Complex Formation Influence on Reaction Rate III

Interaction of Some Isoalloxazines with 8-Chlorotheophylline as Determined by Spectral, Solubility, and Kinetic Methods

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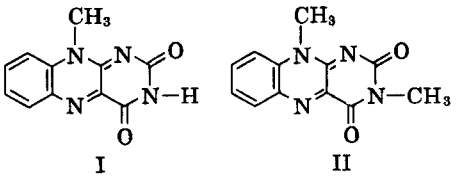
The interactions of 9-methylisoalloxazine and 3,9-dimethylisoalloxazine with 8-chlorotheophylline were studied with the objective of gaining additional information on the effects of complex formation on the reaction rate of flavins and related compounds. The influence of the xanthine on the spectral characteristics and on the rates of hydrolysis of the isoalloxazines was studied. In the case of 9-methylisoalloxazine, the interaction was also investigated by a solubility method. The interactions were shown to be markedly pH-dependent and to involve the ionized 8-chlorotheophylline and unionized isoalloxazine. Good evidence was obtained to indicate that the complexed form of the isoalloxazine was resistant to hydrolytic decomposition. Both isoalloxazines appeared to exhibit the same interactive tendencies toward the xanthine. In both cases, the dissociation constant determined from spectral data differed from that obtained by other experimental techniques and suggested that interaction and stabilization were not solely the consequence of charge-transfer.

A PREVIOUS communication (1) reported that the presence of caffeine exerted a marked stabilizing effect on the base-catalyzed, nonphotolytic, solvolytic decomposition of riboflavin in aqueous solutions. The model that was proposed to explain the observed effect postulated that riboflavin and caffeine interacted to form a 1:1 complex which possessed little or no reactivity toward hydroxide ion. The observed behavior agreed with the proposed model only at the lower concentrations of caffeine. The deviations from the predicted behavior which were apparent at higher concentrations of caffeine and which were thought to be due to the nonideal behavior of caffeine at such concentrations, did not permit a clear-cut realization as to whether the complexed riboflavin was indeed capable of the reaction.

In the present study, a simpler system was chosen for further investigation of this effect. Here, structural analogs of riboflavin, 9-methylisoalloxazine (I) and 3,9-dimethylisoalloxazine (II), were synthesized and utilized as model compounds. These have the same spectral characteristics as riboflavin; furthermore, they appear to undergo the same type of hydrolytic breakdown of the isoalloxazine ring. In addition, they exhibit the same type of interactive tendencies toward many compounds as does riboflavin. These analogs were found to be more suitable than riboflavin to utilize in these studies because of their stabilities toward photolytic decomposition and because the absence of the ribityl side chain precluded certain complicating behaviors which were observed with riboflavin (2).



I

II

8-Chlorotheophylline was chosen as a complexing agent to investigate in this study. It was selected with the anticipation that it would be completely ionized under the conditions of pH that would be encountered. The ionization would be expected to inhibit "self-complexing" and thus eliminate a complicating behavior that was encountered in the caffeine system.

Three different techniques were utilized to investigate and quantitate the interaction of 9-methylisoalloxazine with 8-chlorotheophylline. The influence of 8-chlorotheophylline on the spectral characteristics, solubility, and rate of reaction of 9-methylisoalloxazine was studied. With the intention of seeking further information about the interaction, 3,9-dimethylisoalloxazine was also investigated. Its interaction with 8-chlorotheophylline was studied by the kinetic and the spectral methods. As will be seen, the results showed that: interaction did occur to form a 1:1 complex; the interaction was markedly dependent on the pH of the system; the complexed form of isoalloxazines did not appear to undergo hydrolytic cleavage of the ring; and whereas the values for the equilibrium constants for the interaction between 9-methylisoalloxazine

Received April 2, 1965, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo.

Accepted for publication May 11, 1965.

Presented to the Scientific Section, A.PH.A., Detroit meeting, March 1965.

This investigation was supported by research grant GM-12806 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

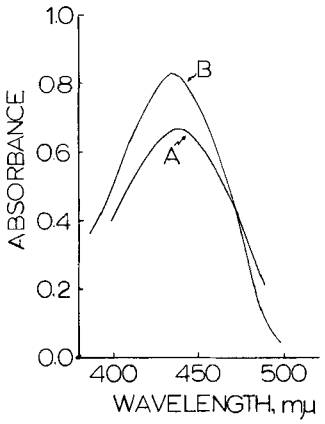


Fig. 1.—A plot showing the influence of 8-chlorotheophylline on the spectrum of 9-methylisoalloxazine. Key: A, 8.81×10^{-5} *M* 9-methylisoalloxazine in the presence of 0.04 *M* 8-chlorotheophylline; B, 8.81×10^{-5} *M* 9-methylisoalloxazine alone.

and 8-chlorotheophylline, and 3,9-dimethylisoalloxazine and 8-chlorotheophylline, determined under identical conditions, agreed well with each other; the spectral value in either case differed significantly from those obtained using other methods.

EXPERIMENTAL

Materials.—9-Methylisoalloxazine was synthesized according to the method of Kuhn (3), m.p. above 330° dec. The pKa was determined spectrophotometrically to be 9.82. 3,9-Dimethylisoalloxazine was prepared in a similar manner, condensing equimolar quantities of *N*-methyl-*o*-phenylenediamine hydrochloride and methylalloxan. The latter was obtained from theobromine according to the procedure described by Biltz (4); m.p. of 3,9-dimethylisoalloxazine 307–308° dec.

All other chemicals were obtained from commercial sources.

Procedure.—*Kinetic Studies.*—The experimental method used was the same as that described previously (1). The rate of disappearance of isoalloxazine was determined both in the presence and absence of 8-chlorotheophylline by following the decrease in absorbance at 430 mμ as a function of time.

Solubility Studies.—Approximately 20 mg. of 9-methylisoalloxazine was placed in each of a series of vials. A volume of a buffered stock solution of 8-chlorotheophylline was added to each vial. Sufficient distilled water was added to each vial to make a total volume of 25 ml. The vials were stoppered and rotated in a constant-temperature bath adjusted at 37° for at least 48 hr. The vials were allowed to stand and an aliquot of the supernatant was withdrawn by means of a pipet fitted with a glass-wool plug. Separation of the isoalloxazine from the xanthine was achieved by partition chromatography. A column was prepared by packing 5 Gm. of Celite previously hydrated with 5 ml. of water in a standard chromatography tube. A 3-ml. quantity of sample was treated with 3 Gm. of Celite, and the resulting mixture was quantitatively transferred to the top of the tube and carefully packed. The isoalloxazine was eluted from the column by using chloroform saturated with water as the eluting solvent. Preliminary experiments showed that the recovery was quantitative and that 8-chlorotheophylline remained on the column. The amount of isoalloxazine in the eluate was determined spectrophotometrically at a wavelength of 430 mμ.

Spectral Studies.—Spectra of 9-methylisoalloxazine in the presence and absence of 8-chlorotheophylline were determined with a Beckman model DB recording spectrophotometer. The influence of 8-chlorotheophylline on the absorbance of 9-methylisoalloxazine and 3,9-dimethylisoalloxazine solutions at 430 mμ was determined with a Beckman model DU spectrophotometer equipped with dual thermal spacers.

The influence of pH on the interaction was assessed by spectral measurements. Solutions were prepared which were constant with respect to the concentrations of isoalloxazine and the xanthine. Sodium hydroxide solution was added to each of the solutions to obtain a range of pH values from approximately 4 to 11. Distilled water was then added to make a constant volume, and the pH of each solution was redetermined. The absorbance of each solution was determined at 430 mμ and compared with the absorbance of a solution of the isoalloxazine alone at the same pH.

RESULTS

Figure 1 shows the visible spectrum of 9-methylisoalloxazine alone and in the presence of 8-chlorotheophylline. The presence of the xanthine resulted in a shift of the 430 mμ peak of the isoalloxazine toward longer wavelengths with a concomitant decrease in the absorbance at 430 mμ. The spectral effect is the same as those observed on interaction of riboflavin with this and other xanthines (1, 5) and is typical of spectral effects encountered in charge-transfer interactions. The magnitude of hypochromism at 430 mμ caused by the xanthine was utilized to indicate the extent of interaction.

The pH profile for the interaction between 9-

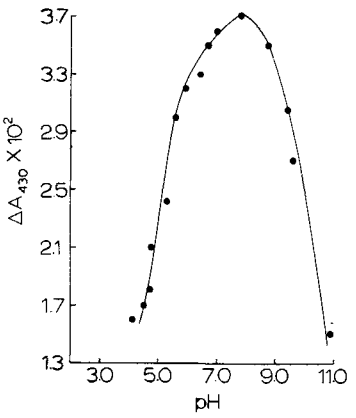


Fig. 2.—A plot illustrating the influence of pH on the interaction between 9-methylisoalloxazine and 8-chlorotheophylline. The concentration of the isoalloxazine was 4.71×10^{-5} *M* and that of 8-chlorotheophylline was 6.88×10^{-3} *M*.

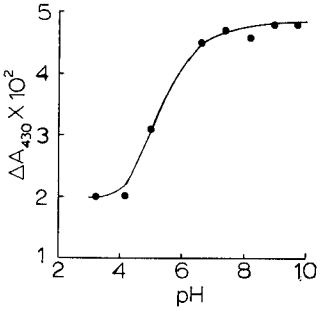


Fig. 3.—A plot showing the influence of pH on the interaction between 3,9-dimethylisoalloxazine and 8-chlorotheophylline. The concentration of the isoalloxazine was 3.59×10^{-5} *M* and that of 8-chlorotheophylline was 0.004 *M*.

methylisoalloxazine and 8-chlorotheophylline is shown in Fig. 2. Here the absorbance change at 430 mμ caused by the addition of 8-chlorotheophylline to the system is plotted as a function of the pH of the system. Little interaction was observed at pH values below 4. As the pH was increased, the extent of interaction increased and maximized at a pH of approximately 7.8. Further increase in pH resulted in reduced interaction until complete inhibition was apparent at pH values above 11. In the pH region that was investigated, the isoalloxazine (pKa = 9.82) existed in solution in the unionized form (ISH) and/or in the ionized form (IS^-). Similarly, 8-chlorotheophylline (pKa = 5.28) was present as the conjugate acid (CTH) and/or conjugate base (CT^-). The relative distribution of species in a particular solution would be dependent on the pH of the solution. Although one would intuitively expect the interactants responsible for complexation to be ionized 8-chlorotheophylline and unionized 9-methylisoalloxazine, the possibility exists that one or more of four equilibria were operative and responsible for the formation of a complex. These include:

$$ISH + CTH = ISH{:}CTH \quad \text{(Eq. 1)}$$

$$ISH + CT^- = ISH{:}CT^- \quad \text{(Eq. 2)}$$

$$IS^- + CTH = IS^-{:}CTH \quad \text{(Eq. 3)}$$

$$IS^- + CT^- = IS^-{:}CT^- \quad \text{(Eq. 4)}$$

The pH profile indicated that Eqs. 1 and 4 were not responsible for interaction. It can be seen that at the pH extreme where ISH and CTH predominated and where IS^- and CT^- predominated, the degree of interaction was negligibly low. The solutions that exhibited maximum interaction were those adjusted to pH values where the interactants of Eqs. 2 and 3 predominated. Mathematical analysis of the concentration dependencies of Eqs. 2 and 3 showed that maximum interaction would be expected to occur in solutions maintained at the pH specified by the following equation:

$$pH = {}^1/_2pK_{ISH} + {}^1/_2pK_{CTH} \quad \text{(Eq. 5)}$$

This pH was calculated to be 7.55. The experimental results were in reasonable agreement with this expected result. The close agreement between theory and experiment confirmed that one or both the equilibria were operative but could not differentiate between the two possibilities. That the interaction involved the unionized isoalloxazine was confirmed by conducting similar studies with 3,9-dimethylisoalloxazine. Here ionization of the isoalloxazine was precluded by replacement of the acidic hydrogen with a methyl group. The influence of pH on this interaction is diagrammatically represented in Fig. 3. It is seen that with the nonionizable isoalloxazine the degree of interaction increased as the degree of ionization of 8-chlorotheophylline increased.

The influence of 8-chlorotheophylline on the absorbance of solutions of 9-methylisoalloxazine and 3,9-dimethylisoalloxazine at 430 mμ was investigated and the results are presented in Fig. 4 in the manner suggested by Benesi and Hildebrand (6). The plot is based on the assumption that a 1:1 interaction occurred to form a complex which followed Beer's law. In such a case it can be shown that

$$(ISH)_t/(A_0 - A_x) = 1/(a_{ISH} - a_C) + K/(a_{ISH} - a_C)C_{CT^-} \quad \text{(Eq. 6)}$$

where

$(ISH)_t$ = the stoichiometric concentration of unionized isoalloxazine
A_0 = absorbance at 430 mμ in the absence of 8-chlorotheophylline
A_x = absorbance at 430 mμ in the presence of 8-chlorotheophylline
a_{ISH} = molar absorptivity of isoalloxazine at 430 mμ
a_C = molar absorptivity of the complex at 430 mμ
K = dissociation constant of the complex
C_{CT^-} = concentration of noncomplexed ionized 8-chlorotheophylline in the system.

Since the concentration of 8-chlorotheophylline was, in all cases, much higher than the concentration of 9-methylisoalloxazine and since 8-chlorotheophylline would essentially exist in the ionized form at the experimental pH which was 9.0, the stoichiometric concentration can be substituted here without

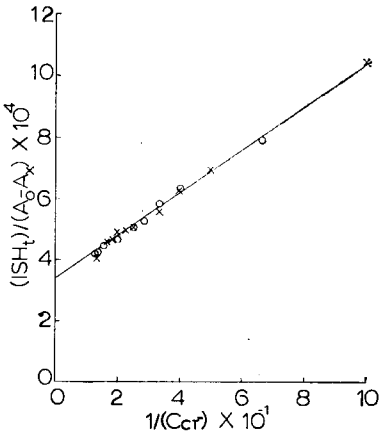


Fig. 4.—A plot showing the Benesi-Hildebrand treatment of the data obtained in spectral studies of the interaction between 9-methylisoalloxazine and 8-chlorotheophylline (○) and between 3,9-dimethylisoalloxazine and 8-chlorotheophylline (×). Concentration of unionized 9-methylisoalloxazine = 4.444×10^{-5} *M* and that of dimethylisoalloxazine = 7.535×10^{-5} *M*.

TABLE I.—EFFECT OF 8-CHLOROTHEOPHYLLINE ON THE RATE OF HYDROLYSIS OF 9-METHYLISOALLOXAZINE IN pH 9.0 BORATE BUFFER[a]

Concn. of 8-Chlorotheophylline × 10^2M	Rate Constant × 10^3 hr.$^{-1}$	$k/k_{app.}$
0.0	5.15	1.0
0.24	4.39	1.17
0.6	3.34	1.54
1.2	2.76	1.87
1.44	2.51	2.05
2.04	2.12	2.43
2.4	1.86	2.77
3.0	1.59	3.25
3.6	1.38	3.73
4.8	1.03	5.0
6.0	0.90	5.74
7.2	0.76	6.78

[a] At 35 ± 0.01°C. and at an ionic strength of 0.2.

TABLE II.—EFFECT OF 8-CHLOROTHEOPHYLLINE ON THE RATE OF HYDROLYSIS OF 3,9-DIMETHYLISOALLOXAZINE IN pH 10.0 BORATE BUFFERS[a]

Concn. of 8-Chlorotheophylline × 10^2M	Rate Constant × 10^2 hr.$^{-1}$	$k/k_{app.}$
0.0	2.25	1.0
0.1	2.06	1.09
0.2	1.87	1.2
0.5	1.52	1.48
1.0	1.13	1.99
1.5	0.94	2.41
2.0	0.79	2.85
2.5	0.72	3.14
3.0	0.57	3.96
3.5	0.52	4.37
4.0	0.46	4.95
5.0	0.41	5.5

[a] At 35 ± 0.01°C. and an ionic strength of 0.2.

appreciable error. The dissociation constants were calculated by the methods of least squares and were found to be $1.92 \times 10^{-2} \pm 0.2 \times 10^{-2}$ mole/L. for 9-methylisoalloxazine–8-chlorotheophylline and 2.05×10^{-2} mole/L. for the 3,9-dimethylisoalloxazine–8-chlorotheophylline interaction.

The influence of 8-chlorotheophylline on the rate of hydrolysis of 9-methylisoalloxazine and 3,9-dimethylisoalloxazine was investigated in 0.05 *M* borate buffer adjusted to an ionic strength of 0.2 and at 35°. The hydrolysis of 9-methylisoalloxazine was investigated at pH 9.0, while that of 3,9-dimethylisoalloxazine was followed at pH 10.0. Both isoalloxazines exhibited a pseudo first-order disappearance at all concentrations of 8-chlorotheophylline employed, and in either case the rate of hydrolysis decreased with an increase in concentration of 8-chlorotheophylline. The results are presented in Tables I and II which show the rate constants for the disappearance of 9-methylisoalloxazine and 3,9-dimethylisoalloxazine at various concentrations of 8-chlorotheophylline. Included in the tables are the ratios of the rate constant observed in the absence of 8-chlorotheophylline to that found in its presence.

If the observed increase in the stability of isoalloxazine in the presence of 8-chlorotheophylline is attributed to the formation of a 1:1 complex and if it is assumed that the complexed form was resistant to hydrolytic decomposition, then it can be shown (1) that

$$k/k_{app.} = 1 + C_{CT^-}/K \qquad \text{(Eq. 7)}$$

where

k = rate constant in the absence of 8-chlorotheophylline
$k_{app.}$ = rate constant in the presence of 8-chlorotheophylline
C_{CT^-} = concentration of noncomplexed ionized 8-chlorotheophylline
K = dissociation constant for complex

According to Eq. 7, a plot of $k/k_{app.}$ *versus* the concentration of noncomplexed ionized 8-chlorotheophylline, which can be approximated by its stoichiometric concentrations, should be linear with a slope equal to the reciprocal of the dissociation constant. Figure 5 shows such plots for 9-methylisoalloxazine and 3,9-dimethylisoalloxazine. The values of K obtained from the slopes are $1.24 \times 10^{-2} \pm 0.02 \times 10^{-2}$ mole/L. for the interaction between 9-methylisoalloxazine and 8-chlorotheophylline and $1.02 \times 10^{-2} \pm 0.05 \times 10^{-2}$ mole/L. for the interaction between 3,9-dimethylisoalloxazine and 8-chlorotheophylline, and at first sight appear to differ from each other. However, the dissociation constant, as obtained from the graph, for the system involving 9-methylisoalloxazine must be corrected for its pH dependence since only the unionized form of the isoalloxazine appears to participate in the interaction. The corrected value of the dissociation constant is 1.08×10^{-2} mole/L.

The solubilities of 9-methylisoalloxazine in the

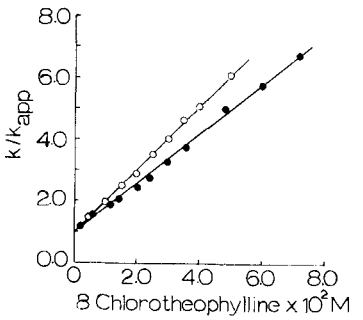


Fig. 5.—A plot showing the influence of 8-chlorotheophylline on the rate of hydrolysis of 9-methylisoalloxazine (●) and 3,9-dimethylisoalloxazine (○) at 35°.

TABLE III.—EFFECT OF 8-CHLOROTHEOPHYLLINE ON THE SOLUBILITY OF 9-METHYLISOALLOXAZINE[a]

Concn. of 8-Chlorotheophylline × 10^2M	Solubility of 9-Methylisoalloxazine × 10^4M	S/S_0
0	3.51	1.0
3.2	4.85	1.38
9.6	6.17	1.76
16.0	7.80	2.22
25.6	11.24	3.20
32.0	12.00	3.42
37.4	14.40	4.09
48.0	18.16	5.17
57.6	20.14	5.74
64.0	24.55	6.99

[a] At 37 ± 0.1°C. and at a pH of 6.5 ± 0.2.

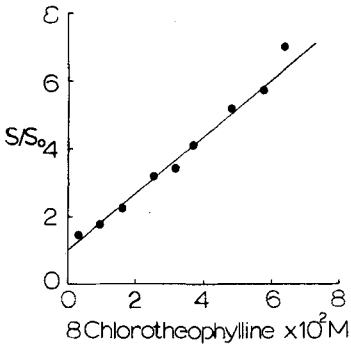


Fig. 6.—A plot showing the influence of 8-chlorotheophylline on the solubility of 9-methylisoalloxazine at 37° in pH 6.5 buffer.

TABLE IV.—DISSOCIATION CONSTANTS FOR 9-METHYLISOALLOXAZINE–8-CHLOROTHEOPHYLLINE AND 3,9-DIMETHYLISOALLOXAZINE–8-CHLOROTHEOPHYLLINE COMPLEXES AT 35°C.

Method of Determination	$K_{diss.} \times 10^2$ mole/L. for the Complex 9-Methylisoalloxazine–8-Chlorotheophylline	3,9-Dimethylisoalloxazine–8-Chlorotheophylline
Kinetic	1.08 ± 0.02	1.02 ± 0.05
Solubility[a]	1.13 ± 0.09	...
Spectral	1.92 ± 0.2	2.05 ± 0.05

[a] Determined at 37°C.

presence and absence of 8-chlorotheophylline are shown in Table III and Fig. 6. Assuming that the increase in solubility was due to interaction to form a 1:1 complex in the solution phase, it can be shown (7) that

$$S/S_0 = 1 + C_{CT}-/K \qquad \text{(Eq. 8)}$$

where

S = solubility in the presence of complexing agent
S_0 = solubility in the absence of complexing agent
$C_{CT}-$, K are as previously defined

As evidenced by Fig. 6, the expected linearity was obtained. The dissociation constant determined from the slope of line was $1.13 \times 10^{-2} \pm 0.09 \times 10^{-2}$ mole/L. The values for the dissociation constants of the complexes as determined by different methods are recorded in Table IV.

DISCUSSION

It was interesting to observe that, for the systems investigated, the spectral method yielded dissociation constants which were different from and significantly larger than those obtained utilizing other experimental techniques. Other workers have shown that the value of an apparent dissociation constant for a complex-forming equilibrium can depend on the experimental method used for the determination. For example, Ross and co-workers (8) showed that the dissociation constant for the interaction between picryl chloride and hexamethylbenzene, as determined from reaction rate studies, differed from that obtained from spectral studies by a factor of 10. Ross and Kuntz (9) determined the dissociation constant for the picric acid–naphthalene complex by a spectroscopic method and obtained a value which was markedly different from that obtained by Moore (10), who utilized a technique based on the partitioning behavior of the interactants between two immiscible liquids. Negoro *et al.* (11) found that the association constant for an interaction between pyrazinamide and *p*-aminosalicylic acid was also dependent on the method of detection. They demonstrated that solubility and calorimetric studies yielded similar values which were larger than that determined by spectroscopic studies. It is not entirely surprising to observe that spectroscopic studies yield information on strengths of interactions which is different from that obtained by methods which measure changes in the magnitude of thermodynamic or kinetic properties of participating species. Spectral changes are rather specific manifestations of charge-transfer interactions. The changes reflect the absorption of electromagnetic radiation by the ground state of a complex in promoting an electron transfer from donor to acceptor. Charge-transfer may or may not be a major component of those forces maintaining the association in the ground state. Other methods which are based on the detection of changes in thermodynamic or kinetic parameters would be expected to detect all types of interactions regardless of whether the forces which are responsible are charge-transfer, dipole–dipole, dispersion, or others.

Rather good evidence was obtained in this study to indicate that the complexed forms of the isoalloxazines were not susceptible to hydrolytic cleavage. Thus, the kinetic behavior that was predicted on the assumption that a 1:1 complex formed which did not undergo hydrolysis held over a thirtyfold range of 8-chlorotheophylline concentration. The close agreement between dissociation constant values calculated from kinetic and solubility data also supports the hypothesis that the complexed form did not decompose. It was previously speculated (1) that charge-transfer interaction might be expected to reduce the electrophilicity of the isoalloxazine, thereby rendering the molecule more resistant to the nucleophilic attack of hydroxide-ion. However, the fact that the spectral studies suggested a degree of interaction that was less than that determined from the kinetic data indicates that some other mechanism of stabilization was operating. Steric hindrance to the attack of hydroxide-ion might be an important factor here, and studies are being conducted to test this possibility.

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