL0 is either 230 or 245 mμ, WVL2 is 327.5 mμ, and WVL3 is 500 mμ. A total of 69 or 75 points is used.

It is aesthetically desirable to draw a smooth curve between experimental points when plotting a spectral curve. This is done by a two-step interpolation. In the first step, points are computed midway between each pair of experimental points by fitting segments of third-order equations to pass through four consecutive points. At the ends of the spectra, segments of second-order equations are fitted to three points. From this first step of the procedure, points at 2.5-mμ intervals are available, half experimental and half interpolated. However, if a supplemental datum was recorded at a particular wavelength, the interpolated value is discarded in favor of the supplemental experimental one. The result of this operation is shown in Figure 2. The open circles on the two curves represent experimental data at 5.0-mμ intervals and the closed squares the interpolated points. It is seen that for curve A, having a moderately sharp curvature, the calculated points fall very close to the true absorption curve, represented by the solid line. In curve B, which contains two very narrow peaks, the calculated points do not lie on the true curve. In this case, supplemental data (open triangles) must be included.

In the second step, an entirely similar procedure using third-order equations is followed to obtain interpolated values between each pair of points at 2.5-mμ intervals. These interpolated points obtained in the second step are indicated by small closed circles in Figure 2B. We now have points at 1.25-mμ intervals. The automatic plotter locates these points and draws straight line segments between them to give the final curve.

## Methods of Calculation

**A. Successive Dissociation of Three Protons.** In a compound like pyridoxal hydrochloride there are two readily dissociable protons, and the three ionic forms may be represented as $H_2P$, HP, and P. In strongly alkaline solution, a third dissociation occurs. We have designated the resulting form as POH, but no chemical significance is to be attributed to this symbol. The four ionic forms and three $K_a$ values may be represented schematically as in eq 1.

$$H_2P \overset{K_{1P}}{\rightleftharpoons} HP \overset{K_{2P}}{\rightleftharpoons} P \overset{K_{3P}}{\rightleftharpoons} POH \qquad (1)$$

The total concentration of all forms of pyridoxal, $C_{tP}$ (equal to the sum of concentrations of the four individual forms), can be expressed as the product of the concentration of any one of the individual ionic forms and some function of the three $K_a$ values and the pH (*e.g.*, eq 2 and 3). The symbol $a_H$ represents the ap-

$$C_{tP} = \alpha C_P \qquad (2)$$

$$\alpha = 1 + a_H/K_{2P} + (a_H)^2/(K_{1P}K_{2P}) + K_{3P}/a_H \qquad (3)$$

parent hydrogen ion "activity" as represented by the pH meter reading

$$a_H = 10^{-pH} \qquad (4)$$

It is assumed that, for dilute solutions, concentrations of all other species may be used in the calculations. Equations 5–7 give the relationships of the concentrations of $H_2P$, HP, and POH to that of form P.

$$C_{H_2P} = (a_H)^2 C_P/(K_{1P}K_{2P}) \qquad (5)$$

$$C_{HP} = a_H C_P/K_{2P} \qquad (6)$$

$$C_{POH} = K_{3P}C_P/a_H \quad (7)$$

**1. Least-Squares Calculation of Molar Extinction Coefficients of Individual Ionic Species.** If it is assumed that the Beer–Lambert law holds for all species, each experimental absorbance, $A$, may be represented by

$$A = \sum_{j=1}^{4} C_j \epsilon_j l \quad (8)$$

where $j$ = 1, 2, 3, and 4 refer to the four ionic species, POH, P, HP, and $H_2P$, respectively, and $l$ is the path length. In the remaining treatment, $l$ is assumed to be always 1 cm. At any one wavelength, the $\epsilon$'s are constant, but the concentrations, $C_j$, vary with pH. To obtain the values of $\epsilon$ at a certain wavelength, we must measure the absorbancies of at least four solutions of different pH and solve a set of simultaneous linear equations. In general, we obtain data for more than four solutions, and use a least-squares method to obtain the best values of the $\epsilon$'s. Since we wish to obtain these values at each of the 69 to 75 selected wavelengths, the least-squares procedure is applied consecutively to the data at each wavelength.

The sum of the squares of the deviation, $U$, for all solutions at all values of pH and at all wavelengths is given by

$$U = \sum_{ik} \omega_k (A_{ik} - \sum_{j=1}^{4} C_{ji}\epsilon_{jk})^2 \quad (9)$$

in which the index $i$ refers to the pH and $k$ to the wavelength. A weighting factor, $\omega_k$, is also introduced. This has been taken as 1.0 at higher wavelengths and as 0.5 at wavelengths between WVL0 and WVL2. In this latter region twice as many points (many interpolated) per wavelength interval were used in the computations, and we wanted to weight the different spectral regions equally. Other weighting factors can be assigned if desired.

The values of the $\epsilon$'s corresponding to the minimum values of $U$ are computed by standard methods. The value of $U$ is an index of the goodness of fit and plays a very important role in the subsequent automatic adjustment of $pK_a$ values. However, it is not a completely adequate index, because there is a possibility that during the adjustment of $pK_a$ values, some extinction coefficients for some species will be negative. This became evident in early attempts to fit the data for pyridoxal when the computer moved the values of $pK_{3P}$ to an incorrectly high value and fitted the data by assuming that the spectrum of POH contained regions of negative absorption.

To diminish the possibility of arriving at a false solution with negative extinction coefficients, a new index of error, $U_a$, was defined

$$U_a = U + \sum_{jik} (C_{tP}\epsilon_{neg})^2 \quad (10)$$

where $\sum_{jik}(C_{tP}\epsilon_{neg})^2$ represents the sum of squares of deviations for all negative values of $\epsilon$. These latter terms are weighted heavily in the summation by multiplication by the square of the total pyridoxal concentration, $C_{tP}$. This procedure is rather arbitrary, but seems to be effective.

**2. Automatic Adjustment of $pK_a$ Values.** Two methods have been employed for the adjustment of the $pK_a$ values.

**a. Program** SWING. If the successive $pK_a$ values differ sufficiently, *e.g.*, by about 3 units, each $pK_a$ can be adjusted independently. The procedure is to first inspect the original spectral data and to estimate within a few tenths of a $pK$ unit the successive $pK_a$ values. Of course, the dissociations must lead to a distinctly perceptible change in the spectrum. These trial values of the $pK_a$'s are supplied to the computer along with the experimental data. The least-squares calculation of molar extinction coefficients is performed and the sum of squares of deviations, $U_a$, is evaluated. Then the first $pK_a$ is altered by an increment of value, $\Delta$ (here $\Delta$ = 0.4). All computations are repeated and a new value of $U_a$ is determined and compared with the first one. Depending upon whether the difference between the two values of $U_a$ is positive, negative, or zero, the $pK_a$ is varied again, either in the same direction or by 0.5 $\Delta$ in the opposite direction. After about 12 cycles of adjustment and recalculation, the value of $\Delta$ falls to below 0.004 and the second $pK_a$ is adjusted next. If any two $pK_a$ values are close enough together to "overlap," it is necessary to repeat the entire calculation a second or third time to ensure a correct answer.

**b. Program** PITMAP. A second method for dealing with "overlapping" dissociation constants is based on the theory developed by Sillén and Ingri.[4] When some of the adjustable constants have a correlation with others, the shape of the pit made by $U_a$ in the multidimensional space of $pK_a$ values becomes so skewed that the direction of the change in constants should be transformed to those of the main axes of the ellipsoid made by the contour lines on the pit surface. Near the minimum point, the shape of the surface is assumed that of a paraboloid in multidimensional space, the equation of which can be established from the values of $U_a$ for $0.5(N + 1)(N + 2)$ systematically chosen points (here, $N$ represents the number of the constants being searched for). The $pK_a$ values corresponding to the minimum point on this surface may be computed directly from the equation so obtained. In a future report, the results of application of this method to data for pyridoxamine and other substances with overlapping $pK_a$'s will be described. The method was used in the present work only for evaluation of equilibrium constants in the systems containing Schiff's bases.

**3. Standard Deviation.** The standard deviation, $\sigma$, of each $pK_a$ value is given by eq 11 where $U_{a0}$ is the

$$\sigma = \left(\frac{2U_{a0}}{N_{df}\delta^2 U_a/\delta pK^2}\right)^{1/2} \quad (11)$$

value of $U_a$ corresponding to the best value of $pK_a$ which has been found; $N_{df}$, the number of degrees of freedom, = (no. of solutions − no. of $pK_a$ values) × (no. of wavelengths used). The standard deviation of the absorbance values is given by eq 12. The value of

$$s = (U_{a0}/N_{df})^{1/2} \quad (12)$$

$\sigma$ is computed by assuming a parabolic form for the variation of $U_a$ with $pK_a$ in the range ±0.01 of the best value of $pK_a$ which is found.

**4. Comparison Plots of Calculated and Observed Spectra. Program** PLOT. The absorbance of a solution can be expressed as the sum of the contributions of each molecular species present (eq 8). Since the concentrations of individual ionic forms and their extinction

coefficients at all wavelengths have been computed, it is a simple matter, using eq 8, to calculate the expected absorbance at all wavelengths for a solution of any pH. If a pH is selected for which the spectrum has been measured experimentally, the experimental data may be plotted along with the calculated curve. A program has been written that does this. If no experimental data are available at exactly the pH selected, a linear interpolation is made between the data for two nearby pH values. In a similar way, the calculated absorbance at any wavelength may be plotted against pH, together with experimental points. If the selected wavelength is not one represented in the input data, an interpolation with a third-order equation is employed.

**B. Reversible Formation of Schiff's Bases.** The various equilibria of acid dissociation and formation of Schiff's bases in the system pyridoxal–alanine may be represented by the following scheme, which will also be applicable in many other cases.

$$H_2P \overset{K_{1P}}{\rightleftharpoons} HP \overset{K_{2P}}{\rightleftharpoons} P \overset{K_{3P}}{\rightleftharpoons} POH \quad (13)$$

$$+$$

$$H_2L \overset{K_{1L}}{\rightleftharpoons} HL \overset{K_{2L}}{\rightleftharpoons} L \quad (14)$$

$$\Big\updownarrow F_{PL} \quad (15)$$

$$H_3PL \overset{K_{1PL}}{\rightleftharpoons} H_2PL \overset{K_{2PL}}{\rightleftharpoons} HPL \overset{K_{3PL}}{\rightleftharpoons} PL \quad (16)$$

P represents pyridoxal, L the amino acid, and PL the Schiff's base. The formation constant, $F_{PL}$, relates the concentrations of the anions of P and of L to the dianion of PL.[8]

For the system 5-deoxypyridoxal–L-leucine we have proposed additional equilibria leading to formation of a complex of unknown structure between the Schiff's base and the amino acids (eq 17).[9] Four functions,

$$PL + L \Big\updownarrow F_{PL_2}$$

$$H_2PL_2 \overset{K_{2PL_2}}{\rightleftharpoons} HPL_2 \overset{K_{3PL_2}}{\rightleftharpoons} PL_2 \quad (17)$$

$\alpha$, $\beta$, $\gamma$, and $\eta$, of the pH and of the acid dissociation constants are introduced.

$$\alpha = 1 + a_H/K_{2P} + (a_H)^2/(K_{1P}K_{2P}) + K_{3P}/a_H \quad (18)$$

$$\beta = 1 + a_H/K_{2L} + (a_H)^2/(K_{1L}K_{2L}) \quad (19)$$

$$\gamma = 1 + a_H/K_{3PL} + (a_H)^2/(K_{2PL}K_{3PL}) + (a_H)^3/(K_{1PL}K_{2PL}K_{3PL}) \quad (20)$$

$$\eta = 1 + a_H/K_{3PL_2} + (a_H)^2/(K_{2PL_2}K_{3PL_2}) \quad (21)$$

If values for all the constants were known, it would be possible to compute the concentrations of the various species as follows. The total concentrations of pyridoxal ($C_{tP}$) and amino acid ($C_{tL}$) for any solution can be expressed by eq 22 and 23. The concentrations of

$$C_{tP} = \alpha C_P + \gamma C_{PL} + \eta C_{PL_2} \quad (22)$$

$$C_{tL} = \beta C_L + \gamma C_{PL} + 2\eta C_{PL_2} \quad (23)$$

Schiff's base ($C_{PL}$) and of a complex of the latter with amino acid (if present) ($C_{PL_2}$) are given by eq 24 and 25 which define the formation constants, $F_{PL}$ and $F_{PL_2}$.

$$C_{PL} = F_{PL}C_PC_L \quad (24)$$

$$C_{PL_2} = F_{PL_2}C_{PL}C_L \quad (25)$$

The value of $C_P$ is obtained through successive approximations. If an excess of amino acid is present, the first estimate of $C_L$ is given by eq 26 and successive estimates of $C_P$ and $C_L$ by eq 27 and 28. Concentrations

$$C_L = C_{tL}/\beta \quad (26)$$

$$C_P = C_{tP}/[\alpha + (\gamma + \eta F_{PL_2}C_L)F_{PL}C_L] \quad (27)$$

$$C_L = C_{tL}/[\beta + (\gamma + 2\eta F_{PL_2}C_L)F_{PL}C_P] \quad (28)$$

of the other three ionic forms of pyridoxal are given by eq 5–7. The concentrations of the various forms of the Schiff's base are given by eq 29–33.

$$C_{H_3PL} = (a_H)^3C_{PL}/(K_{1PL}K_{2PL}K_{3PL}) \quad (29)$$

$$C_{H_2PL} = (a_H)^2C_{PL}/(K_{2PL}K_{3PL}) \quad (30)$$

$$C_{HPL} = a_HC_{PL}/K_{3PL} \quad (31)$$

$$C_{H_2PL_2} = (a_H)^2C_{PL_2}/(K_{2PL_2}K_{3PL_2}) \quad (32)$$

$$C_{HPL_2} = a_HC_{PL_2}/K_{3PL_2} \quad (33)$$

**1. Molar Extinction Coefficients of Individual Ionic Species.** We assume as before that the Beer–Lambert law holds and that the experimental absorbance for a particular solution and a particular wavelength is given by eq 8 where $j$ = 1–11 designate the eleven ionic species, POH, P, HP, $H_2P$, PL, HPL, $H_2PL$, $H_3PL$, $PL_2$, $HPL_2$, and $H_2PL_2$, respectively.

Although 11 light-absorbing ionic species are included, these ordinarily will not all be present in significant amounts. In the system pyridoxal–alanine, there is no evidence for the complex $HPL_2$, and even in the system 5-deoxypyridoxal–leucine, where $HPL_2$ is present, the ionic forms $H_2PL_2$ and $PL_2$ exist in such small quantities that they have a very small effect on the over-all spectrum. Their spectra were assumed, for the purposes of computation, to be identical with those of $H_2PL$ and PL, respectively. Thus, we had to consider only seven of the possible 12 species in either system; for pyridoxal + alanine, POH, P, HP, $H_2P$, PL, HPL, and $H_2PL$; and for 5-deoxypyridoxal + leucine, P, HP, $H_2P$, PL, HPL, $H_2PL$, and $HPL_2$. Since the $\epsilon$'s for POH, P, HP, and $H_2P$ were already known, extinction coefficients remained unknown for only four species. These were obtained by a least-squares method as described previously. It is usually feasible to evaluate a maximum of four unknown sets of extinction coefficients at once, and depending upon the details of the system under investigation, it may be a different set of four for one system than for another. A program has been written which permits one to state conveniently the appropriate simplifications and to specify which sets of $\epsilon$'s (up to four) are to be evaluated.

The error function is defined as in eq 9 except that the second set of terms is multiplied by 100 to discourage strongly the obtaining of false solutions with negative extinction coefficients in some regions.

**2. Adjustment of Values of p$K_a$ and Log $F$. 1. Program GRID.** In seeking the best values for the p$K_a$'s and formation constants we may regard the error func-

(8) D. E. Metzler, *J. Am. Chem. Soc.*, 79, 485 (1957).
(9) D. E. Metzler and K. Nagano, Proceedings of Second IUB Symposium on Chemical and Biological Aspects of Pyridoxal Catalysis, Moscow, Sept 1966, in press.

tion, $U_a$, as a variable which is dependent upon the "trial parameters" (values of the $pK_a$'s and log $F$'s). The minimum value of $U_a$ may be visualized as lying in a multidimensional "pit" in the hypersurface defined by $U_a$ as a function of the trial parameters. We seek to find the values of the trial parameters at the bottom of the pit. In the systems under consideration there is a strong correlation among the trial values of $F_{PL}$ and the two $pK_a$'s, $pK_{2PL}$ and $pK_{3PL}$. Likewise the trial values of $F_{PL2}$ and $pK_{3PL2}$ are strongly correlated. Hence the direct method of varying each parameter in turn (program SWING) cannot be applied.

The simplest approach is to vary each parameter by a certain amount, $\Delta$, both in the positive and negative directions and to evaluate $U_a$ for each of the three resultant trial values. If this is done for all the parameters and for all possible combinations of the altered trial values, an $n$-dimensional grid of $3^N$ values of $U_a$ is obtained, where $N$ is the number of constants being adjusted. If $N = 4$ and we use data for all wavelengths for 30 test solutions, the computation takes almost 10 min and is excessively expensive. It is more practical to vary only three constants at once, the computation time amounting to about 4 min.

The results of the computation are inspected to determine which values of the trial parameters gave a minimum $U_a$, and three difference values of each parameter are chosen, usually more closely spaced than for the first "grid." The procedure can be repeated as long as the new set of trial parameters gives a smaller value of $U_a$ than the minimum value obtained previously. For the first grids we have used values of $\Delta = 0.4$ and for later ones $\Delta = 0.1$. Final refinement of parameters is done with program PITMAP (1b).

**3. Sequence of Computations.** A workable procedure for the solution of problems of the type considered here is as follows. (1) Initially make spectral measurements on several solutions containing a high concentration of the second component, L, and from an examination of the spectra choose initial trial values for the $pK_a$'s of PL. Also measure spectra at a constant pH in a region where formation of PL is extensive for several lower concentrations of component L. This will permit the choice of an initial trial value of $F_{PL}$. (2) Collect for the initial computations the minimum and necessary number of experimental spectra. This will always be at least as great as the number of unknowns ($pK_a$'s, log $F$'s, and spectra of individual ionic forms). (3) Start the computation with the data for lower concentrations of amino acid and adjust $pK_{2PL}$, $pK_{3PL}$, and $F_{PL}$ using program GRID and $\Delta = 0.4$. (4) Repeat using a grid of $\Delta = 0.1$. (5) Use program PITMAP with $\Delta = 0.01$ which will not only refine the three trial parameters but will punch out on cards the spectra of the individual ionic forms of PL which are needed for step 6. (6) Using program PLOT, compare the calculated and experimental plots of absorbance against pH and wavelength. If a good fit is obtained, the problem is solved. (7) If systematic errors are found for different concentrations of amino acid, consider what additional equilibria must be added. No exact procedure can be given for finding the values of the additional equilibrium constants which may be required. The grid method with refinement with program PITMAP in several steps was used in this work. One must always ask whether the spectra obtained are reasonable from a chemical viewpoint.

## Results

**5-Deoxypyridoxal.** The simplest case studied is that of 5-deoxypyridoxal which exists in three ionic forms. From spectra at 24 values of pH from 1.4 to 12.9, the $pK_a$ values of 4.16 and 8.02 were evaluated in 118 sec of computation (Table I). The plots comparing computed and observed absorbances show a nearly perfect fit, the standard deviation of the absorbances, $s$, amounting to only 0.001 absorbance unit. A few extinction coefficients are given in Table II.

**Table I.** Calculated Values of $pK_a$ with Standard Deviations

| | This research $pK_a \pm$ std dev | Lit.[a] | Lit.[b] |
|---|---|---|---|
| 5-Deoxypyridoxal | $4.16 \pm 0.003$ (25°) | 4.17 (25°) | |
| | $8.02 \pm 0.004$ | 8.14 | |
| Pyridoxal | $4.13 \pm 0.008$ (50°) | 4.20 (25°) | 4.23 |
| | $8.37 \pm 0.008$ | 8.66 | 8.70 |
| | $13.04 \pm 0.023$ | 13 | |

[a] D. E. Metzler and E. E. Snell, *J. Am. Chem. Soc.*, 77, 2431 (1955). [b] V.R. Williams and J. B. Nielands, *Arch. Biochem. Biophys.*, **53**, 56 (1954).

**Table II.** Absorption Maxima and Molar Extinction Coefficients of Various Ionic Forms of the Compounds Studied

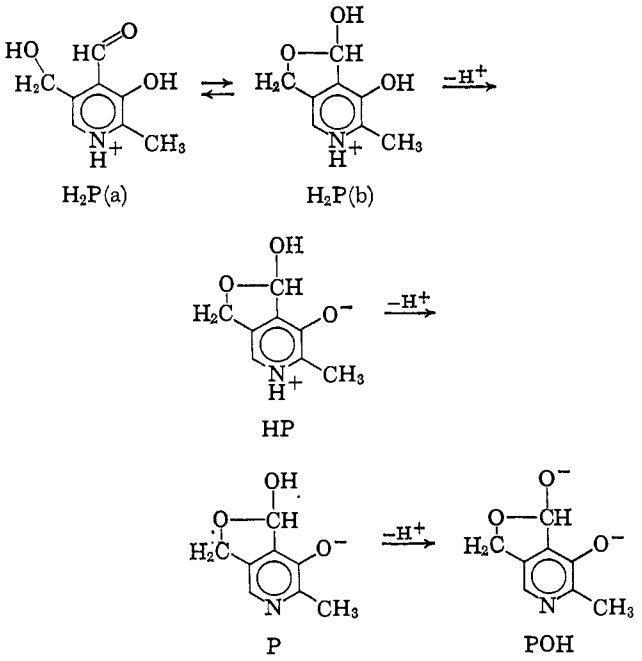
| Ionic form | 5-Deoxypyridoxal, 25° $\lambda_{max}$, mμ | $\epsilon \times 10^{-3}$ | Pyridoxal, 50° $\lambda_{max}$, mμ | $\epsilon \times 10^{-3}$ |
|---|---|---|---|---|
| $H_2P$ | 294 | 6.28 | 288 | 8.61 |
| | 342 | 1.88 | | |
| HP | | | 254 | 5.51 |
| | 324 | 2.97 | 318 | 8.13 |
| | 381 | 4.22 | | |
| P | 263 (shoulder) | | 236 | 9.37 |
| | | 3.48 | 302 | 4.61 |
| | 391 | 6.33 | 394 | 2.97 |
| POH | | | 245 | 8.18 |
| | | | 299 | 7.55 |

**Pyridoxal.** Pyridoxal exists in solution largely as the internal hemiacetal, four ionic forms of which are shown in Scheme I. Each of these is considered to be in equilibrium with a smaller amount of free aldehyde, the structure of which, $H_2P(a)$, is drawn only for form $H_2P$. The structure assigned to POH is, however, not completely certain.

The first two $pK_a$ values have been determined previously at 25°. In this study the spectra and $pK_a$ values have all been measured at 50° because related kinetic studies are being conducted at this temperature.

Spectra were measured for 19 solutions at pH values of 1.03, 3.14, 3.58, 3.91, 4.36, 4.66, 5.60, 6.55, 6.87, 7.52, 7.92, 8.35, 8.65, 9.25, 9.75, 10.16, 12.15, 12.82, and 13.16. No buffers were used, but the pH was adjusted with HCl or KOH. An ionic strength of 1.0 was maintained by addition of KCl. Figure 3 shows the spectra of the four ionic forms. That of form POH is presented for the first time. Figures 4 and 5 show plots of calculated and observed spectra at two selected pH values and spectrophotometric titration curves at three selected wavelengths as drawn by the automatic plotter. The agreement is good, even at the highest

**Scheme I**




pH. The p$K_a$'s are given in Table I and extinction coefficients at the maxima in Table II.

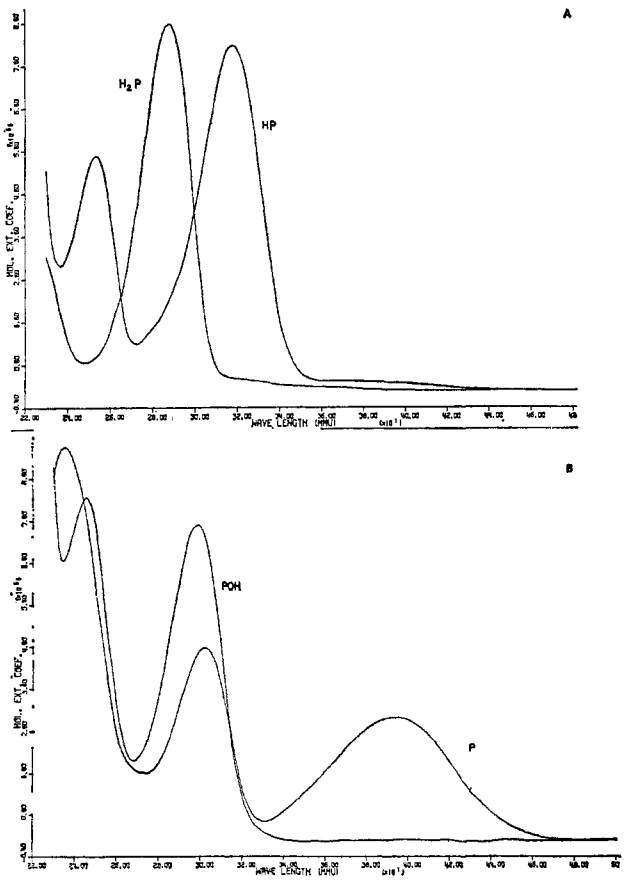



Figure 3. Absorption spectra of the four ionic forms of pyridoxal at 50°: graph A, $H_2P$ and HP; graph B, P and POH.

**Pyridoxylidene-DL-alanine.** This Schiff's base, formed from the rapid, reversible reaction of alanine with pyridoxal, exists in the three ionic forms $H_2PL$, HPL, and PL whose probable structures are shown below.

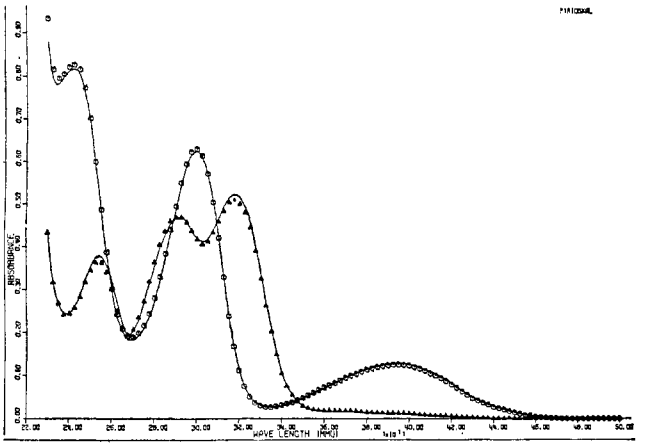


Figure 4. Calculated absorption spectra of solutions of $10^{-4}$ *M* pyridoxal at two pH values plotted together with experimental points, temperature 50°: Δ, pH 4.36; O, pH 13.16.

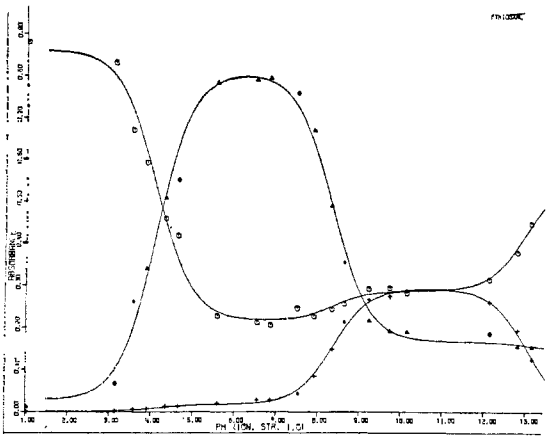
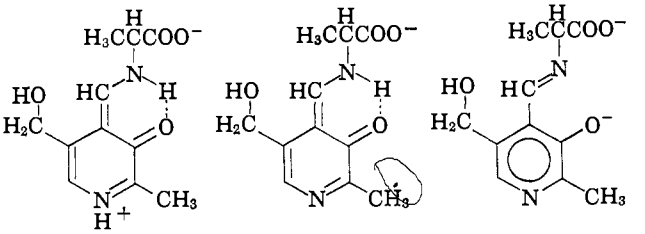


Figure 5. Plots of absorbance *vs.* pH at selected wavelengths for a $10^{-4}$ *M* solution of pyridoxal at 50°: +, 394 mμ; Δ, 318 mμ; O, 288 mμ.

The formation and acid dissociation constants are given in Table III, and the spectra of the three ionic species are shown in Figure 6 and in Table IV. These were evaluated from data for a total of 45 experimental solu-



$$H_2PL \xrightleftharpoons{pK_{2PL}} HPL \xrightleftharpoons{pK_{3PL}} PL$$

tions of pyridoxal concentration $10^{-4}$ *M* and alanine concentrations from 0.05 to 0.45 *M* (see Experimental Section). The comparison plots of observed *vs.* computed spectra, of which those in Figures 7–9 are typical, were all reasonably satisfactory. Figure 7 shows spectra at three values of pH for solutions 0.1 *M* in alanine and $10^{-4}$ *M* in pyridoxal, while the pH profiles in Figures 8 and 9 are for solutions 0.05 and 0.45 *M* in alanine, respectively, and $10^{-4}$ *M* in pyridoxal The existence of forms containing more than one molecule of pyridoxal,

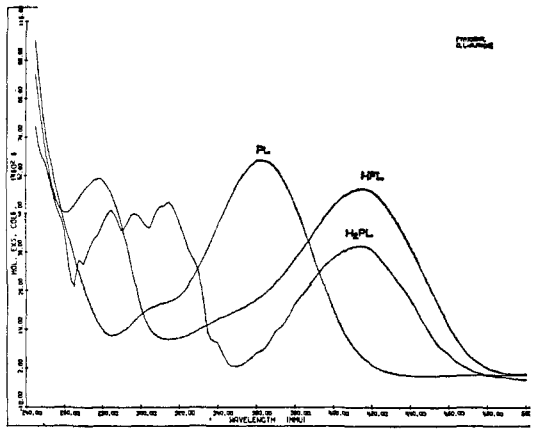



Figure 6. Absorption spectra of the three ionic forms of pyridoxylidene-DL-alanine at 50°. The unevenness of the spectrum of $H_2PL$ results from the fact that this form exists in very small amounts so that experimental errors lead to uncertainty in the spectrum, especially at the lower wavelengths.

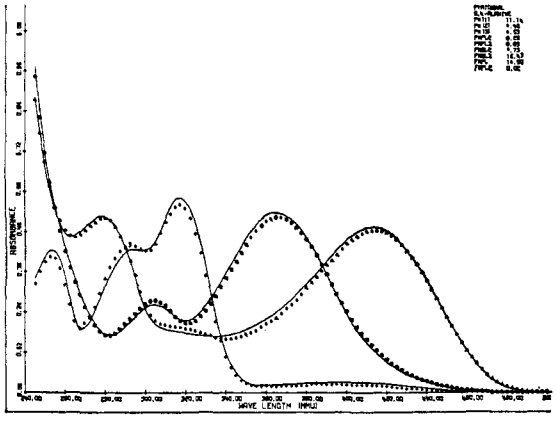


Figure 7. Calculated absorption spectra for solutions of $10^{-4}$ *M* pyridoxal containing 0.2 *M* alanine at three values of pH plotted together with experimental points, temperature 50°: +, pH 4.53; Δ, pH 8.40; O, pH 11.11.

*e.g.*, $P_2L_2$, was ruled out by measurement of 18 solutions containing 100 times higher concentrations of pyridoxal and 0.45 *M* alanine using spacers in the spectrophotometer cells to cut the path length. Calculated and observed spectra agreed equally satisfactorily for these solutions. The standard deviation, *s*, was 0.013 absorbance unit.

**Table III.** Calculated Values of $pK_a$ and *F* with Standard Deviations

| | 5-Deoxypyridoxal + L-leucine, 25°[a] | Pyridoxal + DL-alanine, 50° |
|---|---|---|
| $pK_{2PL}$ | 6.51 ± 0.009 | 6.26 ± 0.018 |
| $pK_{3PL}$ | 11.73 ± 0.023 | 9.91 ± 0.007 |
| $pK_{2PL_2}$ | 7.17 ± 0.13 | |
| $pK_{3PL_2}$ | 13.19 ± 0.006 | |
| $F_{PL}$ | 9.93 ± 0.49 | 14.50 ± 0.20 |
| $F_{PL_2}$ | 0.49 ± 0.01 | |
| $pK_a$ Values of Amino Acid Used for Calculation | | |
| $pK_{1L}$ | 2.33[b] | 2.54 ± 0.008 |
| $pK_{2L}$ | 9.74[b] | 9.40 ± 0.014 |

[a] See ref 9. [b] "The Merck Index of Chemicals and Drugs," 7th ed, Merck and Co., Inc., 1960, p 607.

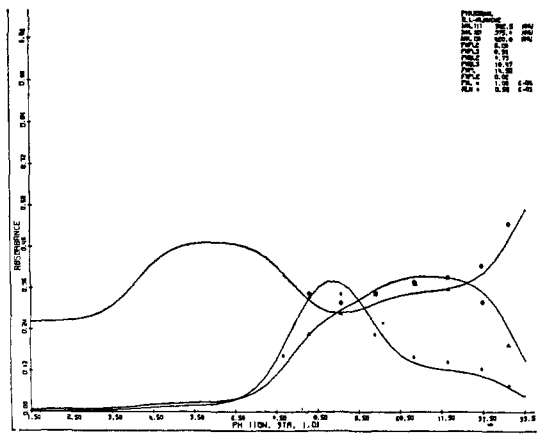


Figure 8. Plots of absorbance *vs.* pH at selected wavelengths for a $10^{-4}$ *M* solution of pyridoxal containing 0.05 *M* DL-alanine at 50°: +, 420 mμ; Δ, 3.75 mμ; O, 302.5 mμ.

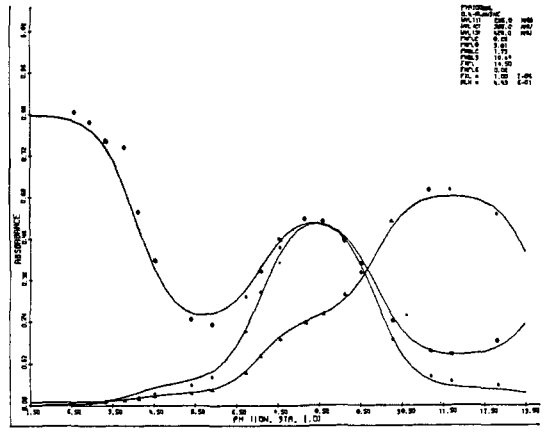


Figure 9. Plots of absorbance *vs.* pH at selected wavelengths for a $10^{-4}$ *M* solution of pyridoxal containing 0.449 *M* alanine at 50°: +, 420 mμ; Δ, 365 mμ; O, 285 mμ.

No buffers were added, and for some solutions the measurement of pH was not completely precise. A few points, *e.g.*, in Figure 9, deviate from the computed curve. During early attempts to analyze the data, other possible species, *e.g.*, $PL_2$ and $HPL_2$, were included in the chemical model. The amounts of such species, if they exist, were found to be immeasurably small.

**Table IV.** Absorption Maxima and Molar Extinction Coefficients of Various Ionic Forms of the Schiff's Bases Studied

| | 5-Deoxypyridoxylidene-L-leucine, 25°[a] | | Pyridoxylidene-DL-alanine, 50° | |
|---|---|---|---|---|
| Ionic form | $\lambda_{max}$, mμ | ε × $10^{-3}$ | $\lambda_{max}$, mμ | ε × $10^{-3}$ |
| $H_2PL$ | 289 | 5.85 | ~300 | 5.3 |
| | 414 | 7.61 | 414 | 4.0 |
| HPL | 284 | 8.48 | 278 | 6.13 |
| | 420 | 5.66 | 416 | 5.81 |
| PL | 343 | 4.92 | ~305 | 2.1 (shoulder) |
| | | | 363 | 6.71 |
| $HPL_2$ | 286 | 7.92 | | |
| | 422 | 5.59 | | |

[a] See ref 9.

**5-Deoxypyridoxylidene-L-leucine.** Results for the system 5-deoxypyridoxal–L-leucine, at 25°, have been reported briefly elsewhere[9] and will be summarized

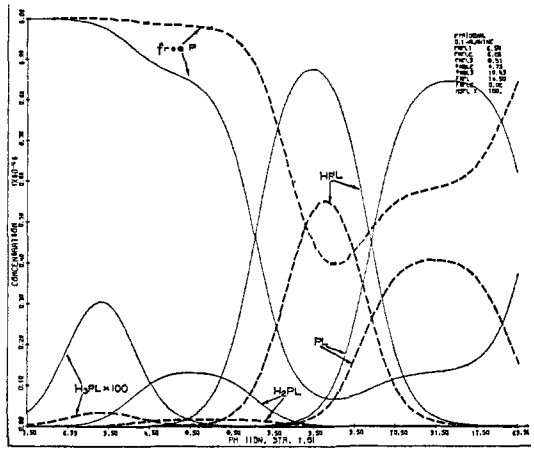



Figure 10. Plot of the computed pH profile of concentrations of free pyridoxal (all forms), $H_3PL$ (multiplied by 100), $H_2PL$, HPL, and PL for solutions of $10^{-4}$ *M* pyridoxal containing 0.449 *M* DL-alanine, ———, and 0.05 *M* DL-alanine, - - - -, at 50°. The concentration of $H_3PL$ was computed on the assumption that $pK_{PL1} = 2.5$, the same as that of alanine. Of course the true value of this p*K* might be quite different and the fit would still be good.

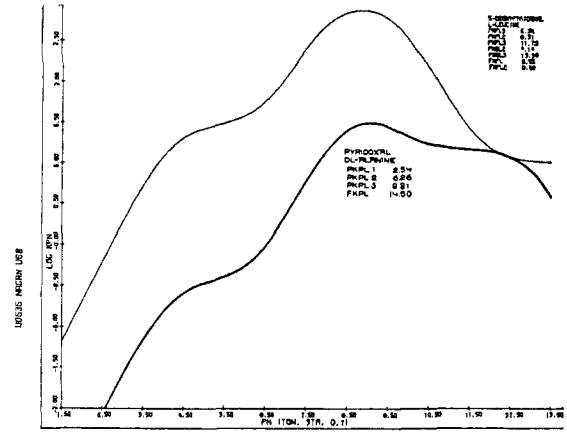


Figure 11. Plots of the logarithm of the apparent formation constant ($K_{PH}$) *vs.* pH for 5-deoxypyridoxylidene-L-leucine at 25° and for pyridoxylidene-DL-alanine at 50°.

here. This system is a favorable one for study because of the high degree of formation of the Schiff's base over a broad range of pH. Initially it was assumed that the three forms of the Schiff's base, $H_2PL$, HPL, and PL, were the only forms present and the equilibrium constants were obtained from the data for solutions containing 0.05 and 0.1 *M* leucine. However, the spectra of solutions containing 0.01 *M* L-leucine could not be predicted accurately using these constants. Introduction of a complex, $HPL_2$, resolved the difficulty. This was found to have a spectrum nearly identical with that of HPL and apparently is a complex of the latter species with the anion of L-leucine. The spectra are shown elsewhere[9] and only the constants are given in Table III. The spectrum of HPL is very similar to that reported previously for pyridoxylidene-DL-valine[8] while that of PL is surprisingly different, possessing an absorption maximum at 343 mμ. The precise spectrum of $H_2PL$ was obtained. It absorbs maximally at 414 mμ and possesses a higher molar extinction coefficient than does HPL.

In considering various problems of equilibria and kinetics, it is useful to know the concentration of each ionic form present at a particular pH. These pH profiles may be computed readily using the auxiliary program designated PROFILE. Figure 10 shows the results of such a program for the pyridoxal–DL-alanine system and alanine concentrations of both 0.449 and 0.1 *M*. This program also computes another useful function, the pH-dependent apparent formation constant which has been designated previously as $K_{pH}$[8] (eq 34). The

$$K_{pH} = \gamma C_{PL}/\alpha C_P \beta C_L \qquad (34)$$

term $\alpha C_P$ represents the total of all free forms of pyridoxal, etc. In Figure 11 a plot of log $K_{pH}$ *vs.* pH is shown for both the pyridoxal–alanine and deoxypyridoxal–leucine systems.

## Discussion

The methods described here permit the automatic evaluation of from one to three successive acid dissociation constants for compounds for which each ionic form possesses a substantially different spectrum, the rapid evaluation of formation constants and acid dissociation constants of complexes or compounds formed reversibly in the presence of a second nonlight-absorbing component, and plotting of complete absorption spectra of all ionic species which contribute significantly to the absorption of light. They should have widespread use. The advantages of this method for obtaining dissociation constants are: (1) a least-squares method assures the best values for dissociation constants and extinction coefficients of individual ionic forms at all wavelengths. (2) The method is rapid. (3) Precise spectra of intermediate ionic forms are provided, whereas previously tedious calculation was often required. (4) A large number of plots of the type displayed in Figures 4, 5, 7, 8, and 9 may be obtained readily and inexpensively These permit a visual judgement of goodness of fit and show at a glance at what wavelengths and under what conditions of pH, concentration, etc., discrepancies exist. (5) Data for $pK_a$'s and extinction coefficients of individual ionic forms are stored on punched cards or by other means in digital form. The data are thus immediately available for use in calculations of chemical equilibria and kinetics in more complex systems.

The method of evaluation of formation constants is superior to the widely used procedure of Ketelaar, *et al.*,[10] because (1) it uses data at many different wavelengths, (2) it yields $pK_a$ values for the compound or complex when its absorption spectrum is altered by association or dissociation of protons, (3) complete absorption spectra of the various forms of the compound or complex are computed, (4) a series of comparison graphs showing experimental data plotted with the computed curves provide a means of judging directly the goodness of fit, and (5) the chemical model can be varied easily, *e.g.*, by introducing additional equilibria, and can readily be tested to detemine whether a better fit is obtained. The method can be applied directly to many chemical and biochemical problems including study of enzyme–substrate complexes and can be used to advantage in handling various kinetic problems as will be discussed in a future paper. The

(10) J. A. A. Ketelaar, C. Van de Stolpe, and H. R. Gersmann, *Rec. Trav. Chim.*, 71, 499 (1951); J. A. A. Ketelaar, C. Van de Stolpe, A. Goudsmit, and W. Dzcubas, *ibid.*, 71, 1104 (1952).

approach should be of value in various problems of metal complex formation.[11]

The transcription of data points by hand from experimental graphs used in this study is tedious and often leads to introduction of errors, the most serious of which must be traced and removed before acceptable results are obtained. However, with the computer programs available, the many users of recording spectrophotometers may immediately employ these methods. A more desirable system will be to collect data directly in digital form, and equipment for doing this is already available commercially. Doubtless, within a few years older methods of handling spectrophotometric data will be completely obsolete. An important advantage of the newer methods will be that complete spectral data can be sent between laboratories over telephone wires. Thus a much more efficient sharing of information will be available.

During the course of this study many questions have arisen for which full answers are not yet available. For example, "How great must be the differences between the spectra of two successive ionic forms in order to allow the evaluation of the $pK_a$'s?" It is clear that each dissociable group must affect the spectrum. Thus, if a compound possesses two groups of identical microscopic dissociation constants, but the dissociation of one group has no effect on the spectrum, the two stepwise dissociation constants of the compound cannot be determined from spectral data alone. In such cases both spectra and titration data are required.

It is necessary in our method to provide to the computer an initial estimate for each $pK_a$. If all of the $pK$ values are well separated it is easy to do this by inspection of the data. Even if the initial guess is poor, both programs SWING and PITMAP lead to a rapid selection of the correct value. However, if the $pK_a$ values are closer, difficulties may be anticipated, especially if "bad" initial guesses are made. We are investigating cases of this type.

An important question is "What is the minimum number of experimental spectra which must be measured in order to obtain precise answers?" It appears that no more than the minimum which are theoretically required (for the determination of $pK_a$'s, three for the first $pK_a$ and two for each successive $pK_a$) are needed, but that a few more are to be recommended. In general, the usual "common sense" judgement of the chemist regarding the number and distribution of pH values of the samples should be followed.

Another question concerns the treatment of experimental errors. Errors in the spectra may vary with the wavelength, being higher where the slope of the spectral curve is steep. Accordingly, it would be desirable to vary the weighting factors, $\omega_k$, with the wavelength for each solution. However, this has not been done in this study. The standard deviations in the $pK_a$'s are very low (Table I) and are considerably less than what we would judge to be the experimental error in the pH measurements. However, the standard error is correctly evaluated and represents a useful index of the reproducibility of the computations. The standard error of the absorbances, $s$ (eq 12), represents an average value for all wavelengths and all solutions and does not indicate whether systematic errors exist in certain regions of wavelength or pH. That such systematic errors do exist for some solutions is obvious from inspection of comparison plots of the type of Figures 4 and 7. If the experimental pH is near a $pK$ value, a small error in pH will lead to large systematic deviations, even though the value of $s$ may be acceptably small. The careful examination of a series of plots of the type of Figures 4, 5, 7, 8, and 9 is extremely valuable in deciding whether observed systematic deviations are within acceptable limits as set, for example, by the precision of pH measurement or whether the assumed chemical model is inadequate.

A particular problem in the evaluation of $pK_a$'s arises when incomplete data are available for a dissociation step. Pyridoxal is an excellent example. No data were obtained above pH 13.16, but $pK_{3P}$ is 13.0. As was mentioned earlier, before steps were taken to discourage the assignment of negative extinction coefficients, the computer preferred to assign a higher value of $pK_{3P}$ and negative extinction coefficients to POH in the region around 390 m$\mu$. But what is the possibility that the true solution is for a $pK_{3P}$ value lower than 13.0 and higher extinction coefficients for POH around 390 m$\mu$? An acceptable but less perfect fit to the data could be obtained by letting $pK_{3P}$ be as low as 12.74. However, the computed spectrum of POH in this case possessed another absorption band at 390 m$\mu$ with $\epsilon$ $0.8 \times 10^3$ (27% of that of P), a result which seems very unlikely on chemical grounds.

Information on spectra and $pK$ values is now available for a small number of different Schiff's bases.[8,12] For all of these the spectrum of form HPL is similar, but striking differences are observed in the spectrum of form PL and in the $pK$ for dissociation of HPL to PL. The basic causes of these differences are not understood, but we believe that the study of other analogs of pyridoxal and of other amino acids will give information which will permit us to understand these variations and to interpret them in the light of possible significance to the events which take place on a surface of enzymes which employ pyridoxal phosphate as a coenzyme.

## Experimental Section

**Chemicals.** Pyrixodal·HCl (Sigma Chemical Co.), DL-alanine (Nutritional Biochemicals Corp.), and L-leucine (methionine-free, Mann Research Laboratories, Inc.) were used without further purification. 5-Deoxypyridoxal was synthesized by Dr. C. Iwata in this laboratory.

**Measurements of pH.** A Beckman Model G pH meter was used. Commercially available standard buffer solutions (Beckman Instruments, Mallinckrodt Chemical Works, or Matheson Coleman and Bell) were used, but later the high pH buffers were replaced by the National Bureau of Standards 0.01 $M$ borax buffer of pH 9.18 at 25° and 9.01 at 50°. All pH values below 12 were determined at 50° by setting the temperature compensator at 25° and standardizing with the dial set at a reading of $^{323}/_{298}$ times the pH of the standard buffer at 50°. The meter reading for the unknown sample was then multiplied by $^{298}/_{323}$. When the pH exceeded 12, the temperature compensator had to be set at 40° (its upper limit) in order to keep the high pH readings on the scale. The meter was adjusted so that the test solutions could be read on the scale. Two pH standards (phosphate at pH 6.83 and borax at pH 9.01) were read at the same temperature and the pH of the test sample was established by a linear extrapolation.

(11) K. Nagano, H. Kinoshita, and Z. Tamura, *Chem. Pharm. Bull.*, (Tokyo), **11**, 999 (1963).

(12) T. C. French, D. S. Auld, and T. C. Bruice, *Biochemistry*, **4**, 77 (1955).

**Test Solutions.** Pyridoxal was studied in unbuffered solutions of ionic strength 1.0. The pH and ionic strength were maintained by addition of suitable amounts of KCl, HCl, and KOH. Deoxypyridoxal was studied in buffered solutions of ionic strength 0.1.

Stock solutions of $2 \times 10^{-4}$ *M* pyridoxal·HCl and 5-deoxypyridoxal were prepared. They could be stored for a month in a refrigerator without any noticeable change in spectrum. Sample solutions were prepared before measurement by mixing 10 ml of the stock solution of the vitamin $B_6$ derivative with a 10-ml aliquot of a suitable buffer solution or with the appropriate amount of HCl, or KOH and KCl.

Mixtures of pyridoxal and DL-alanine were studied in unbuffered solutions of ionic strength 1.0 in order to avoid the effects of buffers on the reaction rates which are also under investigation. The pH and ionic strength were maintained by addition of suitable amounts of KCl, HCl, and KOH. Stock solutions of various concentrations of DL-alanine (0.1, 0.2, 0.4, and 0.898 *M* of ionic strength 2.0) were prepared. Sample solutions were prepared before measurement by mixing 10 ml of the stock solution of pyridoxal maintained at 50° with a 10-ml aliquot of a suitable alanine solution maintained at 50°.

Spectra were obtained for 45 different solutions (eight for 0.05 *M* alanine, five for 0.1 *M* alanine, 17 for 0.2 *M* alanine, and 15 for 0.449 *M* alanine) in the absence of any special buffers at an ionic strength of 1.0. The measured pH values were: 4.13, 4.52, 5.41, 5.92, 6.73, 7.09, 7.57, 8.18, 8.62, 9.13, 9.53, 10.29, 11.21, 11.71, and 12.83 for 0.449 *M* alanine; 4.53, 5.00, 5.35, 5.57, 6.55, 7.05, 7.47, 7.95, 8.40, 8.69, 9.21, 9.23, 9.48, 10.24, 11.11, 12.08, and 13.07 for 0.2 *M* alanine; 7.00, 7.95, 8.61, 12.30, and 13.16 for 0.1 *M* alanine; and 7.66, 8.28, 9.04, 9.87, 10.82, 11.62, 12.45 and 13.10 for 0.05 *M* alanine solutions. The sum of squares of the deviations, $U_a$, for 45 solutions was 0.50.

The interaction of 5-deoxypyridoxal with L-leucine was studied in buffered solutions of ionic strength 0.1 except at very high pH where the ionic strength exceeded 0.1. Stock solutions of $3 \times 10^{-4}$ *M* 5-deoxypyridoxal and of various concentrations of L-leucine (0.015, 0.075, and 0.15 *M* of ionic strength 0.15) were prepared. Sample solutions were prepared by mixing 10 ml of the stock solution of 5-deoxypyridoxal with a 20-ml aliquot of a suitable buffered leucine solution. Spectra were determined for 39 different solutions (13 for 0.01 *M* L-leucine, ten for 0.05 *M* L-leucine, and 16 for 0.1 *M* L-leucine) in the presence of the indicated buffers at an ionic strength of 0.1. The measured pH values were: 3.28, 4.12, 4.40, 4.62, 5.91 (acetate buffers), 7.33, 7.62, 8.83, 9.59, 10.59, 11.00, 11.27 (potassium carbonate buffers), 11.70, 12.21, 12.62, 12.99 (KOH) for 0.1 *M* L-leucine; 3.34, 4.85, 6.17 (acetate buffers), 7.91, 9.81, 10.79, 11.00 (potassium carbonate buffers), 11.71, 12.23, 12.71 (KOH) for 0.05 *M* L-leucine; 5.98, 6.41, 6.73, 7.09, 7.70, 8.18 (potassium phosphate buffers), 8.69, 9.13, 9.71, 10.01, 10.30, 10.80, 11.08 (potassium carbonate buffers) for 0.01 *M* L-leucine solutions. The sum of squares of deviations for 30 solutions was 0.28, which means that the fitness of the calculated spectra to the observed is excellent.

**Titration of DL-Alanine.** Titration of DL-alanine was made at 50° using a Radiometer Model TTT1 automatic pH titrator; 50 ml of 0.02 *M* DL-alanine solution containing 0.02 *N* HCl and 0.98 *M* KCl was prepared. The solution (10 ml) was titrated with 1.0 *N* NaOH solution which was prepared from 50% aqueous solution of analytical grade NaOH and kept in a polyethylene bottle.[13] The data were treated by our own computer program to obtain the following $pK_a$ values: $pK_{1L} = 2.54 \pm 0.008$ and $pK_{2L} = 9.40 \pm 0.014$.

**Spectra.** All measurements were made with a Cary Model 15 recording spectrophotometer with a thermostated cell holder at a scanning rate of 71 mμ/min. The temperature of the solution in the cuvette was measured by use of VECO Model 41A7 thermistor (Victory Engineering Corp.) and associated galvanometric apparatus. Absorbances were read from the charts to the third decimal place and were entered on 80-column FORTRAN coding forms for transfer to punched cards at the computation center.

**Computational Programs.** Programs are written in the language FORTRAN IV for use with the IBM 360-50 computer. The automatic graphing was done by use of the IBM 1627 (Cal-Comp) digital incremental plotter. Complete programs are available upon request.

**Acknowledgments.** The authors are indebted to Mrs. M. Nagano for making most of the experimental measurements, to Dr. C. Iwata for the preparation of the 5-deoxypyridoxal, to Drs. H. Jespersen, G. Scranton, N. Hutton, and other members of the staff of the Iowa State University Computation Center, to Drs. A. Kudo, John Foss, and Jon Applequist, and Mr. Robert Johnson for advice in the writing of the manuscript.

(13) K. Nagano, H. Tsukahara, H. Kinoshita, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **11**, 797 (1963).