Determination of Molecular Binding in Aqueous Solution from Optical Activity Measurements

Interaction of Tryptophan with Alkylxanthines

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Although the feasibility of using ORD for determining stability constants of molecular complexes in organic solvent has already been shown, the present investigation appears to be the first attempt to apply it to an aqueous system. Measurements at 330 mμ show distinct changes in the apparent optical activity of the zwitter ionic form of the amino acid when alkylxanthines were added to its solutions. These readings when analyzed by the iterative procedure previously developed for PMR data have yielded self-consistent stability constant values. Results are given for interaction with caffeine, theophylline, 8-methoxycaffeine, 1,3-dimethyluracil, and sarcosine anhydride. From measurements at several temperatures the heat of binding of tryptophan with caffeine was shown to be −4.0 kcal./mole and that with theophylline to be −4.2 kcal./mole. These results appear to be consistent with results obtained previously on related systems.

ALTHOUGH IT HAS BEEN demonstrated that optical activity measurements can be effectively used to determine stability constants of bound species in essentially nonpolar solvents (1), no attempt appears to have been made to utilize these readings for estimating binding tendencies in aqueous solutions. In nonpolar solutions the binding behaviors arise primarily through hydrogen bonding of the interactants. In aqueous systems the source or sources of the associative tendencies of organic species are still not clearly elucidated (2). The present investigation was concerned with evaluation of the optical activity approach to estimation of stability constants in water based on tryptophan as the primary optically active species. It would appear reasonable to expect that intimate bonding of another organic species on the amino acid will so affect the electrical field polarization in the immediate vicinity of the asymmetric center that significant change in the ORD spectrum may result.

That tryptophan binds with xanthines has not been definitely reported previously but interactions of bovine serum albumin with xanthine molecules have been reported by Guttman *et al.* (3–5). A modification in pharmacological effect of ergotamine tartrate in the presence of caffeine has been suggested to be due to complex formation (6). Since these molecules have an indole group in them, the interactions are likely to be similar to that of tryptophan with caffeine.

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EXPERIMENTAL

Reagents—L-Tryptophan, as supplied by Sankyo Kasei (Tokyo), was recrystallized from ethanol–water before use. Caffeine and theophylline were of USP and NF grades, respectively. L-Tyrosine and L-phenylalanine were from Nutritional Biochemicals and were used without further purification. 8-Methoxycaffeine (Eastman), 1,3-dimethyluracil (Nutritional Biochemicals), sarcosine anhydride (K&K), and tetramethylsuccinamide (C. P. Hall) were all recrystallized from methanol–carbon tetrachloride.

Instrumentation—A Cary 60 spectropolarimeter equipped with a thermostated cell compartment maintained at 25° with a 10-cm. cell was used for spectropolarimetry. Samples were equilibrated in a 25° water bath prior to use. For variable temperature measurements, a 10-cm. Cary 60 thermostatable cell connected to a constant-temperature water bath was used.

Determination of Stoichiometry—To establish the stoichiometry of the complexes under investigation the method of continuous variations (Job's method) (7) was employed. Although this method was developed especially for use with spectrophotometric measurements, it was readily modifiable for use with spectropolarimetric measurements. It was assumed that only a single complex AB_n was formed from A and B, the complexation reaction being written,

$$A + nB = AB_n \qquad \text{(Eq. 1)}$$

Solutions were prepared in which the sum of the number of moles A and B present was maintained constant at S M. These were prepared by making solutions which contained $(1 - x)S$ M of A and xS M of B. It was evident that

$$n = \frac{x}{1 - x} \qquad \text{(Eq. 2)}$$

Molar rotations[1] of A, B, and AB_n in 1-cm. pathlength cells were set equal to r_a, r_b, and r_c, respectively; the difference, R, between the measured rotation and that expected if no interaction took place was then

$$R = l[r_a(C_a - C_c) + r_b(C_b - nC_c) + r_cC_c - r_aC_a - r_bC_f]$$
$$= l(r_c - r_a - nr_b)\ C_c \qquad \text{(Eq. 3)}$$
$$= lr\ C_c$$

where l was the pathlength of the cell in the study in cm. and $C_a - C_c$, $C_b - nC_c$, and C_c, were the concentrations of species A, B, and C, respectively, at equilibrium and $r = r_c - r_a - nr_b$. When C_c was a maximum $(dC_c)/dx = 0$, then $(dR)/(dx) = 0$. Thus R passed through an extremum when C_c was at maximum. In this study total concentration, S, was kept at 3×10^{-2} M, measurements being made at 330 mμ.

Computation of Stability Constants—For the computation of stability constants from ORD measurements, a new iterative method was developed and employed. This method is analogous to that described earlier for the NMR technique (8).

If two species, A and B, interact reversibly in one-to-one stoichiometry,

$$A + B = C \qquad \text{(Eq. 4)}$$

at equilibrium,

$$K = \frac{C_c}{(C_a - C_c)(C_b - C_c)} \qquad \text{(Eq. 5)}$$

Eq. 5 can be rearranged to give

$$KC_aC_b - KC_c(C_a + C_b - C_c) = C_c \qquad \text{(Eq. 6)}$$

When $n = 1$, Eq. 3 may be written

$$R = l(r_c - r_a - r_b)C_c = lrC_c \qquad \text{(Eq. 7)}$$

then

$$C_c = \frac{R}{rl} \qquad \text{(Eq. 8)}$$

Eq. 6 and Eq. 8 give

$$\frac{C_aC_b}{R} = \frac{1}{Krl} + \frac{C_a + C_b - C_c}{rl} \qquad \text{(Eq. 9)}$$

K and r_c were computed from the difference in measured rotation, R, and the initial concentrations of A and B, C_a and C_b, as follows.

Step 1—$(C_aC_b)/R$ was plotted against $(C_c + C_b)$ and the slope of the line, slope_0 was obtained.

Step 2—C_{c1}, the first approximate values of C_c, was calculated employing slope_0 in Eq. 8, *i.e.*, $C_{c1} = \text{slope}_0 \cdot R$.

Step 3—$(C_aC_b)/R$ was plotted against $(C_a + C_b - C_{c1})$ and the slope, slope_1 was obtained.

Step 4—C_{c2} was calculated as in Step 2, *i.e.*, $C_{c2} = \text{slope}_1 \cdot R$.

Step 5—Steps 3 and 4 were repeated until the convergent slope was obtained.

Step 6—K was calculated from the slope and the intercept of the last cycle, and r_c from the final slope, K = slope/intercept, $r_c = r_a + r_b + 1/(\text{slope} \cdot l)$.

[1] Molar rotation stands for optical rotation which will be observed when 1 M solution is placed in 1-cm. pathlength cell, whereas molecular rotation is defined by $[\alpha]^{\lambda}\ M/100$, where $[\alpha]^{\lambda}$ = specific rotation and M = molecular weight.

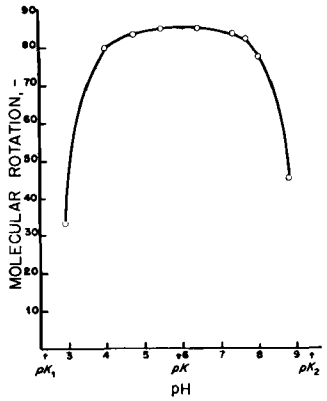

Fig. 1—pH profile of rotations of tryptophan at 330 mμ. Concentration of tryptophan = 4×10^{-3} M.

Optical rotations of tryptophan solutions with varying concentrations of amides were measured at 330 mμ. The concentration of tryptophan was kept constant at 4×10^{-3} M whereas concentrations of caffeine and theophylline were varied between 4×10^{-3} M and 32×10^{-3} M

RESULTS AND DISCUSSION

The observed pH profile of the rotatory activity of tryptophan at 330 mμ is shown in Fig. 1. The rotation was negative and constant in the neighborhood of isoelectric point (pKI), namely between pH 5 and 7. Because of the independence with respect to pH, rotation measurements for subsequent studies were made in this region. The observed optical rotatory dispersion curve for tryptophan alone and those in the presence of alkylxanthines are shown in Fig. 2. As is evident from Fig. 2 the relatively low concentrations of the added solutes produced significant changes in the ORD spectrum, the rotatory activity becoming even more strongly negative and the trough shifting toward shorter wavelength. If the observed shifts can be entirely attributed to formation of a complex, stability constants can be readily calculated from these data.

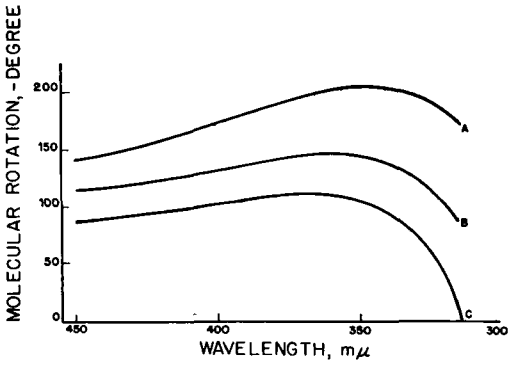

Fig. 2—Optical rotatory dispersion spectra of tryptophan in water (4×10^{-3} M). Key: A, with 3.2×10^{-2} M caffeine; B, with 3.2×10^{-2} M theophylline; C, without alkylxanthines.

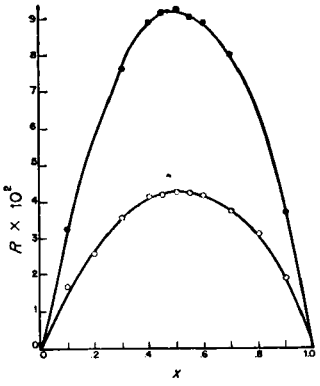

Fig. 3—The difference between the measured rotation and that predicted upon the assumption of no interaction, R, against mole fraction of tryptophan, x, measured at 330 mμ, total concentration S = 3 × 10^{-2} M. Key: ●, tryptophan-caffeine; ○, tryptophan-theophylline.

Optical rotation measurements, moreover, can be used to establish the stoichiometry of the complex species by the method of continuous variations. Plots of the difference between the measured rotation and that expected if no interaction took place, R, against x for tryptophan-caffeine and tryptophan-theophylline at 25° are shown in Fig. 3. For both interactions, $x = 0.5$ appear to correspond to the maximum values of R. The indicated stoichiometric ratio was, therefore, one-to-one.

The stability constant for the tryptophan-caffeine system calculated from ORD measurements in water at 25° by the iterative procedure was 30.1 M In Fig. 4 the initial plot required by the method is shown to illustrate the adherence of the data to the expected relationship. The stability constants for tryptophan complexes with some other amides were computed in the same manner and are listed in Table I. It is of interest to note that 8-methoxycaffeine showed a much stronger binding tendency than caffeine which has been generally considered to be among the stronger complexing agents. 1,3-Dimethyluracil was a much weaker binder and sarcosine anhydride interacted little, possibly because of its unfavorable nonplanar structure and nonaromatic character.

TABLE I—STABILITY CONSTANTS OF TRYPTOPHAN COMPLEX WITH SOME AMIDES AT 25° IN WATER

Compound	Stability Constant, M^{-1}
Caffeine	30
Theophylline	35
8-Methoxycaffeine	50
1,3-Dimethyluracil	5.8
Sarcosine anhydride	≃0
Tetramethyl succinamide	≃0

The higher complexing behavior with theophylline than with caffeine was, however, somewhat unexpected (2). Guttman *et al.* (3) had also found stronger binding tendency of theophylline to bovine serum albumin than caffeine. Since the abnormality was also observed in 8-chlorotheophylline-tryptophan system and was absent in theophylline-indole system [based on ORD studies in methanol and on phase-solubility studies (9)], it may be suspected that 7-proton of theophylline may be interacting with the zwitter ion part of tryptophan. Although the extent of the interaction with the zwitter ion part may be small, it may modify the optical rotation to a certain extent. The observed increase in binding thus may be the sum of interactions of the indole ring and the zwitter ion part of the molecule with theophylline.

The temperature effect on stability constants was studied using a Cary 60 thermostatable sample cell connected with a water bath. Logarithms of the observed stability constants plotted against the reciprocal of the absolute temperature are shown in Fig. 5. $\Delta H°$ calculated from the slope of the straight line was found to be −4.0 kcal./mole for the tryptophan-caffeine system and −4.2 kcal./mole for the tryptophan-theophylline system. $\Delta S°$ was also negative in both instances (−6.7 cal. $mole^{-1}$ and −7.0 cal. $mole^{-1}$ $deg.^{-1}$, respectively).

These values including those in Table I are in agreement with those which may be expected from earlier studies (2, 10). In general it has been shown that largely nonaromatic structures such as in the case of sarcosine anhydride bind very little. 8-

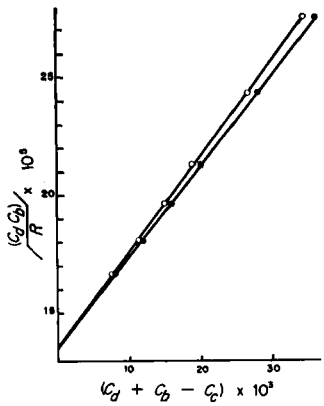

Fig. 4—Plots based on Eq. 9 for tryptophan (4 × 10^{-3} M) — caffeine (4 — 32 × 10^{-3} M) system. Key: ●, 1st approximation; ○, 3rd and final approximation.

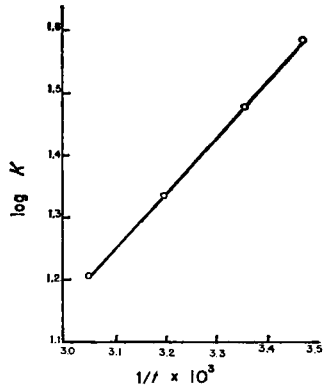

Fig. 5—Temperature dependence of stability constants in tryptophan-caffeine system in water. Tryptophan concentration = 4 × 10^{-3} M; caffeine concentration = 4 — 32 × 10^{-3} M.

Methoxycaffeine, on the other hand, appears to be one of the strongest binders that the authors have found to date.

Partition studies between water and cyclohexane–chloroform (1:1 by volume) have shown that $K = 32\ M^{-1}$ for tryptophan–theophylline complex and $K = 26\ M^{-1}$ for tryptophan–caffeine complex. Although these values are somewhat smaller than those determined by ORD method (35 and 30, respectively), the agreement appears reasonable if one considers the difference in concentrations employed (for partition work original concentration of each species was $1 \times 10^{-2}\ M$). It is believed that phase-solubility method will give only approximate K values for these systems because of the great solubility of tryptophan in water (soly. $= 6 \times 10^{-2}\ M$), yielding a rather complex overall system at saturation.

Experiments based on the ORD method have shown that caffeine interacts with L-tyrosine ($K = 13.4\ M^{-1}$ at 25° in water measured at 315 mμ with tyrosine concentration $= 8 \times 10^{-4}\ M$ and caffeine concentration $= 1 - 5 \times 10^{-2}\ M$). The extent of interaction measured in the same manner between caffeine and L-phenylalanine was much less ($K = 6.3\ M^{-1}$ at 25° in water measured at 320 mμ with phenylalanine concentration $= 3 \times 10^{-3}\ M$ and caffeine concentration $= 1 - 6 \times 10^{-2}\ M$). Preliminary experiments on optical rotatory dispersion studies of tryptophan indicated that tryptophan also interacted with pyrimidines and purines of biological importance.

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Keyphrases

Molecular binding determinations—optical activity measurements
Tryptophan-alkylxanthines—interaction
Complex formation—tryptophan-alkylxanthines
Stability constants—tryptophan-alkylxanthine complexes
Optical rotatory dispersion—analysis

Differential Scanning Calorimetry

Advantages and Limitations for Absolute Purity Determinations

By N. J. DeANGELIS and G. J. PAPARIELLO

A critical evaluation of the effectiveness of absolute purity determination by differential scanning calorimetry (DSC) is presented. Several pure drugs of widely differing structure have been investigated with respect to the detection of known amounts of added impurities of all types. The accuracy of purity values obtained by the DSC technique is shown to fall off rapidly below purities of 99 mole %. Experiments are described which demonstrate that impurities present in solid solution are not detected. Independent purity determinations by phase solubility and quantitative thin-layer chromatography are shown to be in good agreement with DSC values for samples that are 99% pure or better. A technique for extending the useful range of DSC purity determination to about 95% is given.

CALORIMETRIC METHODS of absolute purity determination by measurements related to melting and freezing point depressions have been successfully applied to the analysis of organic compounds that melt without decomposition for many years. However, prior to the availability of the Perkin-Elmer differential scanning calorimeter (DSC), these methods had not been widely used in pharmaceutical laboratories. With the introduction of this type of instrumentation, there has been increased interest and application of purity measurements of this type (1).

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