Theoretical Analysis of Comparative Studies of Complex Formation

Solubility, Spectral, and Kinetic Techniques

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The stoichiometric ratios and the equilibrium constants describing the extent of formation of complexes are the basic information required in studies of complex formation between two species. Some of this information can be obtained by measuring the apparent solubility, the absorption spectrum, or the reactivity of one species (the substrate) as a function of the concentration of the other species (the ligand). The stability constant evaluated by these methods, assuming that a single complex of 1 : 1 substrate–ligand ratio is present, can be related to the actual constants of the system. Analyses of some of the complex systems most likely to be encountered show that the three experimental approaches may not always yield the same numerical result and that comparative studies with several techniques may yield valuable information concerning the natures of the complexes. The solubility, spectral, and kinetic methods for studying complexes are, in general, subject to about the same degree of nonselectivity in their responses to multiple complexes and interactions.

THE CONCEPT of complex formation has been adopted as a simple hypothesis that can account for nonadditive behavior in the physical and chemical properties of solutions of two or more species. With this hypothesis it becomes possible to utilize quantitative measures of these properties to describe the extent of interaction between the species and to investigate the nature of the interaction product, or complex. Many definitions of "a complex" have been proposed, but for the purpose of this paper it will not be necessary to limit sharply the chemical nature of the species, and the techniques to be discussed may be applicable to the study of reactions that may not be accepted as complexation reactions. Throughout this paper complex formation is considered to be a reversible chemical reaction in which the rate of attainment of equilibrium is much greater than any rates involved in the measuring processes. The system is, therefore, considered to be at equilibrium.

The basic purpose of studies of complex formation is to provide a comprehension of the properties of complexes, including their structure and reactivity. Since the reversibility of complex formation is the fundamental aspect relating all of these processes, the general reaction may be written

$$mS + nL \rightleftharpoons S_mL_n$$

where S represents the substrate and L is the ligand. (The substrate is the compound whose apparent properties are measured.) Given adequate evidence that a complex is present in a system, the first information to be sought is its stoichiometry, *i.e.*, the values of m and n. It is probable that in many (perhaps most) systems more than one complex is formed, and the stoichiometries of all species are desired. Note also that it is entirely conceivable that two or more complexes may co-exist with the same stoichiometry but different structures (1). (A single complex species will possess a unique average molecular and electronic configuration.)

The strength or stability of a complex is specified in terms of its stability (association, formation) constant. The over-all stability constant K_{mn} for the complex formation reaction is written

$$K_{mn} = \frac{[S_mL_n]}{[S]^m[L^n]} \qquad \text{(Eq. 1)}$$

where brackets signify molar concentrations, and K_{mn} is the constant applicable to the solvent system and temperature employed. The standard state of the solute is taken to be the infinitely dilute solution in the experimental solvent. Often the concentrations of S and L are sufficiently low that they do not affect the value of the stability constant. An alternative description of the stability of S_mL_n is available in the step stability constant; the assumption is that S_mL_n is formed from $S_mL_{(n-1)}$ by reaction with one L, or from $S_{(m-1)}L_n$ by reaction with one S. Rossotti and Rossotti (2) review methods for the determination of stability constants.

Most of the molecules of pharmaceutical and biological interest are of complicated structure and contain numerous functional groupings. Such molecules will, therefore, possess multiple interaction sites for complex formation. A given system of substrate, ligand, and solvent can be described as a member of one of the following two classes.

(*a*) Only one complex species is formed. This

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complex may be a resultant of multiple interactions. The possibility is admitted that the complex contains only substrate or only ligand molecules.

(*b*) Two or more complex species are formed. The several complexes may be formed by means of different types of interaction forces or by the same interactions differently oriented.

In the studies of complexation equilibria and their relation to enzyme specificity behavior from these laboratories, solubility measurements, absorption spectroscopy, and rate measurements for the determination of complex stability have been employed. These are all well-known techniques, but few investigators have systematically applied more than one of them to a complexation system (3–5). The three methods do not always yield the same numerical result (taking into account the expected experimental uncertainty), and the authors' analysis of these differences may be of value to others.

The usual procedure is to assume that a single complex of one-to-one stoichiometry is responsible for the observed effects. If the data suggest that this simple assumption is untenable, another will of course be made in its place, but the observations are not always susceptible to such an interpretation. The problem, therefore, is to find the relationship between the apparent 1:1 stability constant (as evaluated by each of the experimental methods) and the actual parameters of the system. This analysis will be carried out for each technique as applied to the systems most likely to be encountered. A comparison of the three methods will then be given.

ONE COMPLEX PRESENT

Solubility Method.—*1:1 Complex.*—The theory and practice of the solubility method have recently been reviewed in detail (6), and only a brief outline will be given here. The experimental operation entails the addition of an equal weight (in excess of its normal solubility) of the slightly soluble substrate into each of several vials. A constant amount of solvent is added to each container, then successively increasing portions of the relatively soluble ligand are added to these vessels, which are closed and brought to solubility equilibrium at constant temperature. The solution phases are analyzed for their total concentration of S, no matter what its molecular state may be. A phase diagram is constructed by plotting, on the vertical axis, the total molar concentration of S found in the solution, S_t, against the total molar concentration of L added, L_t. Here only the formation of soluble complexes is considered; these produce a phase diagram consisting of a smooth curve with a positive slope. In general, if the solution contains but one complex, S_mL_n, the concentrations at any point on the curve can be expressed (see *Appendix* for an explanation of the symbols):

$$\begin{aligned} [S] &= S_0 \\ [S_mL_n] &= (S_t - S_0)/m \\ [L] &= L_t - n[S_mL_n] \end{aligned} \qquad \text{(Eq. 2)}$$

since the concentration of free S is maintained constant by the presence of solid substrate. Consider the case in which $m = n = 1$. Then Eq. 1 for K_{11} is combined with Eq. 2 to give

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0} + S_0 \qquad \text{(Eq. 3)}$$

showing that the plot of S_t *versus* L_t is a straight line with intercept S_0 on the vertical axis; the slope of this line is $K_{11}S_0/(1 + K_{11}S_0)$, leading to

$$K_{11} = \frac{\text{slope}}{S_0(1 - \text{slope})}$$

In general, the apparent 1:1 stability constant is evaluated from solubility measurements by means of Eq. 4; if a single 1:1 complex is present, the apparent K_{11} (symbolized K'_{11}) determined in this manner is equal[1] to the actual K_{11}.

$$K_{11}' = \frac{\text{slope}}{\text{intercept}\,(1 - \text{slope})} \qquad \text{(Eq. 4)}$$

2:1 Complex.—If, more generally, $n = 1$ but m assumes any value, Eqs. 1 and 2 give

$$S_t = \frac{mK_{m1}S_0{}^mL_t}{1 + K_{m1}S_0{}^m} + S_0 \qquad \text{(Eq. 5)}$$

The phase diagram is linear as long as the complex contains only 1 molecule of L. If the slope is greater than unity, then at least one species must be present in which m is greater than 1, for it is clearly impossible for 1 mole of L to take more than 1 mole of S into solution if the complex is of the 1:1 type. On the other hand, a slope smaller than 1 does not necessarily mean that a 1:1 complex is formed, though this assumption is usually made. More definite statements concerning the order with respect to S cannot usually be made since the presence of solid substrate is responsible for maintaining a constant activity of S in the system.

If a single 2:1 complex is present, the slope is given by Eq. 6.

$$\text{slope} = \frac{2K_{21}S_0{}^2}{1 + K_{21}S_0{}^2} \qquad \text{(Eq. 6)}$$

If this quantity is less than 1, the system will be interpreted as a probable 1:1 complex, and an apparent K'_{11} will be calculated from Eq. 4. The actual nature of this quantity is found by combining Eqs. 4 and 6,

$$K_{11}' = \frac{2K_{21}S_0}{1 - K_{21}S_0{}^2} \qquad \text{(Eq. 7)}$$

where K_{11}' is the apparent 1:1 stability constant. (If the true stoichiometry were known, it would be a simple matter to evaluate the correct constant, K_{21}, but this information will seldom be available.)

This discussion has ignored the route of formation of S_2L. This can conceivably occur in three ways: $2S + L = S_2L$; $S_2 + L = S_2L$; $SL + S = S_2L$. The first of these has been employed in the preceding discussion. The other possibilities require the presence of another complex, and can be treated with methods developed later for these more complicated systems.

1:2 Complex.—When a complex is present that is second-order in L, the solubility diagram will not be linear but will show a positive curvature (6). Such a curvature would be recognized and would prevent evaluation of an apparent K_{11}'. If, however, the system contains both 1:1 and 1:2 com-

[1] This is not exactly true, of course, for the general solvent effect of S and L on the constant has been neglected; these relatively minor effects may be responsible for small differences, but can be ignored as long as S_t and L_t remain fairly small. The "statistical" or "contact" complexes formed as a result of random distribution of the molecules have also been neglected (7, 8).

plexes, the deviation from linearity may be unnoticed and misinterpretation may result. This system will be analyzed in a later section.

Substrate Dimer.—Suppose S undergoes reaction to form the dimer S_2 with dimerization constant K_{SS}. Then the total concentration $S_t = [S] + 2[S_2]$, or $S_t = S_0 + 2K_{SS}S_0^2$. Obviously the apparent complexation constant evaluated from the phase diagram will be zero, but the intercept will give $S_0 + 2K_{SS}S_0^2$ rather than S_0. No solubility experiment will reveal this anomaly, however.

Spectral Method.—*1:1 Complex.*—If the molar absorptivities of the complex and the substrate differ at the same wavelength, it may be possible to determine the stability constant spectrophotometrically. It is assumed that Beer's law is followed by all species. Then at a concentration S_t of substrate, in the absence of ligand, the solution absorbance is

$$A_0 = a_S b S_t \qquad \text{(Eq. 8)}$$

In the presence of ligand at total concentration L_t, the absorbance of the solution containing the same total substrate concentration is

$$A_L = a_S b[S] + a_L b[L] + a_{11} b[SL]$$

which, combined with the material balance on S, gives

$$A_L = a_S b S_t + a_L b L_t + \Delta a b[SL]$$

where $\Delta a = a_{11} - a_S - a_L$. By measuring the solution absorbance against a reference containing ligand at concentration L_t, the measured absorbance becomes

$$A_L' = a_S b S_t + \Delta\, ab[SL] \qquad \text{(Eq. 9)}$$

Combining Eqs. 8 and 9 with the stability constant definition leads to

$$\Delta A/b = K_{11}\Delta a[S][L]$$

where $\Delta A = A_L' - A_0$. Utilizing the expression $[S] = S_t/(1 + K_{11}[L])$, this becomes

$$\Delta A/b = \frac{K_{11}S_t\Delta a[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 10)}$$

This equation can be put into several linear forms, one of these being Eq. 11, which is similar to the equation used by Benesi and Hildebrand (9, 10) to determine stability constants spectrophotometrically.

$$b/\Delta A = 1/K_{11}S_t\Delta a[L] + 1/S_t\Delta a \qquad \text{(Eq. 11)}$$

If [L] can be approximated by L_t, a plot of $b/\Delta A$ *versus* $1/L_t$ will be linear. The stability constant K_{11} is taken as the ratio *intercept/slope* of this plot. This is the operational definition of the spectrally measured 1:1 stability constant. Note that the types of interaction or their distribution in the complex are irrelevant, the only requirement in the application of the method being that Δa is not equal to zero.

No approximations have been introduced in the derivation of Eq. 11, but it is necessary to assume $[L] = L_t$ in its application. This assumption is equivalent to supposing that $1 + K_{11}[S] = 1$, because of the relationship $L_t = [L](1 + K_{11}[S])$. The assumed equality $[L] = L_t$ is, therefore, sensitive to the magnitude of the stability constant and to the substrate concentration. If K_{11} is quite large, it is essential to hold S_t to a small value if Eq. 11 is to be applied.

As noted above, Eq. 11 is exact, but its use requires an approximation. It is possible to introduce the approximation during the derivation (2), leading to the equation

$$bL_t/\Delta A = (S_t + L_t)/\Delta a S_t + 1/\Delta a K_{11}S_t$$

It can be shown that the application of this equation requires conditions that are similar to those adopted in the use of Eq. 11. Throughout this paper the spectral method will be discussed in terms of Eq. 11.

2:1 Complex.—In this system the equation corresponding to Eq. 9 is written

$$A_L' = a_S b S_t + \Delta ab[S_2L]$$

where $\Delta a = a_{21} - 2a_S - a_L$. The concentration of free substrate is given by

$$[S] = \frac{S_t}{1 + 2K_{21}[S][L]}$$

The resulting equation in its reciprocal form is

$$b/\Delta A = 1/K_{21}S_t\Delta a[S][L] + 2/S_t\Delta a \qquad \text{(Eq. 12)}$$

so the apparent spectral constant is

$$K_{11}' = 2K_{21}[S] \qquad \text{(Eq. 13)}$$

Note, however, that the plot should not theoretically be linear, since the slope is a function of [S]. This complication will be treated in more detail in later sections dealing with multiple complexes.

Substrate Dimer.—If S dimerizes with a change in spectrum, the quantity ΔA will be independent of L_t. The apparent stability constant will be zero. Beer's law will not be followed by the substrate if the dimer's molar absorptivity is not twice that of the monomer.

Kinetic Method.—*1:1 Complex.*—The kinetic method, as it has been most frequently applied, utilizes a reduction in rate of a reaction of S when L is present to obtain information about the nature of the complex; the basic assumption is that the decreased reactivity is the result of complexation, the complexed S being less reactive than free S. The kinetic scheme can be represented

$$S + L \overset{K_{11}}{\rightleftharpoons} SL$$

$$S + R \overset{k_S}{\rightarrow} \text{products}$$

$$SL + R \overset{k_{11}}{\rightarrow} \text{products}$$

If, as is usually the case, a reagent R is involved in the reaction, k_S is the second-order rate constant (often determined under pseudo first-order conditions with reagent in excess). It is assumed that R does not form complexes with S or L. The theoretical rate equation is

$$v = k_S[S][R] + k_{11}[SL][R] \qquad \text{(Eq. 14)}$$

and the experimental rate equation is

$$v = k_{obs.}S_t \qquad \text{(Eq. 15)}$$

where $k_{obs.}$ is the pseudo first-order rate constant. Setting Eqs. 14 and 15 equal and dividing through by [R] and S_t,

$$k_S' = k_S f_S + k_{11} f_{11} \qquad \text{(Eq. 16)}$$

where k_S' is the apparent second-order rate constant, f_S is the fraction of S in the uncomplexed form, and f_{11} is the fraction present as SL. The stability constant K_{11} is combined with the definitions of these fractions, giving Eq. 17.

$$f_S = \frac{1}{1 + K_{11}[L]} \qquad f_{11} = \frac{K_{11}[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 17)}$$

In the special case that $k_{11} = 0$, Eq. 16 can be expressed in the forms Eqs. 18 and 19.

$$k_S' = k_S f_S \qquad \text{(Eq. 18)}$$

$$k'_S/k'_S = K_{11}[L] + 1 \qquad \text{(Eq. 19)}$$

According to Eq. 18, a plot of k_S' *versus* f_S is linear, passing through the origin, with slope k_S. Prior knowledge of K_{11} is required to calculate f_S. Equation 19, however, can be plotted without this knowledge if the equality $[L] = L_t$ may be made. Then the slope of the plot of k_S/k_S' *versus* L_t gives K_{11}.

If $k_{11} \neq 0$, the general Eq. 16 must be used. Since $f_S + f_{11} = 1$, this can be written

$$k_S - k_S' = f_{11}(k_S - k_{11}) \qquad \text{(Eq. 20)}$$

Introducing the definitions $r_{11} = k_{11}/k_S$ and $q_{11} = 1 - r_{11}$ permits Eq. 20 to be transformed to Eq. 21.

$$k_S - k_S' = q_{11} k_S f_{11} \qquad \text{(Eq. 21)}$$

or

$$k_S - k_S' = \frac{q_{11} k_S K_{11}[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 22)}$$

Equation 22 can be placed in the following three forms amenable to linear graphing:

$$\frac{1}{k_S - k_S'} = \frac{1}{q_{11} k_S K_{11}[L]} + \frac{1}{q_{11} k_S} \qquad \text{(Eq. 23)}$$

$$\frac{[L]}{k_S - k_S'} = \frac{[L]}{q_{11} k_S} + \frac{1}{q_{11} k_S K_{11}} \qquad \text{(Eq. 24)}$$

$$\frac{k_S - k_S'}{[L]} = -K_{11}(k_S - k_S') + q_{11} k_S K_{11} \qquad \text{(Eq. 25)}$$

Throughout this paper the kinetic method will be treated in terms of Eq. 23, which predicts that a plot of $1/(k_S - k_S')$, or of $k_S/(k_S - k_S')$, *versus* $1/[L]$ should be linear. The kinetically determined 1:1 stability constant is then defined as the ratio *intercept/slope* of this plot. From the intercept value the quantity q_{11}, and ultimately k_{11}, can be evaluated.

Equation 19 has frequently been employed for the estimation of stability constants from rate measurements. This procedure is not recommended, however, for the reason made evident in Fig. 1. In this figure Eq. 19 is plotted for three hypothetical systems having q_{11} values of 0.5, 0.9, and 1.0; in each instance the true $K_{11} = 25.0\ M^{-1}$. Only the topmost line should be straight, since Eq. 19 is valid only when $q_{11} = 1.0$ and in fact the other lines do exhibit a slight negative curvature. But if these were experimental points based on ordinary rate data, rather than calculated theoretical points, it is probable that these curves would be interpreted as straight lines.[2] The slopes of these lines, which are

[2] As K_{11} is made larger, the curvature in these plots becomes more evident.

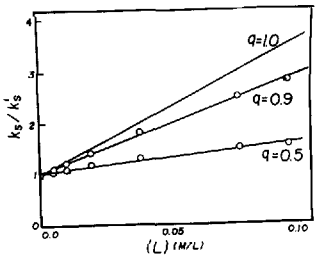

Fig. 1.—Plots of Eq. 19 for systems containing a single 1:1 complex with stability constant $K_{11} = 25.0\ M^{-1}$ and the q values shown.

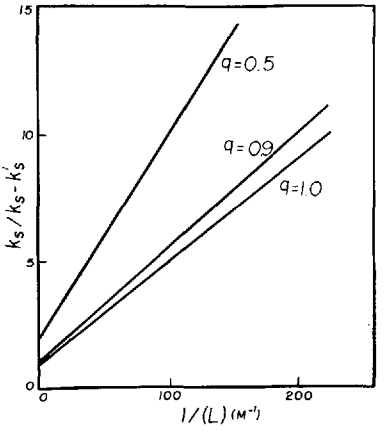

Fig. 2.—Plots of the data shown in Fig. 1 according to Eq. 23.

the apparent 1:1 stability constants according to Eq. 19, are 25 (for $q_{11} = 1.0$), 18 (for $q_{11} = 0.9$), and 5.4 (for $q_{11} = 0.5$). It is suggested that Eqs. 23, 24, or 25 be used in analyzing kinetic data. The same data plotted in Fig. 1 have been replotted in Fig. 2 according to Eq. 23; the apparent 1:1 stability constant is 25 M^{-1} in each case.

2:1 Complex.—In dealing with this system it becomes necessary to take into account various possible fates of the complex S_2L. It may undergo reaction with R to give products from one S molecule, releasing the other unreacted, or both S molecules may react, or 2 molecules of R may be required, etc. The simplest assumption will be adopted, that $k_{21} = 0$, recognizing that this places a limit on the applicability of the result. Then the basic equation, corresponding to Eq. 16, is

$$k_S' = k_S f_S \qquad \text{(Eq. 26)}$$

The fraction $f_S = [S]/S_t$, while $S_t = [S] + 2[S_2L]$. Combining these equations with the definition of K_{21} gives

$$f_S = 1/(1 + 2K_{21}[S][L])$$

which, with Eq. 26, leads to

$$k_S' = k_S/(1 + 2K_{21}[S][L]) \qquad \text{(Eq. 27)}$$

Equation 27 shows that k_S', at a given ligand concentration, is a function of substrate concentration; in other words, the apparent rate constant will vary with time as the reaction proceeds. If measurements

are made for only a small portion of the total reaction time, it is quite possible (taking into account ordinary experimental uncertainties) to overlook the variability of k_S' and to interpret the system as belonging to the 1:1 class. Equation 27 can be converted to the usual plotting form:

$$1/(k_S - k_S') = 1/2k_S K_{21}[S][L] + 1/k_S \quad \text{(Eq. 28)}$$

The kinetically evaluated K_{11}' is equal to the ratio *intercept/slope*, or

$$K_{11}' = 2K_{21}[S] \quad \text{(Eq. 29)}$$

Substrate Dimer.—If S dimerizes, and the dimeric form is essentially unreactive, the apparent constant k_S' will be related to the substrate concentration by the equation $k_S' = k_S(1 + 2K_{SS}[S])$. The apparent constant will, therefore, vary with time during a reaction. Since k_S' is not dependent upon the ligand concentration, however, the apparent 1:1 stability constant will be zero.

TWO COMPLEXES PRESENT

Solubility Method.—*Two 1:1 Complexes.*—It is possible that two complexes of 1:1 stoichiometry but different structures may co-exist. If one distinguishes between these by representing them as SL and LS, the solubility conservation equations may be written

$$S_0 = [S]$$

$$S_t = [S] + [SL] + [LS]$$

$$L_t = [L] + [SL] + [LS]$$

These are combined with the stability constants to give

$$S_t = \frac{(K_{SL} + K_{LS})S_0L_t}{1 + (K_{SL} + L_{LS})S_0} + S_0 \quad \text{(Eq. 30)}$$

which has the same form as Eq. 3 for a single 1:1 complex. Applying Eq. 4 shows that

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 31)}$$

Thus the apparent 1:1 stability constant evaluated by solubility measurements gives the sum of the individual constants. This can be generalized to any number of 1:1 complexes. Note that the slope of the phase diagram cannot exceed unity as long as only 1:1 complexes are present.

1:1 and 2:1 Complexes.—The step stability constants are defined as

$$K_{11} = [SL]/[S][L]$$

$$K_{(21)} = [S_2L]/[S][SL]$$

The development follows the lines already indicated. The equation of the phase diagram is

$$S_t = \left[\frac{K_{11}S_0 + 2K_{11}K_{(21)}S_0^2}{1 + K_{11}S_0 + K_{11}K_{(21)}S_0^2}\right] L_t + S_0 \quad \text{(Eq. 32)}$$

Combining the slope of this plot with Eq. 4:

$$K_{11}' = \frac{K_{11} + 2K_{11}K_{(21)}S_0}{1 - K_{11}K_{(21)}S_0^2} \quad \text{(Eq. 33)}$$

That a mathematically equivalent expression would be obtained if the over-all stability constant had been employed can be seen from the equality $K_{21} = K_{11}K_{(21)}$.

1:1 and 1:2 Complexes.—Combining the step constants K_{11} and $K_{(12)}$ with the material balance equations gives

$$S_t = \left[\frac{K_{11}S_0 + K_{11}K_{(12)}S_0[L]}{1 + K_{11}S_0 + 2K_{11}K_{(12)}S_0[L]}\right] L_t + S_0 \quad \text{(Eq. 34)}$$

which, with Eq. 4, leads to

$$K_{11}' = \frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_0[L]} \quad \text{(Eq. 35)}$$

According to Eq. 34 the phase diagram should show a positive curvature; but if the 1:1 complexing is much more extensive than the 1:2 type, this nonlinearity may not be noticed. Methods are available to analyze this system, when it is recognized, to obtain the individual stability constants (6).

1:1 Complex and Ligand Dimer.—The equation of the phase diagram is easily developed as before:

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0 + 2K_{LL}[L]} + S_0 \quad \text{(Eq. 36)}$$

where $K_{LL} = [L_2]/[L]^2$. The phase diagram will exhibit a negative curvature, but if the curve is mistaken for a straight line the apparent 1:1 stability constant that will be evaluated is given by Eq. 37.

$$K_{11}' = \frac{K_{11}}{1 + 2K_{LL}[L]} \quad \text{(Eq. 37)}$$

1:1 Complex and Substrate Dimer.—In this case the equation of the phase solubility diagram is

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0} + S_0 + 2K_{SS}S_0^2$$

The slope of the straight line is the same as that which would be observed in the absence of dimer formation, but the intercept is different. The apparent constant is

$$K_{11}' = \frac{K_{11}}{1 + 2K_{SS}S_0} \quad \text{(Eq. 38)}$$

Spectral Method.—*Two 1:1 Complexes.*—If the two complexes SL and LS are formed and at least one of them possesses a molar absorptivity different from free S, a spectral change will be observed. The analysis follows that given for a single 1:1 complex. The concentration of free substrate is related to the other system variables by Eq. 39.

$$[S] = \frac{S_t}{1 + (K_{SL} + K_{LS})[L]} \quad \text{(Eq. 39)}$$

The reciprocal form of the equation for this system is

$$b/\Delta A = \frac{1}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})[L]} + \frac{K_{SL} + K_{LS}}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})} \quad \text{(Eq. 40)}$$

(where $\Delta a_{SL} = a_{SL} - a_S - a_L$ and $\Delta a_{LS} = a_{LS} - a_S - a_L$), showing that the apparent 1:1 stability constant is given by

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 41)}$$

This result has been pointed out by several authors (1). Even if one of the complexes has an absorption spectrum identical with that of the free substrate,

K_{11}' will be given by Eq. 41. This may be intuitively pictured as the result of a depletion of free S by formation of this second complex, even though it is not spectrally distinctive.

1:1 and 2:1 Complexes.—This system should not, theoretically, yield a linear reciprocal plot; yet, as Johnson and Bowen have shown, the experimental plots may well appear to be linear (11). The analysis may be conducted as in earlier examples, leading to

$$\Delta A/b = K_{11}[S][L](\Delta a + \Delta a' K_{(21)}[S])$$

where $\Delta a = a_{11} - a_S - a_L$ and $\Delta a' = a_{21} - 2a_S - a_L$. For the present purpose the substrate concentration is written as

$$[S] = \frac{S_t}{1 + K_{11}[L](1 + 2K_{(21)}[S])}$$

These equations are combined to give

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a + \Delta a' K_{(21)}[S])[L]} + \frac{1 + 2K_{(21)}[S]}{S_t(\Delta a + \Delta a' K_{(21)}[S])} \quad \text{(Eq. 42)}$$

from which it is seen that the apparent spectrally measured constant is

$$K_{11}' = K_{11} + 2K_{11}K_{(21)}[S] \quad \text{(Eq. 43)}$$

This conclusion is not always valid, however; a fuller discussion is given under the next system.

1:1 and 1:2 Complexes.—Again it is evident that the plot should be nonlinear, but Johnson and Bowen (11) have found that the curvature may be overlooked. The development of the appropriate equation is similar to that in the preceding example; the equation is

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])[L]} + \frac{1 + K_{(12)}[L]}{S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])} \quad \text{(Eq. 44)}$$

where $\Delta a_{11} = a_{11} - a_S - a_L$ and $\Delta a_{12} = a_{12} - a_S - a_L$. The apparent stability constant is

$$K_{11}' = K_{11} + K_{11}K_{(12)}[L] \quad \text{(Eq. 45)}$$

That Eq. 44 is not the equation of a straight line must be kept in mind, however, and it may be expected that the range of ligand concentration over which the system is studied may affect the results. Suppose the ligand concentration is made very large, so that $\Delta a_{12}K_{(12)}[L] \gg \Delta a_{11}$; then Eq. 44 becomes

$$b/\Delta A = 1/\Delta a_{12}K_{11}K_{(12)}[L]^2 + 1/\Delta a_{12}S_t$$

and the apparent constant is

$$K_{11}' = K_{11}K_{(12)}[L] \quad \text{(Eq. 46)}$$

The necessary condition for Eq. 46 to be approached is a function not only of [L], but also of the quantities Δa_{11}, Δa_{12}, and $K_{(12)}$. This conclusion agrees with the calculations of Johnson and Bowen (11), who designed hypothetical systems to demonstrate these effects. (These remarks apply also to the system described by Eq. 42, which is, however, not as sensitive to these effects because [S] is usually much smaller than [L].) Thus, the apparent stability constant may vary with the wavelength at which the absorbance measurements are made.

1:1 Complex and Ligand Dimer.—This situation is made rather complicated because the assumption that $L_t = [L]$, made hitherto in the spectral analysis, is not valid; a very appreciable fraction of the uncomplexed ligand may exist as the dimer. This system does not appear to be amenable to a useful treatment according to the manner of the earlier examples.

1:1 Complex and Substrate Dimer.—This system does not lead to a useful analysis. The extent of dimerization will depend upon the ligand concentration (unlike the case, discussed earlier, where S_2 was the only complex present) because the free substrate concentration is a function of ligand concentration.

Kinetic Method.—*Two 1:1 Complexes.*—Proceeding as for a single complex, this basic equation is obtained

$$k_S' = k_S f_S + k_{SL} f_{SL} + k_{LS} f_{LS} \quad \text{(Eq. 47)}$$

which, since $f_S + f_{SL} + f_{LS} = 1$, leads to

$$k_S - k_S' = q_{SL}k_S f_{SL} + q_{LS}k_S f_{LS} \quad \text{(Eq. 48)}$$

where the q's are defined as before. Expressions for the fractional compositions are found by combination of the material balance and stability constant equations,

$$f_{SL} = \frac{K_{SL}[L]}{1 + K[L]} \qquad f_{LS} = \frac{K_{LS}[L]}{1 + K[L]}$$

where $K = K_{SL} + K_{LS}$. Substituting these into Eq. 48 and rearranging gives the linear form

$$\frac{1}{k_S - k_S'} = \frac{1}{(q_{SL}K_{SL} + q_{LS}K_{LS})[L]} + \frac{K_{SL} + K_{LS}}{(q_{SL}K_{SL} + q_{LS}K_{LS})} \quad \text{(Eq. 49)}$$

showing that K_{11}' is given by Eq. 50.

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 50)}$$

This result will be obtained even if one of the complexes has a reactivity equal to that of the free substrate (*i.e.*, if one of the q's equals zero).

1:1 and 2:1 Complexes.—Suppose that the S_2L complex is unreactive. Then the basic equation of the system is

$$k_S' = k_S f_S + k_{11} f_{11}$$

But the fractions f_S and f_{11} are functions of [S], so k_S' will vary during the course of the reaction, as pointed out in connection with Eq. 27. If this variability should not be evident, the usual kinetic treatment will be made. The above equation is transformed into

$$k_S - k_S' = k_S(q_{11}f_{11} + f_{21})$$

where $f_{11} = [SL]/S_t$ and $f_{21} = 2[S_2L]/S_t$. This leads finally to Eq. 51.

$$\frac{1}{k_S - k_S'} = \frac{1}{k_S K_{11}[L](q_{11} + 2K_{(21)}[S])} + \frac{1 + 2K_{(21)}[S]}{(q_{11} + 2K_{(21)}[S])k_S} \quad \text{(Eq. 51)}$$

This is not the equation of a straight line, but, if

k_S' appears to be constant during a reaction (perhaps because only a few per cent of total substrate is allowed to react during the observation period), then Eq. 51 will be essentially linear. The apparent stability constant will be

$$K_{11}' = K_{11} + 2K_{11}K_{(21)}[S] \qquad \text{(Eq. 52)}$$

If, however, $2K_{(21)}[S] \gg 1$, the apparent constant will be $K_{11}' = 2K_{11}K_{(21)}[S]$.

1:1 and 1:2 Complexes.—The basic equation is

$$k_S' = k_S f_S + k_{11} f_{11} + k_{12} f_{12} \qquad \text{(Eq. 53)}$$

which can be transformed to

$$k_S - k_S' = q_{11} k_S f_{11} + q_{12} k_S f_{12}$$

By means of the stability constant definitions and the material balance on S_t, this is converted to

$$\frac{1}{k_S - k_S'} = \frac{1}{k_S K_{11}[L](q_{11} + q_{12}K_{(12)}[L])} + \frac{1 + K_{(12)}[L]}{(q_{11} + q_{12}K_{(12)}[L])k_S} \qquad \text{(Eq. 54)}$$

This is not the equation of a straight line, but under many circumstances it will probably yield an essentially linear plot. Equation 54 has the same form as Eq. 44 for the spectral treatment of this system, and the earlier comments apply. The apparent stability constant can range from

$$K_{11}' = K_{11} + K_{11}K_{(12)}[L] \qquad \text{(Eq. 55)}$$

to

$$K_{11}' = K_{11}K_{(12)}[L] \qquad \text{(Eq. 56)}$$

depending on the relative magnitudes of q_{11} and $q_{12}K_{(12)}[L]$; thus, the value of K_{11}' may be dependent upon the quantities q_{11} and q_{12}.

1:1 Complex and Ligand Dimer.—As in the spectral method, this system does not give a simple analytical solution. If such a system is detected, perhaps the best way to treat it would be to determine by an independent method the ligand dimerization constant, then to calculate [L] as a function of L_t, and finally to treat the system as containing the single 1:1 complex (thus using Eq. 23), with the calculated monomer concentration taking the place of total concentration in constructing the graph. This procedure uses the approximation $L_t = [L] + 2[L_2]$; *i.e.*, consumption of ligand by formation of SL is ignored.

1:1 Complex and Substrate Dimer.—The experimental rate constant is a function of [S] and, therefore, varies during the reaction. This system cannot be conveniently analyzed.

DISCUSSION

Criteria for System Classification.—The operational definitions of the apparent 1:1 stability constants may be summarized as follows:

Solubility.—Plot S_t *versus* L_t; then

$$K_{11}' = \frac{\text{slope}}{\text{intercept } (1 - \text{slope})}$$

Spectral.—Plot $b/\Delta A$ *versus* $1/L_t$; then

$$K'_{11} = \textit{intercept/slope}$$

Kinetic.—Plot $1/(k_S - k_S')$ *versus* $1/L_t$; then

$$K_{11}' = \textit{intercept/slope}$$

The results of the preceding analyses, giving K_{11}' in terms of stability constants and concentrations, are gathered in Table I. The earlier discussion should be consulted for details concerning assumptions, approximations, and limits of applicability of these relationships. With their aid, it would be appropriate to consider how the comparative study of complexation systems with several techniques may yield information inaccessible with a single probe.

The usual order of investigation of a complex system will be (*a*) the determination of the stoichiometrics of all complexes present in significant concentrations or proportions, (*b*) the evaluation of stability constants for these complexes, (*c*) ultimately the determination of the structure and chemical and physical properties of each complex. Several criteria can be suggested to help in establishing stoichiometries and stability constants.

TABLE I.—THEORETICAL EQUIVALENTS OF APPARENT STABILITY CONSTANTS DETERMINED ASSUMING 1:1 COMPLEXATION

Complexes Present	K_{11}' as Found from— Solubility	Spectra	Kinetics
None	0	0	0
L_2	0	0	0
S_2	0	0	0
SL	K_{11}	K_{11}	K_{11}
S_2L	$\frac{2K_{21}S_0}{1 - K_{21}S_0^2}$	$2K_{21}[S]$	$2K_{21}[S]$
SL + LS	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$
$SL + S_2L$	$\frac{K_{11} + 2K_{11}K_{(21)}S_0}{1 - K_{11}K_{(21)}S_0^2}$	[a]	[a]
$SL + SL_2$	$\frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_0[L]}$	[a]	[a]
$SL + L_2$	$\frac{K_{11}}{1 + 2K_{LL}[L]}$	[b]	[b]
$SL + S_2$	$\frac{K_{11}}{1 + 2K_{SS}S_0}$	[b]	[b]

[a] Variable; see text for discussion. [b] See text.

Relative Values of K_{11}' by the Solubility, Spectral, and Kinetic Techniques.—Table I shows the rationale for this criterion. If a finite value of K_{11}' is obtained (concerning this point see the later discussion), its relative value by the three methods may allow a partial assignment of stoichiometric types. Thus, if all three methods yield the same numerical value, the system probably contains only 1:1 complexes. The possibility exists, however, that identical values can be observed with two methods by a coincidental combination of constant and concentrations. This can easily be detected as pointed out below.

Dependence of K_{11}' on Initial Total Substrate Concentration by the Spectral and Kinetic Techniques.—When a complex S_mL_n is present for which m is greater than 1, K_{11}' by the spectral and kinetic methods will be a function of substrate concentration. K_{11}' should be determined with at least two appreciably different initial substrate concentrations. A significant dependence of K_{11}' on substrate concentration means that at least one complex is present with m larger than 1. The functional form of this dependence may yield further information. Because of this dependence, the substrate concentration should be specified when complex stability constants are reported.

Dependence of K_{11}' on Ligand Concentration by the Solubility Technique.—In each of the three techniques the ligand concentration is the independent variable. As noted earlier, linear spectral plots may be observed even though a curve is theoretically to be expected, and similar results will apply in the kinetic method. The solubility method offers the best chance to detect a dependence of K_{11}' on ligand concentration. If a positive curvature is noted in the phase diagram at least one complex is present of the form SL_n, where n is greater than 1. Negative curvature may indicate dimerization (or higher aggregate formation) of the ligand, as in the system $SL + L_2$. A linear phase diagram does not prove that there are no complexes of these types, for certain combinations, as, for example, the system $SL + SL_2 + L_2$, may give rise to an essentially linear curve over wide ranges of ligand concentration (12).

Dependence of k_S' on Time.—When a complex is present with two or more S molecules per complex molecule, the apparent rate constant should vary with time. In order to detect this variation it may be necessary to follow the reaction for at least two half-lives. If variability of k_S' is not observed the conclusion cannot be positive that all complexes contain only one S molecule, because of the assumptions made concerning the fate of the higher order complex, but this is a reasonable tentative inference.

Dependence of K_{11}' on Wavelength in the Spectral Technique.—This criterion has been emphasized by Johnson and Bowen (11). If K_{11}' varies with the wavelength, at least one higher order complex is present. The theoretical reason for this dependence was pointed out in connection with Eq. 46.

Independent Evidence Relating to Stoichiometry and Stability.—Some of these additional sources are: estimate of stoichiometry from isolable complexes or from the solubility phase diagram (6), Beer's law behavior of pure substrate and ligand, liquid–liquid partition studies of substrate and ligand to detect and measure the extent of self-aggregation processes, and spectral studies leading to stoichiometric ratios (*e.g.*, the method of continuous variations).

These criteria will obviously not be capable of defining the nature of all complexation systems, but they should help considerably in this problem. The possibility that systems may be encountered that are more complicated than those in Table I is very real and must be kept in mind.

It is most important to realize that when the spectral K_{11}' is smaller than either the solubility or the kinetic constant this does not constitute evidence that only the charge-transfer portion of the complex interactions is being measured. If only 1:1 complexes are present, the three methods will yield the same apparent stability constant no matter what the distribution of forces responsible for maintaining the complexes. As long as one of these complexes possesses a changed absorption spectrum this will be true, even if the other complexes cause no spectral change. The same kind of argument applies to the kinetically determined K_{11}', if only 1:1 complexes exist, and at least one of these has an altered reactivity, the apparent K_{11}' will be equal to the sum of all the true 1:1 constants. The general statement may be made that if reliable K_{11}' values for a system differ when determined by the three methods, some complexes are present other than 1:1 combinations of substrate and ligand.

The reliability of stability constants evaluated spectrophotometrically as evidence for the existence of complexes has been explored by Person (13), who suggests that as a practical guide a 1:1 stability constant must be equal to or greater than $0.1/L_{max.}'$ where $L_{max.}'$ is the highest ligand concentration used in the study, in order for the constant to be considered significantly different from zero. Suppose, for example, that the upper limit of ligand concentration in a spectral study is 0.2 M; then the borderline value of K_{11}' is 0.5 M^{-1}. A value smaller than this cannot be taken as evidence for complexing. Similar guides could be formulated for other techniques. Throughout this paper the authors have supposed that nonzero values of stability constants can be demonstrated.

Capabilities of the Solubility, Spectral, and Kinetic Methods.—The solubility method is considered by many to possess the disadvantage of nonselectivity in that it measures the results of all types of interactions. But the foregoing analysis shows that the spectral and kinetic methods are also subject to this type of nonselectivity, and, in the mathematical terms of the analysis as represented in Table I, it may be held that the solubility method is actually more selective than the other techniques. The solubility method possesses two real drawbacks: it is primarily limited to slightly soluble solid substrates, and the substrate concentration cannot be varied. In those systems where the ligand is not too soluble the second disadvantage may be eliminated by reversing the system, treating S as L and vice versa. It is of course not possible to extrapolate a solublity K_{11}' to zero concentration of substrate.

Solubility studies are carried out at constant [S], and spectral studies are at constant S_t. Part of the difficulty in analyzing spectral data follows from this difference, but the capability of varying S_t when desired is an advantage of the spectral method. (It is possible to perform some spectral complexation studies at solubility equilibrium, thus setting [S]

$= S_0$ and letting S_t vary throughout the run; this may simplify some analyses.) The great disadvantage of the spectrophotometric method is of course that a spectral change must occur upon complexation, but when a change is observed the method is very convenient, especially since it provides wavelength as an additional variable. In a general sense, the spectral technique is neither more nor less selective, when applicable, than are other methods. The mathematics developed for spectral studies can be applied to any other physical property that is directly proportional to a species concentration; examples are refractive index (14), optical rotation (15), and fluorescence intensity.

The kinetic method is carried out with neither [S] nor S_t held constant (though if initial rates were measured S_t could be considered the constant factor). Mathematically it is similar to the spectral method; but it possesses the advantage that it is applicable even if no spectral change occurs, and the disadvantage that it does not include a convenient variable corresponding to wavelength. (The parallel to wavelength is complex reactivity, but this cannot easily be altered without changing the system.) Throughout this paper the inhibition of rates by complex formation has been taken as the basis for the analysis, but the complex may in some systems exhibit an enhanced reactivity, and this phenomenon also can serve for study of the complex equilibrium (16, 17). The outstanding potential advantage of the kinetic technique is its capability for providing information about the reactivity, and thus the structure, of the complex. This capability has not yet been exploited, although some attempts have been made to utilize it (5), and further studies in the chemistry of organic complexes may find its application valuable.

Conclusions.—The application of more than one experimental technique is advisable in the study of complexation systems. By comparing the apparent stability constants evaluated by the several methods on the basis of an assumed 1:1 stoichiometry between substrate and ligand, it may be possible to establish, in part, the stoichiometries of the complexes present. If the solubility, spectral, and kinetic techniques yield essentially identical values for the apparent 1:1 stability constant (and if certain other criteria suggested in this paper are satisfied), it may be concluded that only 1:1 complexes between substrate and ligand are present. If the three techniques do not give the same value, the manner in which they differ and their dependence on variables of the system may permit a further classification. On the basis of the mathematical analysis it is concluded that the solubility, spectral, and kinetic methods for studying complex formation are about equally nonselective in their responses to multiple complexes, with the solubility method perhaps possessing a slight advantage in specificity.

APPENDIX

S, substrate molecule
L, ligand molecule
S_mL_n, general formula for complex
S_t, total (formal) concentration of S
L_t, total (formal) concentration of L
S_0, equilibrium molar solubility of substrate monomer in absence of L
$[i]$, molar concentration of species i
$f_s = [S]/S_t$, fraction of S in uncomplexed form
$f_{mn} = m[S_mL_n]/S_t$, fraction of S in form of S_mL_n
$K_{mn} = [S_mL_n]/[S]^m[L]^n$, over-all stability constant for the complex S_mL_n
$K_{(mn)} = [S_mL_n]/[S][S_{m-1}L_n]$ or $[S_mL_n]/[S_mL_{n-1}][L]$, step stability constant for S_mL_n
K'_{11}, apparent stability constant assuming 1:1 stoichiometry
k_S, specific rate constant for a reaction of S
k'_S, apparent rate constant for S in presence of L
k_{mn}, specific rate constant for a reaction of S_mL_n
$r_{mn} = k_{mn}/k_S$, relative reactivity of S_mL_n
$q_{mn} = 1 - r_{mn}$
b, cell path length
a_S, molar absorptivity of S
a_{mn}, molar absorptivity of S_mL_n
A, absorbance

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