Simultaneous Kinetic Analysis of Multicomponent Mixtures

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Two systems of multicomponent mixtures are analyzed by simultaneous kinetic methods using a minicomputer on-line with a stopped-flow spectrometer. The data handling techniques include variable rates of data acquisition, linear least-squares regression analysis, centering the data, and reparameterization of matrices. One system observes the rate of dissociation of metal–Zincon complexes at 620 nm allowing the simultaneous analysis of Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+} at 10^{-6} to 10^{-5} M concentrations. The other system provides an analysis of 10^{-5} to 10^{-4} M epinephrine, norepinephrine, and L-Dopa mixtures by monitoring the reactions of their aminochromes with ascorbic acid at 480 nm.

In simultaneous kinetic analysis, the concentrations of several components in a mixture are determined by utilizing different rates of reaction of the individual components. Time is a variable that can be measured with high precision and this method can be an attractive alternative to the physical separation of components or to chemical procedures based on equilibrium measurements. The use of differentiating reaction rates for the analysis of multicomponent mixtures has been explored in previous work done in this laboratory (*1–15*). Simultaneous first-order reactions are most suitable for analytical applications and it is important that the reactions be well behaved, that is, of well-defined order without side reactions or other complications. In the present work, two new chemical systems are tested. Both are observed using stopped-flow spectrophotometry which is advantageous because of the speed of the analyses and the ease of data handling. One system uses an intensely colored metallochromic indicator for the simultaneous determination of traces of Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+}. In the other system, a mixture of catecholamines is analyzed. Improved data acquisition and calculation methods are used for the simultaneous kinetic analysis of these mixtures.

It has been pointed out previously (*1, 2, 4*) that multicomponent analyses can be achieved if the entire response signal (i.e., absorbance vs. time) is used. Similarly two-component analyses employing hundreds of data points (readily available with computerized systems) can give much more accurate and precise determinations than graphical methods based on the signal after all but the slowest component has reacted, or initial rate methods for the fastest component, or two-point methods (*6*). Methods which use on-line computer processing of data from a stopped-flow spectrophotometer (*3*) followed by a linear least-squares fit of the regression equation also on-line (*4*) permit the rapid determinations of mixtures (*4, 5*). However, in using previous regression analysis computations, empirical weighting factors were introduced in an attempt to account for the differences in weight of the measured data in terms of the magnitude of the signal and the larger number of points for slower reacting components. These empirical factors were helpful in performing the calculations but were not optimal factors. In the present work we consider the weighting factors in more detail and show that for most cases these factors can be eliminated and the analyses can be improved by making other changes in the data acquisition and handling procedures. (1) A variable rate of data acquisition is employed based on the reaction rate constant of each component. (2) The technique of "centering the data" (*7*) is used to reduce the absolute size of the numbers in the calculations by considering only the signal which is time dependent. This also reduces the order of the matrices used by one, eliminating constant background signals from the simultaneous equations. (3) A correlation matrix (*7*) is used in the calculations in order to obtain the best solution for all components.

Regression Equations. Previously the chemical systems used gave a common product, P, and the reactions of A, B, and C with excess reagent were monitored by the increase in the absorbance of P. With on-line computation it is no more difficult to treat a system where each reactant and each product are different and each has a measurable absorbance. Thus, with the metallochromic indicator used in the present study, the molar absorptivities (ϵ) of A, B, and C are all different. It would be possible to analyze mixtures where some reactions caused absorbance increases and others caused absorbance decreases. The generalized reactions where k_a, k_b, and k_c are pseudo-first-order rate constants are given in Equation 1.

$$A \xrightarrow{k_a} P_A \tag{1a}$$

$$B \xrightarrow{k_b} P_B \tag{1b}$$

$$C \xrightarrow{k_c} P_C \tag{1c}$$

The concentrations of products in terms of the initial concentrations of the reactants, A_0, B_0, and C_0 at any time t_j (time after mixing) is given by Equations 2 and 3.

$$P_A + P_B + P_C = A_0 a_j + B_0 b_j + C_0 c_j \tag{2}$$

$$a_j = (1 - e^{-k_a t_j}) \tag{3a}$$

$$b_j = (1 - e^{-k_b t_j}) \tag{3b}$$

$$c_j = (1 - e^{-k_c t_j}) \tag{3c}$$

The observed absorbance at any time, Y_j, is given by Equations 4 and 5,

$$Y_j = a_j Y_A + b_j Y_B + c_j Y_C + Y_X \tag{4}$$

$$Y_A = A_0(\epsilon_{P_A} - \epsilon_A)l \tag{5a}$$

$$Y_B = B_0(\epsilon_{P_B} - \epsilon_B)l \tag{5b}$$

$$Y_C = C_0(\epsilon_{P_C} - \epsilon_C)l \tag{5c}$$

where Y_X is a time-independent absorbance, l is the cell path, and ϵ is the molar absorptivity of the indicated species. The Y_A, Y_B, and Y_C values can be positive or negative, depending on the nature of the spectra of the reactants and products and the wavelengths used. The objective is to determine the correct values of A_0, B_0, and C_0 by obtaining the best fit of the observed Y_j values in the response signal and by using an optimal set of data points. The rate constants must be free of synergistic effects so that a_j, b_j, and c_j can be calculated from the independently measured rate constants and the time after mixing.

Regression Analysis. *Least Squares.* The data consist of n pairs of observations (Y_j, t_j, j = 1 to n) taken over a prescribed period of time. The differences between the observed absorbance values and the expected absorbance values are called residuals, μ_j (Equation 6).

$$\mu_j = Y_j - Y_{j,\text{calcd}} = Y_j - (a_j Y_A + b_j Y_B + c_j Y_C + Y_X) \quad (6)$$

If the errors in the measurements are random, then the residuals also will be random so that each residual will belong to a Gaussian distribution with a standard deviation, σ_j, centered about the true residual, $\bar{\mu}_j = 0$.

The probability of finding all the residuals within their respective regions of $\bar{\mu}_j$ to $\bar{\mu}_j + \bar{\sigma}_j$ is maximized by adjusting the parameters Y_A, Y_B, Y_C, and Y_X such that they minimize $\sum_{j=1}^{n}(\mu_j^2/\sigma_j^2)$. If all the σ_j values are equal then it is only necessary to minimize $\Sigma\ \mu_j$ with respect to the parameters. However, if this is not the case, then a weighting factor $w_j = (1/\sigma_j^2)$ is defined, and the goal then becomes to minimize the sum of the weighted squares of the residuals.

$$\sum_{j=1}^{n} w_j \mu_j^2 = \text{minimum} \quad (7)$$

Weighting Factor. The variance, σ_j^2, of each data point (σ_j and μ_j are in absorbance units) is calculated from the propagation of errors in absorbance and time measurements, i.e.,

$$\sigma_j^2 = \left(\frac{\partial \mu_j}{\partial Y_j}\right)^2 \sigma_Y^2 + \left(\frac{\partial \mu_j}{\partial t_j}\right)^2 \sigma_t^2 \quad (8)$$

where σ_j^2 is the variance of the jth residual, σ_Y is the standard deviation of the absorbance measurements, and σ_t is the standard deviation of the ability to predict the time of the reaction. The value of σ_Y is determined from time-independent absorbance measurements and is for all practical purposes constant over a fairly large range of absorbance values (*8*). The value of σ_t is the uncertainty of the time of the reaction due to the stopped-flow system and will appear as a random error for ensemble averaged runs. The expression for the weighting factor then becomes:

$$w_j = \frac{1}{(Y_A e^{-k_a t_j} k_a + Y_B e^{-k_b t_j} k_b + Y_C e^{-k_c t_j} k_c)^2 \sigma_t^2 + \sigma_Y^2} \quad (9)$$

The effect of w_j is to de-emphasize the data taken during the period of time in which the error of time measurement is significant. (As discussed later, the uncertainty in the time measurement is related to the reproducibility of the flow velocity.) If the experiment is designed such that σ_t and k_a are small enough (i.e. $\Sigma k_n Y_n < 1$ for this system) then the first term in the denominator becomes negligible when compared to σ_Y^2 and the weighting factor assumes a constant value.

$$w_j = \frac{1}{\sigma_Y^2} \quad (10)$$

If this is the case, it is not necessary to consider w_j in the regression analysis. However, for fast reactions such as those of Cd–Zincon, the w_j value is not constant and Equation 9 is helpful.

In previous work (*3–5*) an empirical weighting factor was used in an attempt to counteract the effects of overemphasis of data for particular components due to the data rate or the magnitude of the signal. While this approach met with some success, its justification is dubious and we recommend instead the use of variable data acquisition rates and the procedure of conditioning the matrix in solving the linear least-squares equations.

Solution of Linear Least Squares. The method of minimizing $\Sigma w_j \mu_j^2$ with respect to the adjustable parameters is straightforward and yields an exact answer when these parameters are linearly related to each other (*4*). The partial derivatives of the residual equation with respect to each parameter must be zero at the minimum and provide the normal equations which can be solved simultaneously for the least-squares best estimates of the true values of the parameters. Solving the normal equations is most convenient when they are written in matrix form. Matrix inversion is required and can lead to serious round-off errors in the calculations if the experiment is poorly designed and the matrix is not first conditioned to prevent errors due to either the matrix aproaching singularity or the terms of the matrix from being of widely differing orders (*7*).

Conditioning the Matrix. Several steps can be taken to ensure that round-off errors will have little effect. First, the model and the data can be "centered" to reduce the number of parameters to be estimated, reduce the absolute sizes of the numbers used in the calculations, and emphasize the spread and the distribution of the matrix terms about their mean rather than their absolute values (*7*).

Equation 4 can be rewritten as:

$$(Y_j - \bar{Y}) = Y_A(a_j - \bar{a}) + Y_B(b_j - \bar{b}) + Y_C(c_j - \bar{c}) \quad (11)$$

where

$$\bar{a} = \frac{\sum_{j=1}^{n} a_j}{n}; \quad \bar{b} = \frac{\sum_{j=1}^{n} b_j}{n}; \quad \bar{c} = \frac{\sum_{j=1}^{n} c_j}{n} \quad (12)$$

and

$$\bar{Y} = \frac{\sum_{j=1}^{n} Y_j}{n} = Y_A \bar{a} + Y_B \bar{b} + Y_C \bar{c} + Y_X \quad (13)$$

This is now the centered model to be used to solve for Y_A, Y_B, and Y_C. The values of Y_X can then be determined by:

$$Y_X = \bar{Y} - Y_A \bar{a} - Y_B \bar{b} - Y_C \bar{c} \quad (14)$$

A second procedure responsible for conditioning the matrices is to reparameterize the centered model of the regression equation (*7*). This assures that all the terms of the matrix to be inverted will have values that lie between −1 and +1. When the numbers all are of this order, the adverse effects of round-off errors are minimized.

Multiple Data Rates. Because the rate constants for a multicomponent system may be widely different, multiple data rates are used and data acquisition is designed so that an equitable number of data points are taken during four half lives (93% of the absorbance change) of each reaction. This adjusts the averages of $\bar{Y}$, $\bar{a}$, $\bar{b}$, and $\bar{c}$ so that they fall within a reasonable range for all three reactions. The number of data points taken at each rate is given by Equation 15 and the actual rate is given by Equation 16.

$$n_x = \frac{N}{L} \sum_{i=x}^{L} \frac{(I_x - I_{x-1})}{I_i} \quad (15)$$

$$r_x = \frac{n_x}{I_x - I_{x-1}} = \frac{N}{L} \sum_{i=x}^{L} \frac{1}{I_i} \quad (16)$$

where N = total number of data points (250 in the present work), L = total number of reacting components, x = number of the component as listed in order from largest to smallest rate constant, n_x = number of data points taken at the xth

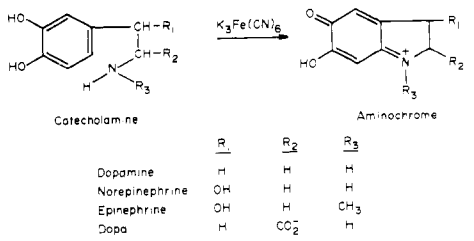

Figure 1. Structures of catecholamines and their aminochrome formation reaction

rate, I = interval of time defined by four half lives of the designated component = $(4(0.693))/k$, r_x = rate of data acquisition for the xth component.

Expressed in terms of the rate constants for three components the number of data points at each rate and the rates are:

$$n_1 = \frac{N}{3}\left(\frac{k_1 + k_2 + k_3}{k_1}\right) \qquad r_1 = \frac{N}{3}\left(\frac{k_1 + k_2 + k_3}{4(0.693)}\right)$$

$$n_2 = \frac{N}{3}\left(\frac{k_1 - k_2 - k_3}{k_1} + \frac{k_3}{k_2}\right) \qquad r_2 = \frac{N}{3}\left(\frac{k_2 + k_3}{4(0.693)}\right)$$

$$n_3 = \frac{N}{3}\left(\frac{k_2 - k_3}{k_2}\right) \qquad r_3 = \frac{N}{3}\left(\frac{k_3}{4(0.693)}\right)$$

Simultaneous Kinetic Analysis of Zn^{2+}, Cd^{2+}, and Hg^{2+}. The group 2B metals often occur together and their simultaneous determination at trace levels would be desirable in a number of different types of samples. All three metal ions react with Zincon (*9*), a metallochromic indicator, forming highly colored 1:1 complexes (ϵ of 10 000 to 20 000 M^{-1} cm^{-1} at 620 nm). The dissociation rates of these complexes are pH-dependent and can be made to be sufficiently different to permit the determination of each metal in the presence of the other two as well as in the presence of most other metal ions. The reaction system used for the simultaneous kinetic analysis is given in Equation 17,

$$\text{M(Zincon)} + \text{CyDTA} \rightarrow \text{M(CyDTA)} + \text{Zincon} \qquad (17)$$

where the CyDTA is added in excess. In earlier work from this laboratory (*2*), these metal ions were among some 30 elements which were differentiated kinetically using the rates of dissociation of their CyDTA complexes. The Zincon system shifts the kinetic observations to the visible spectral region, which is freer from interference, and permits more sensitive as well as more selective determinations. Each of the three metal–Zincon complexes has a different molar absorptivity so that the general situation is that described in Equations 1 to 5.

Simultaneous Kinetic Analysis of Epinephrine, Norepinephrine, and L-Dopa. The most important endogenous catecholamines are dopamine, epinephrine (adrenalin), and norepinephrine (noradrenalin). The structures are shown in Figure 1. Epinephrine is particularly associated with the adrenal medulla so that the plasma and urinary levels of this hormone are used to assess the functional status of the adrenal medulla. Hyperfunction, especially in patients with hypertension, is associated with pheochromocytoma, a tumor of the chromaffin tissue. About 90% of all such tumors originate in the adrenal medulla and are characterized by increased secretion of epinephrine. Norepinephrine, produced mainly at adrenergic nerve endings, is an indicator of peripheral sympathetic activity. Extra adrenal pheochromocytomas are characterized by increased norepinephrine secretion (*10*). These catecholamines are present in urine in a free form and a form conjugated primarily as ethereal sulfates which is easily hydrolyzed by heating with acid. The analysis of epinephrine and norepinephrine from blood or urine requires the separation of these components from the sample matrix typically using either alumina (*11*, *12*) or ion-exchange resins (*13*, *14*). The two catecholamines in the free form are selectively eluted from alumina with acetic acid or from cation exchange resin with a borate solution at pH 8.4.

Many techniques have been developed for differential analysis of the eluate including fluorimetric (*12*, *15*, *16*) colorimetric (*17*), gas chromatography (*18*), and liquid chromatography (*19*) procedures. The most widely used method is the formation of fluorescent trihydroxyindole species. The catecholamines are oxidized typically by $K_3Fe(CN)_6$ (see Figure 1) and strong base is added to produce the fluorescent species. A multiple wavelength analysis using two sets of excitation and emission wavelength settings allows the determination of the individual concentrations of epinephrine and norepinephrine in a mixture upon solving simultaneous equations (*12*). Methyl Dopa and possibly L-Dopa used for the treatment of Parkinson's disease could be present in the eluate and produce inaccurate results. This error could be eliminated by the simultaneous kinetic analysis of Dopa, norepinephrine, and epinephrine. While it would be advantageous to follow the formation or disappearance of the fluorescent species to maximize the sensitivity, a simultaneous kinetic analysis of such a mixture appears to be impractical because of the complex relationship between fluorescence and concentration for species with significant absorbance at the excitation wavelength using conventional spectrofluorimeters.

A two-point method of simultaneous kinetic analysis of epinephrine and dopa has been performed using the colorimetric method of observing the rates of oxidation of these catecholamines. The first-order rate constant for L-Dopa was reported as 18.8 s^{-1} and for epinephrine was 0.258 s^{-1} for the method used (*20*). These rate constants are separated by a factor of 73.

We present a method for the simultaneous kinetic analysis of norepinephrine, epinephrine, and L-Dopa which monitors the decomposition by ascorbic acid of the acid-stabilized amino chrome derivatives of the catecholamines. The respective rate constants are 0.65, 0.28, and 0.032 s^{-1}, so that this system is a worthwhile test of the recommended data handling methods.

EXPERIMENTAL

Apparatus. A stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.) interfaced (*3*) to an 8K 16-bit computer (Model 2115A Hewlett-Packard Co., Palo Alto, Calif.) was used to obtain all rate data. The digital logic of the interface activates a solenoid which causes gas pressure to force a piston to push the drive syringes forward when signalled by the experimenter. Reagents (0.2 mL of each per push) flow through a mixing chamber, an optical flow cell (2 cm), and into a stopping syringe. Just before the stopping syringe reaches its limit of travel, a microswitch is tripped and within 1 μs sends a flag to the interface. The interface then initiates an analog-to-digital conversion every 100 μs resulting in a flag being sent to the computer from the ADC at the end of each conversion. The data rate is determined by counting the appropriate number of flags before loading the value from the ADC into memory. The ADC (AN5200 Series, Analogic Company, Waltham, Mass.) is a 12-bit successive approximation type and requires about 35 μs for a complete conversion.

Other input/output devices include a Teletype, a paper tape reader, a high speed paper tape punch (to preserve the data if desired), and a storage oscilloscope (Tektronix 601) interfaced by a HP1255A DAC to give absorbance vs. time and other plots.

Reagents. Zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene), obtained from Sigma Chemical Co., was standardized spectrophotometrically using $Zn(ClO_4)_2$ solution which had been standardized by CyDTA titration. CyDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate) from LaMont Laboratories was recrystallized from acidic solutions. Solutions of $Hg(ClO_4)_2$, $Cd(ClO_4)_2$, and $Cu(ClO_4)_2$ were prepared and

standardized. Borate buffer was prepared from boric acid and NaOH.

L-Epinephrine, L-norepinephrine, and L-Dopa (L-β-3,4-dihydroxyphenylalanine) were obtained from Sigma Chemical Co. and used without further purification. Ascorbic acid was obtained from Matheson, Coleman and Bell and the potassium ferricyanide came from Baker Chemical Co.

Time after Mixing. The actual time of the reaction after mixing, t_j, is determined from two measurements.

$$t_j = t_n + \Delta t$$

The time measured from the beginning of data acquisition, t_n, has an uncertainty of 0.5 μs determined by the 1-MHz clock rate. The time, Δt, required to extrapolate the first-order plot back to obtain the true value of Y_A is constant for an individual run, but appears as a random variable for ensemble-averaged runs. The value of Δt is measured to be 3 ms with a standard deviation of 0.2 ms for a given adjustment of the stopped-flow system. The standard deviation of measuring t_j, σ_t, is 0.2 ms for ensemble-averaged runs.

The age of the solution after mixing (typically 6 ms) consists of the time required to flow from the mixer to the beginning of the flow cell (~3 ms) and the average time of solution in the flow cell during the flow (~3 ms). If the data acquisition were initiated precisely when the flow stops, then Δt would be 6 ms. In our typical operation the trigger is initiated about 3 ms before the flow actually stops so that Δt = 3 ms under the experimental conditions. The uncertainty in t_j is largely a matter of the reproducibility of the flow velocity which causes Δt to vary from run to run. Naturally the flow velocity depends on the gas pressure driving the piston and this is kept constant. However, it has been observed (*21*) that the flow velocity can be dependent upon the position of the piston and of the push syringes. Therefore for very fast reactions where the σ_t value may be important, it is best to operate the push syringes from similar positions.

Programming. The software consists of a Fortran main program with assembly language subroutines for data acquisition, plotting data on a storage oscilloscope, and controlling a high speed punch to preserve the data on paper tape (see Figure 2). The procedure to perform simultaneous kinetic analysis using this system requires that the rate constants of the individual components first be measured under identical experimental conditions as used for the analysis of mixtures. These rate constants are input to the computer via the teletype in order from largest to smallest. The computer then calculates the data rates and number of data points at each rate as described earlier for a total of 250 data points and proceeds with the data acquisition when a run is initiated by the operator.

The operator may ensemble average any number of runs (typically three) in order to improve the signal-to-noise. The data are plotted and the range of data points to be used for the analysis is input to the computer. The computer then centers the data, calculates the reparameterization factors, determines the appropriate matrices, inverts the correlation matrix, and finally calculates the least-squares best estimates of the parameter values. The residuals are calculated and plotted on the oscilloscope to allow inspection for bias in the calculated fit due to the presence of side reactions or change in reaction conditions.

The program previously reported (*4*), REDKAN, was written in Basic, an interpretive language. As the present program developed, the convenience of programming in Basic soon became too costly in execution time as well as in the use of available core memory. The efficiency of using a Fortran compiler system, however, greatly increases the capability to extract quickly up to five parameters from a large data base. This becomes increasingly important when an iterative procedure, such as the nonlinear regression program, is used.

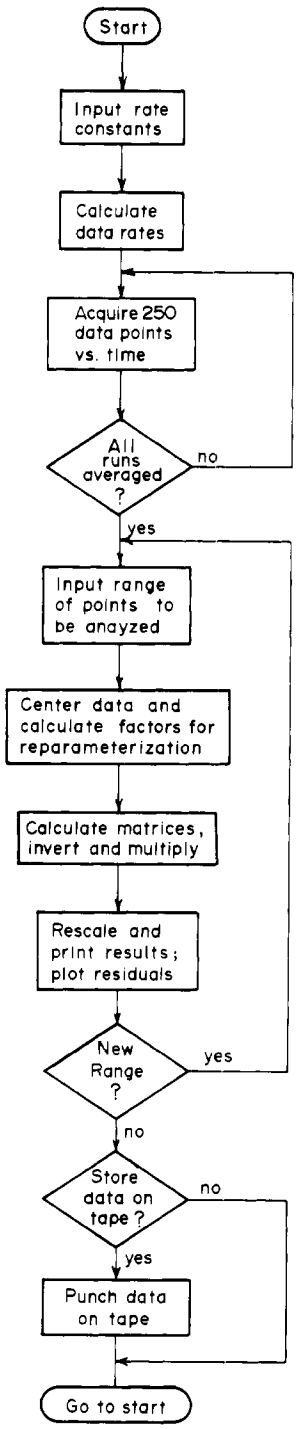

Figure 2. Flow chart of program to calculate initial concentrations of multiple components undergoing parallel first-order reactions

RESULTS

Kinetics of Metal-Zincon Dissociation Reactions. Rush and Yoe (*9*) described a colorimetric method for the determination of zinc and copper using Zincon. Although the method was very sensitive, an ion-exchange separation was required because Zincon forms blue complexes with Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Cd^{2+}, and Pb^{2+}. We have found that the reaction given in Equation 17 divides the metal ions into at least two groups of kinetic reactivity. Zincon complexes of Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} dissociate slowly, while the reactions of Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} are fast. Table I summarizes the first-order rate constants for the more reactive group at pH 8.5 in the presence of 0.02 M NaCl. The Pb^{2+} complex reacts too quickly under these conditions to be used for kinetic analysis, however, Pb^{2+} could be determined from initial absorbance jump measurements. The method proposed will simultaneously determine the concentrations of Zn, Cd and Hg. The reaction mixture is observed at 620 nm, following the disappearance of the metal–Zincon complexes, which all have different molar absorptivities (Table I). Other metal ions are much slower to react or have neg-

Table I. Molar Absorptivities of Zincon Complexes and Their First-Order Rate Constants in Their Reactions with CyDTA

Zincon-complex	ϵ_{620nm}, [a] M^{-1}	k_{obsd}, s^{-1}
Zincon	175	...
Cu^{2+}	11 400	very slow
Zn^{2+}	17 100	0.41
Hg^{2+}	20 100	6.2
Cd^{2+}	12 800	75
Pb^{2+}	7 400	~500

Conditions in the reaction cell

[Zincon] = 4×10^{-4} M
[CyDTA] = 10^{-3} M
[Borate] = 0.02 M
[NaCl] = 0.02 M
pH = 8.5

[a] Apparent ϵ for the Durrum 2-cm cell which have been found to be about 30% lower than those measured on Cary 14.

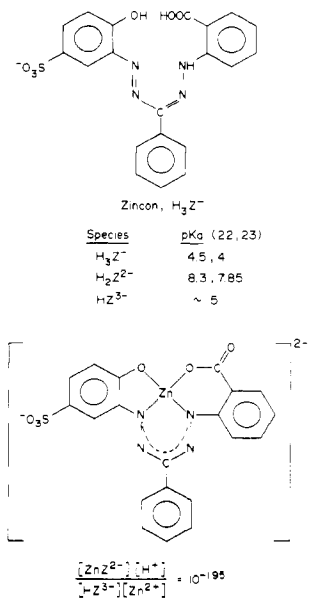

Figure 3. Structure of Zincon and ZnZ^{2-} complex with formation constant

ligible color reactions with Zincon under these conditions. Hence, another metal ion such as Cu^{2+} can be analyzed as a fourth component as we have done in the test system.

The formula for Zincon is given in Figure 3 along with its pK_a values which correspond to the successive ionization of the carboxyl hydrogen about pH 4, the phenolic hydrogen about pH 8, and the imine hydrogen in concentrated NaOH (22). Ringbom and co-workers (23) showed that three protons are displaced in forming the 1:1 zinc complex and suggest the hydroxy species $Zn(OH)(HZ)^{2-}$. Reilley and coworkers (22) found a similar proton loss but suggested a ZnZ^{2-} complex instead. The latter species is more reasonable in view of its spectral properties and the reported preparation of many solid compounds of the formazyl type where the imino hydrogen ion is replaced by metal ions (24, 25). Accordingly, this structure for ZnZ^{2-} is given in Figure 3. The conditional stability constant of this complex as pH 9 is 10^7 (22).

The rates and mechanisms of metal ion complexes with ligands such as Zincon have not been reported previously and their behavior is of interest. The rate of disappearance of ZnZ^{2-} is totally independent of the CyDTA concentration (Table II) but has a complex hydrogen ion dependence (Figure 4). The observed first-order dissociation rate constant, k_{obsd},

Table II. Effect of [CyDTA] on Dissociation Rate Constant of Zincon Complexes

10^3 M [CyDTA]	Zn k_0	Cd k_0	Hg k_1
1	0.33	73	3.3
5	0.33	79	4.4
10	0.34	81	5.4

pH 8.9, temp 25 °C. [metal] = 5×10^{-6} M; [Zincon] = 1.4×10^{-4} M; [Borate] = 0.02 M; [$NaClO_4$] = 0.1 M.

Table III. Effect of pH on Dissociation Rate Constant of Zincon Complexes

pH	Zn k_0	Cd k_0	Hg k_0
8.0	0.68	74	0.97
8.3	0.55	...	...
8.9	0.33	73	3.3
9.5	...	...	7.8
10.0	0.25	88	10.2

[metal] = 5×10^{-6} M; [Zincon] = 1.4×10^{-4} M; [Borate] = 0.02 M; [$NaClO_4$] = 0.1 M; [CyDTA] = 10^{-3} M, temp 25 °C.

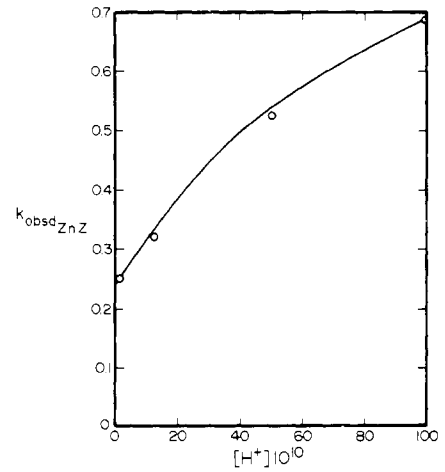

Figure 4. Dependence of dissociation rate constant for ZnZ^{2-} on the concentration of hydrogen ion

increases with $[H^+]$ but then tends to level off as $[H^+]$ increases. Since there is no evidence of a stable protonated ZnZ^{2-} species in this pH range (22), the leveling effect must be due to a kinetic limitation as shown in the mechanism in Equation 18,

$$ZnZ^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ZnZ^{2-}]^* \xrightarrow{k_2} \left.\begin{array}{l} \\ \\ \downarrow k_3[H^+] \end{array}\right\} \xrightarrow[\text{CyDTA}]{\text{rapid}} ZnCyDTA^{2-} + HZ^{3-} \qquad (18)$$

where $[ZnZ^{2-}]^*$ is a reactive intermediate. The fully or partially dissociated species formed as a result of the steps involving k_2 or $k_3[H^+]$ react rapidly with CyDTA. Using the steady-state approximation for the reactive intermediate gives the rate expression in Equation 19.

$$\frac{-d[ZnZ^{2-}]}{dt} = \frac{k_1(k_2 + k_3[H^+])[ZnZ^{2-}]}{k_{-1} + k_2 + k_3[H^+]} \qquad (19)$$

The intercept in Figure 4 corresponds to $k_1k_2/(k_{-1} + k_2)$ and equals 0.235 s^{-1}. As the $[H^+]$ increases, the first rearrangement becomes the rate-determining step (k_1 = 1.12 s^{-1}).

As seen in Table II, the reaction rates of Cd–Zincon and Hg–Zincon have a slight CyDTA dependence. However, the

Table IV. Effect of [Cl^-] on Dissociation Rate Constant of Zincon Complexes

[Cl^-]	Zn k_0	Cd k_0	Hg k_0
0	0.33	73	3.3
0.01	...	...	11.3
0.02	0.34	77	17.1
0.03	...	...	23.1
0.04	0.35	88	28.9

[metal] = 5×10^{-6} M; [Zincon] = 1.4×10^{-4} M; [Borate] = 0.02 M; [$NaClO_4$] = 0.1 M; [CyDTA] = 10^{-3} M; pH = 8.9, temp = 25 °C.

observed rates did not have a Zincon dependence and therefore these complexes must undergo some nucleophilic attack by CyDTA as well as the solvent dissociation pathway.

Table III shows that the pH effect on the Hg–Zincon reaction is opposite to that of the Zn–Zincon reaction. The spectrum of the mercury complex changes with pH and many mercury complexes are known to add hydroxide ion in this pH range. The observed first-order rate constant is plotted against OH^- concentration in Figure 5. A similar behavior is found for chloride ion as seen in Table IV and Figure 6. The suggested mechanism for both the OH^- and Cl^- acceleration (neglecting direct CyDTA attack) is given in Equation 20 where X = OH^- or Cl^-.

$$\begin{array}{l} HgZ^{2-} \xrightarrow{k_4} \\ \qquad + X^- \\ K_X \quad \downarrow \uparrow \qquad \text{Products} \\ HgZX^{3-} \xrightarrow{k_5} \end{array} \tag{20}$$

This leads to the expression for k_{obsd} given in Equation 21

$$k_{obsd} = \frac{k_4 + k_5 K_X [X^-]}{1 + K_X [X^-]} \tag{21}$$

where the rate depends on $k_{obsd}[HgZ]_{Total}$ and $[HgZ]_{Total} = [HgZ^{2-}] + [HgZX^{3-}]$. At higher concentrations of X^-, the k_{obsd} value approaches k_5. The curves in Figures 5 and 6 fit this equation where $K_X = 4.7 \times 10^4$ M for OH^- and 5.4 M^{-1} for Cl^-, and $k_5 = 12.4$ s^{-1} for OH^- and 144 s^{-1} for Cl^-.

Acceleration of the rate of dissociation of $Hg^{II}CyDTA^{2-}$ complexes by OH^- and by halide ions has been observed (*26*) with similar evidence for stable $Hg(CyDTA)X^{3-}$ complexes. In the CyDTA complexes, OH^- forms a stronger complex than Cl^- by one order of magnitude compared to four orders of magnitude difference for the Zincon complexes. On the other hand Cl^- has a much bigger kinetic effect with the Zincon complex than with the CyDTA complex.

The Cd–Zincon complex is intermediate in behavior in comparison with the Zn and Hg complexes, showing a slight effect due to Cl^- (Table IV) and some evidence for an OH^- effect by pH 10 (Table III).

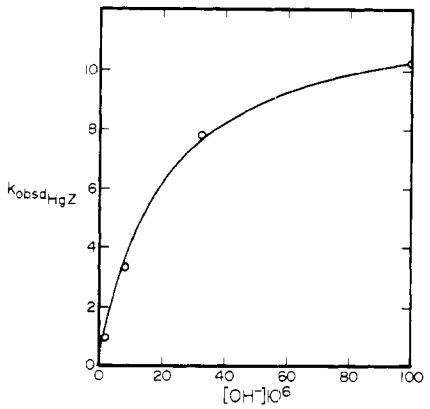

Figure 5. Dependence of dissociation rate constant for HgZ^{2-} on the concentration of hydroxide ion

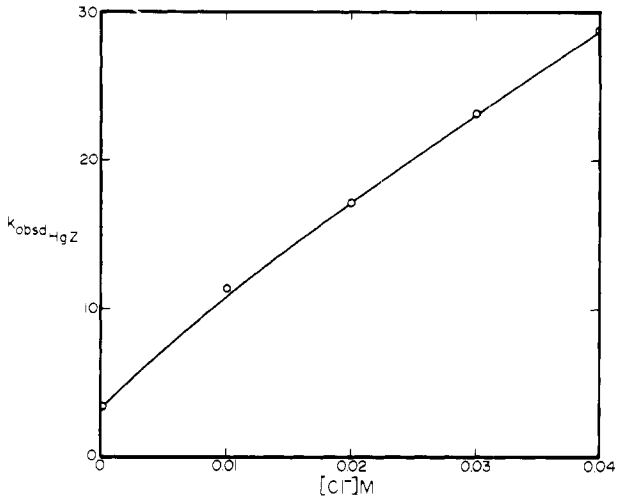

Figure 6. Dependence of dissociation rate constant for HgZ^{2-} on the concentration of chloride ion

In order to obtain predictable rate constants, the pH and halide ion concentrations need to be controlled. It is advisable to determine the rate constants experimentally in the solution composition equivalent to that of the sample or to add reagents to give a controlled composition.

Determination of Zn, Cd, Hg, and Cu Using Zincon. Solutions containing 1.4×10^{-4} M Zincon, 0.02 M borate, 0.02 M NaCl, and metal ion (Zn^{2+}, Hg^{2+}, Cd^{2+}, and Cu^{2+}) concentrations listed in Table V are reacted with solutions of 0.02 M borate, 0.02 M NaCl, and 10^{-3} M CyDTA. The observed first-order rate constants are determined from calibration runs of the single components and the ratio of rate constants for Cd:Hg:Zn are 185:15:1. The results of the simultaneous kinetic analysis are (shown graphically in Figures 7a to 7d) listed in Table V. Each of the metal ion concentrations is measured in the presence of other metal ions whose total concentration is in excess by a factor of up to eighteen. The calculated value

Table V. Results for Four-Component Mixtures Determined by Simultaneous Kinetic Analysis Using the Zincon Method

	10^6 M of Metal Ions in the Reaction Cell							
Mixture	$[Cd]_{added}$	$[Cd]_{found}$	$[Hg]_{added}$	$[Hg]_{found}$	$[Zn]_{added}$	$[Zn]_{found}$	$[Cu]_{added}$	$[Cu]_{found}$
A	10.6	10.6	5.1	5.5	1.04	1.05	1.04	0.95
B	5.30	5.72	1.02	0.80	10.4	10.4	2.08	2.22
C	1.06	0.92	10.2	10.0	5.2	5.35	3.12	3.09
D	3.18	3.26	3.06	2.96	7.28	7.22	4.16	4.14
E	7.42	7.17	3.06	3.13	3.12	3.10	5.20	5.19
F	3.18	3.11	7.14	7.11	3.12	3.01	6.24	6.24
Precision	±0.16		±0.05		±0.06		±0.04	
Accuracy	±0.16		±0.17		±0.06		±0.04	

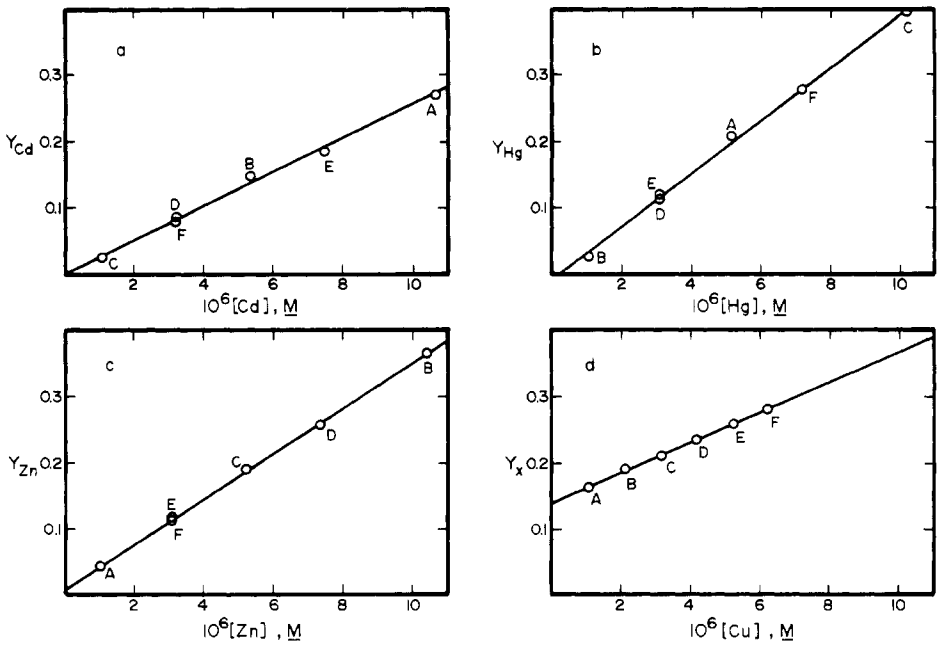

Figure 7. Beer's law plots of results of simultaneous kinetic analysis for Cd^{2+}, Hg^{2+}, Zn^{2+}, and Cu^{2+}. The letters A, B, C, D, E, and F refer to the solution compositions given in Table V

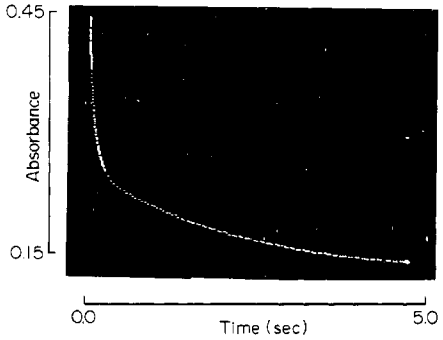

Figure 8. Absorbance vs. time plot for a mixture of Cd^{2+}, Hg^{2+}, Zn^{2+} where $[Cd^{2+}] = [Hg^{2+}] = [Zn^{2+}] = 3 \times 10^{-6}$ M

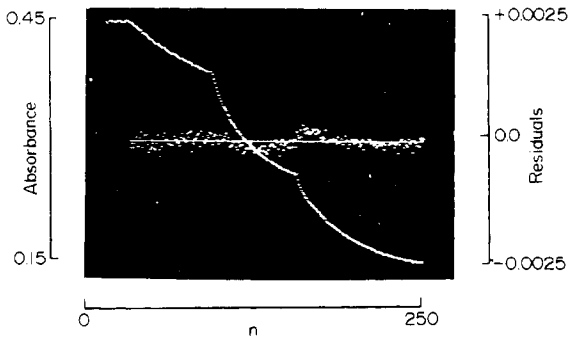

Figure 9. Absorbance vs. number of the data point, n, for same data in Figure 8. Also shown is a plot of the residuals for the simultaneous kinetic analysis

of Y_X (see Figure 7d) is the time independent absorbance due to the presence of excess Zincon and unreacted metal Zincon complexes. The intercept is determined by the absorbance of the excess Zincon and the difference between Y_X and the intercept is assumed to be the absorbance due to the Cu^{2+} Zincon complex. The analysis results give a sensitivity of at least 8 ppb for Zn, 35 ppb for Cd, and 70 ppb for Hg under conditions where Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, and Fe^{3+} do not interfere.

Plots of absorbance vs. time (Figure 8) and absorbance vs. number of the data point (Figure 9) for a representative run are shown. A plot of the residuals also is included on Figure 9 and illustrates several points. First, the standard deviation of the absorbance measurements determines the scatter in the residuals. This scatter does appear to be constant over the entire range of absorbance values measured and provides evidence that the assumption that σ_Y is constant is correct. Second, the standard deviation of the residuals about the line (which represents $\bar{\mu}_j = 0$) will be larger than σ_Y due to the bias of the residuals about the line. This is a result of attempting to fit data to a mathematical model containing terms which are imperfect. However, a small amount of bias is to be expected in this case because the rate constants used have a finite uncertainty. As long as the bias remains small and the rate constants well separated, the effect of the bias on the results is minimal. If conditions should exist such that the rate constants may vary significantly under reaction conditions as opposed to the calibration conditions, then a nonlinear regression is needed. Such a program has been developed and used successfully for a two-component system where Y_A, Y_B, Y_X, k_a, and k_b are the parameters determined.

Determination of Catecholamines. The reaction used in this analysis is the first-order reduction of the aminochromes of norepinephrine, epinephrine, and L-Dopa by ascorbic acid. Solutions of the catecholamine mixtures are reacted with at least a tenfold excess of $K_3Fe(CN)_6$ buffered at pH 7.0 by phosphate buffer. The reaction is allowed a few seconds to reach completion before the pH is adjusted to 4.75 with acetic acid. The final concentration of the individual aminochromes ranges from 10^{-5} M to 10^{-4} M ([phosphate] = 0.025 M, and $[K_3Fe(CN)_6] = 10^{-3}$ M). This solution is mixed in the stopped-flow with an equal volume of a 0.2 M ascorbic acid solution (pH 4.75) and the absorbance decrease at 480 nm is measured.

The analysis of response curve from the reactions of the aminochromes derived from norepinephrine, epinephrine, and L-Dopa is performed similarly to that described for the Zincon system.

The first-order regression analysis residual plots for the reactions of the aminochrome derivatives of norepinephrine, epinephrine, and L-Dopa with ascorbic acid show a significant amount of bias due to the presence of an induction period. The calculated rate constants of the individual norepinephrine,

Table VI. Comparison of Results Obtained by Simultaneous Kinetic Analysis of Mixtures with Those Obtained by Kinetic Analysis of Individual Components

Catecholamine		ϵ 10^3 M^{-1} cm^{-1}	Intercept	R^2
Noradrenalin	mixture	2.7 ± 0.2	−0.023 ± 0.01	0.988
	individual	3.00 ± 0.05	−0.006 ± 0.004	0.998
Adrenalin	mixture	3.6 ± 0.1	0.012 ± 0.005	0.997
	individual	3.44 ± 0.03	0.003 ± 0.002	0.9995
L-Dopa	mixture	3.17 ± 0.05	0.016 ± 0.003	0.998
	individual	2.95 ± 0.05	0.0005 ± 0.003	0.998

Table VII. Results of Simultaneous Kinetic Analysis of Mixtures of Catecholamines by Monitoring Reactions of Aminochromes with Ascorbic Acid

	10^5 M					
Mixture	$[NE]_{exp}$	$[NE]_{calcd}$	$[E]_{exp}$	$[E]_{calcd}$	$[Dopa]_{exp}$	$[Dopa]_{calcd}$
A	2.50	2.05	5.00	5.04	0.50	0.56
B	0.50	1.00	2.50	2.45	5.00	5.06
C	5.00	5.20	0.50	0.54	2.50	2.47
D	1.50	1.63	1.50	1.42	3.50	3.43
E	1.50	1.53	3.50	3.41	1.50	1.53
F	3.50	3.65	1.50	1.50	1.50	1.46
G	5.00	4.84	2.50	2.62	0.50	0.47

NE = Norepinephrine; E = Epinephrine.

epinephrine, and L-Dopa aminochrome reactions are 0.65, 0.28, and 0.032 s^{-1}, respectively, giving a ratio of 20.3:8.8:1.

The results of the simultaneous kinetic analysis of mixtures of these catecholamines are calculated from Beer's law plots. The intercepts and the molar absorptivities calculated from the slopes of these plots are compared to those obtained by the analysis of the individual components in Table VI. The residual plot of the simultaneous kinetic analysis of this mixture shows some bias due to the induction period. The effect of the bias appears to be independent of the composition of the mixtures listed in Table VII and mainly affects the slopes and intercepts of the Beer's law plots while proportionality is maintained. However, the precision and limits of concentration ratios over which the proportionality is maintained is degraded by increased bias. The actual concentrations and those calculated from the slopes and intercepts are listed in Table VII. As expected, the results for the fastest reacting specie, norepinephrine, are the most affected by the bias since the induction period is most significant during its reaction time. Another factor which degrades the analysis is that the rate constants of the reactions of aminochromes derived from norepinephrine and epinephrine are different by only a factor of 2.3. Nevertheless, the analysis gives an uncertainty of 3×10^{-6} M for norepinephrine, 7×10^{-7} M for epinephrine, and 5×10^{-7} M for L-Dopa.

Nonlinear Regression Analysis. Because no linear relationship of the parameters can be established if one or more of the rate constants are included as one of the adjustable parameters, an exact solution is not possible. Instead, an iterative procedure must be used. The error surface may be searched for the minimum by a method of steepest descent (*7*), or, as has been used in this laboratory, the least-squares best estimates of the parameters may be obtained by using the Taylor expansion series linearization. The latter technique leads to normal equations for which estimates of the errors in the parameters can be solved (*27, 28*). In this manner, new estimates are obtained and the process is repeated until the estimated errors become sufficiently small.

Programs for using this process on-line have been developed and used in this laboratory successfully. Excellent results have been attained for a two-component system as long as the rate constants remain separated by a factor of five and a good signal-to-noise ratio (S/N = 20) is maintained. Another technique developed by Marquardt (*29*) combines steepest descent and linearization techniques to solve problems which have difficulty in converging but this was not deemed necessary in the present application.

Recommendations. Our general recommendations for simultaneous kinetic analysis of multiple components are as follows.

(1) Multiple data rates are recommended to give approximately the same number of data points per four half lives of each component.

(2) Weighting factors are not recommended for small absorbance changes unless the product of the absorbance change times the rate constant for all components is greater than the ratio of σ_Y/σ_t (i.e., the desirable condition is that $k_n Y_n \ll \sigma_Y/\sigma_t$).

(3) Calibration rates should be run with pure components under exactly the same conditions of the analysis in order to obtain accurate rate constants for linear least-squares analysis.

(4) Linear least-squares regression analysis with up to five total components is possible for on-line operation using minicomputers. The technique of centering the data and the use of a correlation matrix are recommended.

(5) Tests in which the residuals are displayed against the data points are recommended in order to detect bias caused by interferences.

(6) In general the ratio of the concentrations of the components to be analyzed should not greatly exceed the ratio of their rate constants unless the data are extremely precise.

(7) The limits in regard to the total number of components which can be analyzed for any system depend upon the range of the rate constants and the total absorbance signal for all components. Thus, it should be possible to analyze for four fast-reacting components separately from four slow-reacting components using a single run, if this were desirable.

(8) Nonlinear least squares can be used for a small number of components if the rate constants of the pure components under the reaction conditions are not known accurately.

(9) It is critical that there be no synergistic effects and that the kinetics of the system be well defined.

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Simultaneous Kinetic and Spectral Analysis with a Vidicon Rapid-Scanning Stopped-Flow Spectrometer

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A rapid-scanning vidicon spectrometer system is reported with improved stopped-flow mixing, spectral characteristics, and speed of data handling. This system is evaluated in regard to determinant errors (stray light, resolution, vidicon lag time) and random errors in absorbance measurements. Weighting factors are determined from the uncertainties predicted by the propagation of errors of the measurements. The system is programmed to give (1) weighted linear least-squares regression analysis of first-order reactions at multiple wavelength, (2) repetitive spectra of reactions or transient intermediates, and (3) weighted least-squares regression analysis of two parallel first-order reactions using the response surface (the absorbance measured simultaneously as a function of time and wavelength). The system is tested by monitoring the dissociation reactions of Hg(II) and Zn(II) Zincon complexes under conditions where the rate constants and spectral changes are similar so that a three-dimensional response improves the accuracy and precision in the determination of the metal ion concentrations.

Multiple component analysis performed by the direct measurement of the physical properties of the components or their reaction products must meet several general requirements. (1) The number of measurements obtained at different values of the independent variable (i.e., wavelength, time, etc.) must be at least equal to the number of components to be determined. (However, additional data may improve the precision.) (2) The relative contributions of the components at each measurement must not be redundant (i.e., there must be at least as many nondegenerate equations as there are components). (3) The absolute and relative contribution of each component to the measurements must be large enough to achieve the sensitivity desired.

One such technique is the analysis at multiple wavelengths in which the response such as absorbance, fluorescence, or phosphorescence (*1–3*), is measured as a function of wavelength. When the responses of the individual components overlap one another, simultaneous equations must be solved. Another technique of multiple component analysis, which has been investigated extensively in this laboratory, is simultaneous kinetic analysis (*4–8*). The absorbance of a mixture of components undergoing parallel first-order reactions is measured at one wavelength as a function of time. The initial concentrations of the individual components are calculated by a least-squares fit of all the data to a linear sum of exponential response curves (*6*).

Array detectors have led to new techniques of rapid scanning spectrometry (*9–12*). Vidicon systems are particularly useful for visible and ultraviolet absorption studies. A number of computerized vidicon systems have been developed (*13, 14*) and can be used to obtain spectral and kinetics information simultaneously (*13, 15, 16*). Other types of computer-coupled rapid-scanning stopped-flow systems have been developed (*17, 18*).

In the present work, an improved vidicon rapid-scanning stopped-flow system is constructed [the basic design is that given by Milano, Pardue, Santini, Margerum, and co-workers (*13, 16*)]. It is used to measure rate constants at multiple wavelengths, to measure transient spectra, and to perform multicomponent analysis. The absorbance of reaction systems undergoing parallel first-order reactions is measured as a function of two independent variables, wavelength and time, simultaneously. A three-dimensional output results in a response surface which contains all the rate and spectral information within the boundaries of the experiment. Wilson and Miller used three-dimensional plots of luminescence vs. time vs. wavelength to give time-resolved and component-resolved phosphorescence spectra (*3*). Their data were obtained using a photomultiplier with a motor-driven wavelength