Keyphrases

Polypropylene-polyisobutylene alloy—stability
Heat effect—polypropylene alloy
UV radiation effect—polypropylene alloy
Thermal analysis—differential
Tensile properties—polypropylene alloy
IR spectrophotometry—analysis.

Characterization of Complex Formation Between Small Molecules by Membrane Permeation Measurements

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A membrane permeation technique is described which permits characterization of complex formation between small molecules. The method is applicable if the components of the complex diffuse across a nonporous membrane at markedly different rates. The procedure may be used (*a*) to determine the stoichiometry and stability constant of a complex, (*b*) to verify values of stability constants obtained by other methods, (*c*) to check assumptions concerning the stoichiometry of a complex, and (*d*) to determine the degree of complexation of a compound in systems complicated by the existence of two or more simultaneous equilibria, where other methods of determination may fail or be very time consuming. The membrane permeation technique has been applied to the characterization of the complex formation between salicylamide and caffeine at concentrations where appreciable self-association of caffeine occurs.

MANY TECHNIQUES have been developed in recent years for the characterization of drug complexes. Among the methods which have been most commonly used for the analysis of drug interactions are partitioning (1), spectrophotometric (2), equilibrium dialysis (3), dialysis rate (4, 5), and solubility techniques (6). All of the methods available for the study of complex formation are subject to certain limitations peculiar to the particular technique. Some of these limitations which were encountered in the present study are: the partitioning method requires that at least one of the uncomplexed species does not partition into one of the two solvent phases; the spectrophotometric method cannot be applied to systems involving relatively weak molecular interactions and to those which do not manifest a pronounced change in spectral characteristics upon complex formation; the dialysis techniques are limited to the study of interactions between drugs which diffuse across a dialysis membrane and macromolecules which do not; the solubility method does not permit a determination of the stoichiometry of the complex with respect to the less soluble component, if the complex itself is very soluble.[1] Connors and Mollica (8) have recently pointed out that some of the experimental approaches mentioned above may not always yield the same values for an equilibrium constant and that comparative studies with several techniques may yield valuable information concerning the nature of a complex.

Complex formation between salicylamide and caffeine has been detected recently in this laboratory. The salicylamide–caffeine complexing system was used in studies concerning the effect of complex formation on drug absorption, which are

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[1] It is sometimes possible to reverse the system and thereby determine the stoichiometric ratio of the complex (7).

reported elsewhere. Complexation of an appreciable fraction of salicylamide by caffeine requires a relatively high concentration of caffeine at which self-association of the latter occurs (9). While the solubility method could be used to characterize the interaction between salicylamide and caffeine at relatively low concentrations of caffeine, it was felt that an extrapolation of these data to higher concentrations would be unjustified in view of the self-association of caffeine at higher concentrations, and the possible formation of salicylamide–caffeine complexes of stoichiometries other than 1:1. It became desirable, therefore, to develop a technique which permits a determination of the extent of complexation of salicylamide by caffeine at the concentrations and in the buffer system actually employed in the absorption study. Advantage was taken of the fact that salicylamide permeates relatively rapidly across a nylon membrane while caffeine permeates much more slowly. This difference in permeation rates was not sufficient to determine equilibrium concentrations by procedures analogous to equilibrium dialysis. However, it was possible to estimate the extent of salicylamide complexation in a solution of salicylamide and caffeine on one side of the nylon membrane by using varying concentrations of salicylamide on the other side of the membrane, and thereby determining the direction of the concentration gradient of free salicylamide and the concentration at which this gradient is essentially zero.

EXPERIMENTAL

Membrane Permeation Procedures—One hundred and ten milliliters of a solution of 0.384% salicylamide and 2.2% caffeine in pH 5 Krebs-Henseleit Acetate Ringer solution (KHAR)[2] was placed in a 125-ml. conical flask. Ten milliliters of a solution of salicylamide (varying concentrations in KHAR) was placed in a nylon bag.[3] The bag was suspended in the solution contained in the 125-ml. flask. The flasks were agitated at 37° in a water bath shaker and 0.5-ml. samples were withdrawn from each dialysis bag after 1, 4, and 7 hr. The samples were adjusted to pH 1 with HCl and diluted with 0.1 *N* HCl as necessary. One milliliter of diluted sample was added to 5 ml. of Trinder reagent (11) and the absorbance of this solution was measured at 525 mμ using as the blank a solution consisting of 1 ml. 0.1 *N* HCl and 5 ml. Trinder reagent. It was established that caffeine, if present in the solution, does not interfere with this assay for salicylamide.

Similar experiments were carried out with caffeine alone and salicylamide alone and only the solvent inside the nylon bag. Salicylamide was assayed as described above and caffeine was assayed spectrophotometrically at 273 mμ, using KHAR, pH 5.0, as the diluent.

In order to determine the rate of permeation of caffeine in the presence of salicylamide (*i.e.*, to assess a possible difference in the permeation rate of free and complexed caffeine), nylon bags, containing only KHAR were suspended in solutions of either 0.388% caffeine or 0.388% caffeine and 0.543% salicylamide in KHAR. A sample of 0.5 ml. was removed from each nylon bag after 11 hr. of equilibration and assayed for salicylamide and caffeine. This experiment was performed in duplicate. The assays were carried out as described above except that the absorbance at 273 mμ due to salicylamide was subtracted from the total absorbance to obtain that due to caffeine. It was determined that, in the concentration range at which the measurements were carried out, the absorbances of salicylamide and caffeine were additive. Since nylon and many other materials used to make membranes take up salicylamide and other drugs from aqueous solution, it is essential that the membrane is equilibrated with the drug solution before it is used in a permeation experiment. In this study, the nylon bags were immersed in salicylamide solution (same concentration as used in the subsequent experiment) for 24 hr. prior to an experiment. There was no measurable uptake of drug when these pretreated bags were filled with fresh salicylamide solution. The time required for adequate equilibration of a membrane must be determined experimentally for each system. Failure to equilibrate the membrane may result in artifacts and possible misinterpretation of data.

Determination of the Stability Constant of the Salicylamide–Caffeine Complex by the Solubility Method—The solubility method described by Higuchi and Zuck (6) was used to determine the stability constant of the salicylamide–caffeine complex at 37° in pH 5 KHAR.

RESULTS

The results of the membrane permeation study are shown in Fig. 1. There is a pronounced difference between the diffusion rates of salicylamide and caffeine across the nylon membrane, as illustrated in the upper portion of the figure. While salicylamide diffuses across the nylon membrane quite rapidly, there was practically no diffusion of caffeine across the membrane in 7 hr. Even after 11⅔ hr. the concentration ratio (inner–outer solution) of caffeine was less than 0.02 while that of salicylamide had increased to 0.56.

The permeation rate of caffeine in the presence of a concentration of salicylamide sufficient to complex about 50% of the caffeine did not differ measurably from that of caffeine alone. It must be noted, however, that the sensitivity of the measurement was limited due to the extremely slow permeation of caffeine and the interference of salicylamide with the caffeine assay, which necessitated a rather large correction of the absorbance at 273 mμ to account for the considerably higher concentrations of salicylamide present. Thus, minor differences between the relative permeation of caffeine alone and in the presence of salicylamide could not be excluded. These initial findings demonstrated that it is possible

[2] This is similar to Krebs-Henseleit Bicarbonate Ringer solution (10) except that an equimolar amount of acetate buffer was substituted for the bicarbonate.

[3] Tomac Nylon Bags, American Hospital Supply Corp., Evanston, Ill.

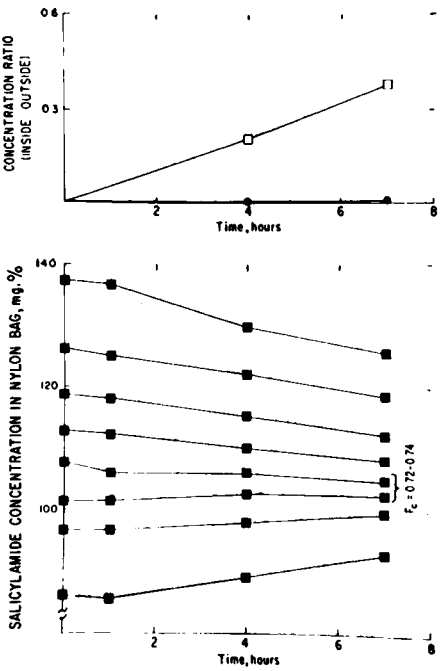

Fig. 1—Determination by the membrane permeation method of the extent of salicylamide complexation by caffeine. Upper part, ratio of drug concentration inside the nylon bag (initially only pH 5 buffer) to that outside (initially, 0.384% salicylamide or 2.2% caffeine in pH 5 buffer) as a function of time. □, salicylamide; ●, caffeine. Lower part, concentration of salicylamide inside the nylon bag as a function of time. Data are from experiments in which initially only salicylamide solution (varying concentrations) was inside the nylon bag and a solution of 0.384% salicylamide and 2.2% caffeine was outside. All data points are averages of two determinations.

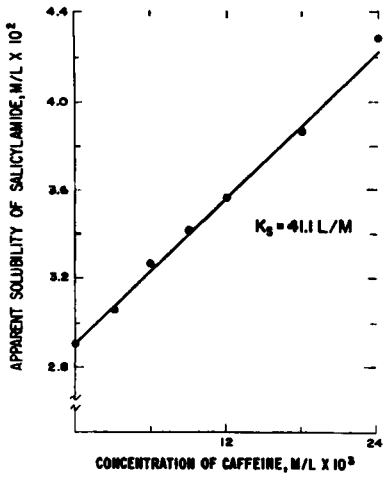

Fig. 2—Total solubility of salicylamide in pH 5 buffer at 37° as a function of total caffeine concentration. Each point represents the average of two determinations.

to maintain an essentially constant concentration of caffeine (both free and complexed) on one side of a nylon membrane for a period of time sufficient to afford considerable transfer of free salicylamide. Consequently, the series of experiments depicted in the lower portion of Fig. 1 was carried out. A solution containing 0.384% salicylamide and 2.2% caffeine was placed into conical flasks and nylon bags containing salicylamide in concentrations ranging from 0.085 to 0.135% were suspended in this solution. The concentrations of salicylamide inside the nylon bag increased or decreased with time, depending on the initial concentration, thereby reflecting the concentration gradient of uncomplexed salicylamide across the membrane. The results show that there was no significant change in the concentration of salicylamide in the solution inside the nylon bag at initial salicylamide concentrations ranging from 0.100 to 0.106%. On the basis of a total salicylamide concentration of 0.384% outside the nylon bag and a concentration of 0.100 to 0.106% of free salicylamide inside the nylon bag, it can be calculated that F_c, the fraction of total salicylamide complexed in the presence of 2.2% caffeine, ranged from 0.72 to 0.74. The value of 0.73 for F_c was used in all subsequent calculations.

The results of the solubility method as applied to the salicylamide–caffeine system are shown in Fig. 2. There was a linear increase in the apparent solubility of salicylamide with increasing concentrations of caffeine. Assuming a 1:1 stoichiometry of the salicylamide–caffeine complex, and neglecting the possibility of some slight dimerization of caffeine at these concentrations, a stability constant of 41.1 l./mole at 37° in pH 5 KHAR was obtained (12).

DISCUSSION

The membrane permeation method may be used to characterize complex formation in systems consisting of two components which exhibit rather pronounced differences in permeation rate across a suitable membrane. A major advantage of the method is that the extent of complexation of a drug or other compound can be determined directly in solutions of any desired composition, without having to determine the stoichiometry of the complex, the stability constant, or the nature of the equilibria involved. It is possible, for example, to determine the extent of drug complexation in systems involving several simultaneous equilibria as long as the drug to be studied diffuses across the membrane appreciably more rapidly than the components with which it interacts. In addition, the membrane permeation method does permit the determination of stoichiometric ratios (when these are simply 1:1, 2:1, *etc.*, and not a mixture) and stability constants in systems characterized by a single complexation equilibrium.

The membrane permeation experiments showed that the fraction of total salicylamide complexed (F_c) in a solution containing 0.384% salicylamide and 2.2% caffeine in pH 5 KHAR at 37° is 0.72–0.74. Using the stability constant obtained by the solubility method (Fig. 2) and neglecting the self-association of caffeine which is known to occur at higher concentrations of this drug (9), it can be calculated (*Reference 6* and *Appendix*) that F_c should be 0.79. A more realistic estimate of F_c can be obtained by taking into account the degree of self-association of caffeine. From the data of

Guttman and Higuchi (9) it was possible to determine a stability constant for caffeine dimerization at 37° in distilled water.[4] This constant, 8 l./mole, was used in the calculations described here since it was found that the stability constant for caffeine dimerization was not measurably different in water and in Ringer's solution, as judged by the partitioning behavior of caffeine (13). Calculations using Method II of the *Appendix*, which incorporates the stability constant of the salicylamide–caffeine complex as determined by the solubility method and the stability constant for caffeine dimerization, yielded a value for F_c of 0.67. Thus, the F_c value of 0.72–0.74 obtained by the membrane permeation method was intermediate between the values calculated from the 1:1 stability constant of the salicylamide–caffeine complex which were uncorrected, and corrected for caffeine dimerization, respectively.[5]

While the results described in the preceding paragraph are consistent with the assumption of a 1:1 stoichiometry of the salicylamide–caffeine complex, a more rigorous test of this assumption is obtained by calculating the stability constant of the complex based on a 2:1 (salicylamide–caffeine) stoichiometric ratio.[6] If the results of the solubility method and the membrane permeation method yield widely different values, a 2:1 stoichiometry can be ruled out. Values for the stability constant of the salicylamide–caffeine complex obtained by both methods assuming either a 1:1 or 2:1 stoichiometry are listed in Table I. The method of calculating the stability constants from the membrane permeation data is described in the *Appendix*. It is evident from the data in Table I that there was no agreement between the stability constants obtained by the two methods assuming a 2:1 stoichiometric ratio, but that there was reasonably good agreement of the stability constants based on a 1:1 ratio. The complexity and multiplicity of the simultaneous equilibria involved are probably responsible for the lack of a very close agreement between the 1:1 stability constant obtained by the solubility method at low caffeine concentration and that obtained by the membrane permeation method at high caffeine concentration.

A more detailed understanding of the applicability of the membrane permeation technique for distinguishing between a 1:1 and a 2:1 stoichiometry of a complex can be obtained by a review of Fig. 3, which is a theoretical plot of the fraction of total salicylamide complexed (F_c) as a function of total salicylamide concentration, with the total caffeine concentration held constant at 0.113 moles/l. (2.2%). The plots were constructed by assuming complete solubility of the complex in the concentration range shown, and a stoichiometric ratio of 2:1 and 1:1, respectively. The curves shown in the figure were calculated by procedures

TABLE I—STABILITY CONSTANTS OF THE SALICYLAMIDE–CAFFEINE COMPLEX FROM MEMBRANE PERMEATION AND SOLUBILITY MEASUREMENTS ASSUMING 1:1 OR 2:1 STOICHIOMETRY

Method	$K_{1:1}$, l./mole	$K_{2:1}$, l.²/mole²
Membrane permeation, uncorrected	29	17×10^2
Membrane permeation, corrected for caffeine dimerization	53	33×10^2
Solubility	41	4.4×10^2

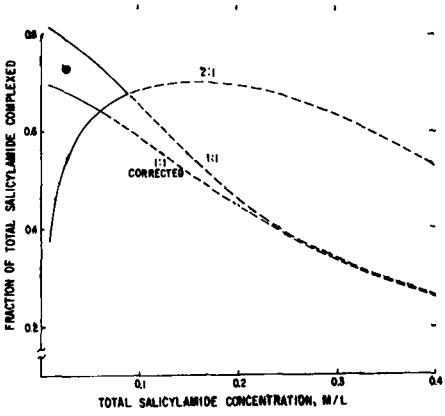

Fig. 3—Theoretical plot of the fraction of total salicylamide complexed (F_c) as a function of total salicylamide concentration. F_c was calculated from the stability constant determined by the solubility method. Numbers in the figure refer to assumed stoichiometric ratios (salicylamide–caffeine). The term "corrected" refers to a correction of the calculation of F_c which takes into account the dimerization of caffeine. Total caffeine concentration was held constant at 0.113 moles/l. The dashed portions of the curves indicate extrapolations of the theoretical calculations beyond the solubility limit of uncomplexed salicylamide. The solid circle represents the experimental value determined directly by the membrane permeation technique.

outlined in the *Appendix* and are based on the stability constants listed in Table I which were obtained by the solubility method. It is apparent that the most pronounced difference in F_c values occurs at the low salicylamide concentrations and that the curves converge as the salicylamide concentration is increased. These curves intersect (*i.e.*, the F_c values are equal) when the solution is saturated with respect to free salicylamide.[7] This is consistent with the fact that the solubility method cannot distinguish between a 1:1 and a 2:1 stoichiometry of an *A-B* complex on the basis of the total solubility of *A* (12). As a matter of theoretical interest, the curves were drawn beyond the solubility limit. The continuing decline of F_c with increasing concentration of total salicylamide reflects the decreasing availability of caffeine for complex formation. Of interest, and perhaps surprising, is the fact that F_c initially increases with total salicylamide concentration in the case of a hypothetical stoichiometric ratio of

[4] The stability constant for caffeine dimerization at 37° was determined by a graphical extrapolation of the constants reported by Guttman and Higuchi (9) at 0, 20, 30, and 35°.

[5] The small difference between the experimental results and those predicted from theoretical calculations based on a 1:1 stoichiometric ratio and corrected for caffeine dimerization cannot be ascribed to the fact that tetramer formation of caffeine was neglected in the calculations. Correction for the latter would actually increase somewhat the difference between the experimental and theoretical results.

[6] The linearity of the solubility phase diagram (Fig. 2) indicates that the stoichiometry with respect to the caffeine component is 1 in this concentration range (12).

[7] Actually, since the curve representing a 2:1 stoichiometric ratio is not corrected for caffeine dimerization, there is a slight difference between the actual solubility limit and the intersection of the "1:1 corrected" curve with the "2:1" curve.

2:1. This reflects, apparently, the fact that the concentration of salicylamide is the limiting factor initially and that caffeine becomes limiting only after an appreciable extent of caffeine complexation is attained. The most important conclusion to be derived from the relationships shown in Fig. 3 is that a distinction between 2:1 and 1:1 stoichiometry can be made most readily at the lower concentrations of salicylamide and that the sensitivity of the membrane permeation method for making such a distinction decreases as the solubility limit of salicylamide is approached. The data point in Fig. 3, which represents the results of the membrane permeation experiments, shows that the determination of F_c for salicylamide was carried out at a concentration at which a clear distinction between a 1:1 and a 2:1 stoichiometric ratio can be made.

APPENDIX

Calculation of the Extent of Complexation of Compound A by Compound B Where B is Subject to Dimerization[8]—The extent of complexation of Compound A by Compound B can be represented by F_c, the fraction of total A which is complexed. The following three methods may be used to determine F_c at relatively low concentrations of B (where dimerization is negligible) and at relatively high concentrations of B (where appreciable dimerization occurs).

Method I—Negligible dimerization of B, 1:1 stoichiometry of the complex.

$$A + B \rightleftharpoons A\text{-}B$$

The designation A-B refers to the complex of A with B. The 1:1 stability constant ($K_{1:1}$) of this equilibrium is defined as:

$$K_{1:1} = \frac{[A\text{-}B]}{[A][B]} \qquad \text{(Eq. 1)}$$

where the brackets refer to molar concentrations. F_c, the fraction of total A in solution which is complexed, is defined as follows:

$$F_c = \frac{[A\text{-}B]}{[A] + [A\text{-}B]} \qquad \text{(Eq. 2)}$$

Equations 1 and 2 can be combined to give the following expression:

$$F_c = \frac{K_{1:1}[B]}{K_{1:1}[B] + 1} \qquad \text{(Eq. 3)}$$

In order to use Eq. 3 to calculate F_c, the concentration of free B must be determined. The total concentrations of A species and B species, A_t and B_t, respectively, are defined as:

$$A_t = [A] + [A\text{-}B] \qquad \text{(Eq. 4)}$$

$$B_t = [B] + [A\text{-}B] \qquad \text{(Eq. 5)}$$

A substitution of Eqs. 4 and 5 into Eq. 1 yields upon rearrangement:

$$K_{1:1}[B]^2 + (1 + K_{1:1}A_t - K_{1:1}B_t)[B] - B_t = 0 \qquad \text{(Eq. 6)}$$

The quadratic formula applied to Eq. 6 then can be used to calculate $[B]$. This in turn permits the calculation of F_c by use of Eq. 3.

[8] Applied to the present study, A denotes salicylamide and B represents caffeine.

Method II—Partial dimerization of B, 1:1 stoichiometry of the complex. Assuming that appreciable dimerization of B occurs, the following simultaneous equilibria are applicable:

$$A + B \rightleftharpoons A\text{-}B$$

$$B + B \rightleftharpoons B\text{-}B$$

where B-B refers to the dimer. The stability constant of the dimer (K_d) is defined as follows:

$$K_d = \frac{[B\text{-}B]}{[B]^2} \qquad \text{(Eq. 7)}$$

As in the case of negligible dimerization of B, F_c can be calculated from Eq. 3. However, $[B]$ must now be determined on the basis of the following relationship:

$$B_t = [B] + 2\,[B\text{-}B] + [A\text{-}B] \qquad \text{(Eq. 8)}$$

This definition of B_t, when combined with Eqs. 1, 4, and 7, yields upon rearrangement:

$$(2K_{1:1}K_d)[B]^3 + (K_{1:1} + 2K_d)[B]^2 + (1 + K_{1:1}A_t - K_{1:1}B_t)[B] - B_t = 0 \qquad \text{(Eq. 9)}$$

Thus, a knowledge of the stability constants of the two simultaneous equilibria ($K_{1:1}$ and K_d) and the total concentrations of A species and B species (A_t and B_t, respectively) allows calculation of $[B]$ from this cubic equation. Methods of solving this type of equation are available[9] (14) and consequently F_c can be calculated by use of Eq. 3.

Method III—Negligible dimerization of B, 2:1 stoichiometry of the complex.

$$2A + B \rightleftharpoons A_2\text{-}B$$

In this equilibrium, the symbol A_2-B represents the 2:1 complex. The 2:1 stability constant is defined as follows:

$$K_{2:1} = \frac{[A_2\text{-}B]}{[A]^2[B]} \qquad \text{(Eq. 10)}$$

In this case the fraction of total A in solution which is complexed (F_c) is defined as:

$$F_c = \frac{2[A_2\text{-}B]}{[A] + 2[A_2\text{-}B]} \qquad \text{(Eq. 11)}$$

Equations 10 and 11 can be combined to give the following expression for F_c:

$$F_c = \frac{2K_{2:1}[A][B]}{1 + 2K_{2:1}[A][B]} \qquad \text{(Eq. 12)}$$

In order to use Eq. 12 it is necessary to determine $[A]$ and $[B]$ from known or experimentally determinable factors. A_t and B_t are defined for this case as follows:

$$A_t = [A] + 2[A_2\text{-}B] \qquad \text{(Eq. 13)}$$

$$B_t = [B] + [A_2\text{-}B] \qquad \text{(Eq. 14)}$$

The following expression for A can be obtained by combining Eq. 10 with Eqs. 13 and 14 and rearranging:

$$0.5K_{2:1}[A]^3 + (K_{2:1}B_t - 0.5K_{2:1}A_t)[A]^2 + 0.5[A] - 0.5A_t = 0 \qquad \text{(Eq. 15)}$$

[9] A digital computer was used in this study to solve the cubic equation.

Solution of this cubic equation yields the value of $[A]$. The value of $[B]$ can be determined from the following two relationships:

$$[A_2\text{-}B] = \frac{A_t\text{-}[A]}{2}$$

$$[B] = B_t - [A_2\text{-}B]$$

With $[A]$ and $[B]$ thus calculated, F_c can now be determined using Eq. 12.

Calculation of Stability Constants from Membrane Permeation Data—The calculation of the stability constants from membrane permeation data is shown here for the case where the calculations are corrected for the dimerization of B. The calculations which are not corrected are merely a simplification of these. The symbols used are the same as those in the previous section.

Complex of 1:1 Stoichiometry—The membrane permeation method provides a value for the concentration of uncomplexed A, $[A]$. The total concentrations A_t and B_t are known. From these values the stability constant ($K_{1:1}$) may be calculated as follows:

$$[A\text{-}B] = A_t - [A]$$

$$[B] = B_t - [A\text{-}B] - 2[B\text{-}B] = B_t - [A\text{-}B] - 2K_d[B]^2$$

$$2K_d[B]^2 + [B] - B_t + [A\text{-}B] = 0$$

The value of $[B]$ can be determined by using the quadratic formula. Then:

$$K_{1:1} = \frac{[A\text{-}B]}{[A][B]}$$

Complex of 2:1 Stoichiometry—Again the membrane permeation experiment provides a value for $[A]$. Then:

$$[A_2\text{-}B] = \frac{A_t\text{-}[A]}{2}$$

$$[B] = B_t - [A_2\text{-}B] - 2[B\text{-}B] = B_t - [A_2\text{-}B] - 2K_d[B]^2$$

$$2K_d[B]^2 + [B] + [A_2\text{-}B] - B_t = 0$$

The determination of $[B]$ then permits calculation of the stability constant:

$$K_{2:1} = \frac{[A_2\text{-}B]}{[A]^2[B]}$$

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Keyphrases

Complex formation—small molecules
Membrane permeation, nylon—complex formation determination
Salicylamine-caffeine—complexing system
Diffusion rates—salicylamide, caffeine
Colorimetric analysis, salicylamide—spectrophotometer
UV spectrophotometry—analysis, caffeine
Stability constants—equations