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# Mechanisms of Formation and Equilibria of 4a and 5 Adducts of an Isoalloxazine. Reaction of 10-(2′,6′-Dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate with Sulfite in Aqueous Media[†]

Thomas C. Bruice,* Laszlo Hevesi,[‡] and Seiji Shinkai[‡]

ABSTRACT: The reaction of 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate (I) in aqueous sulfite–bisulfite buffers (30°, $\mu = 2.0$) yields an equilibrium mixture of I plus 4a- and 5-sulfite adducts (**4a**, **5**$^-$, and **5H** of eq 1). From the pH dependence of the relative concentration of **4a** and the kinetically apparent acid dissociation constant of **5H** (*i.e.*, **5H** → **5**$^-$ with p$K_{a_5}$) the pH and buffer independent equilibrium constant ($K_e$ = [**5H**]/[**4a**]) has been calculated to be $4.12 \times 10^{-2}$. Therefore, at 30° in aqueous solution, the 4a adduct is thermodynamically favored over the neutral 5 adduct. From studies of the dependence of the equilibrium constants $K_x$ = [**4a**]/[I] and $K_y$ = [**5**$^-$]/[I] upon pH and total sulfite buffer concentration ($[S]_T$) it was determined that $K_x$ was proportional to $[HSO_3^-]^{1.0}$ and $K_y$ proportional to $[SO_3^{2-}]^{1.0}$. These dependencies of $K_x$ and $K_y$ upon concentrations of buffer species establish that the forward reactions from I to **4a** and **5**$^-$ have in their rate expressions the terms $[HSO_3^-]$ and $[SO_3^{2-}]$, respectively, in excess over the retrograde reactions of **4a** → I and **5**$^-$ → I. The kinetics for approach to equilibrium in the conversion of I to products (**4a**, **5**$^-$, and **5H**) were studied under the pseudo-first-order conditions of [buffer] ≫ $[I_0]$. The pseudo-first-order rate constants ($k_{obsd}$) were found to be dependent upon three terms (eq 14); the first contained the product $[HSO_3^-][SO_3^{2-}]$, the second $[SO_3^{2-}]$, and the third was independent of buffer species but dependent upon the mole fraction of a reactant of p$K_{app}$ (p$K_{a_5}$) assignable to dissociation of **5H** → **5**$^-$. With the knowledge of the dependence of the equilibrium ratios [**4a**]/[I] and [**5**$^-$]/[I] upon $[HSO_3^-]$ and $[SO_3^{2-}]$, the rate terms were assignable (Scheme I) to: general acid (by $HSO_3^-$) catalysis of nucleophilic attack of $SO_3^{2-}$ upon I to yield **4a** and by microscopic reversibility general base (by $SO_3^{2-}$) catalysis of conversion of **4a** → I and unassisted nucleophilic attack of $SO_3^{2-}$ upon I to yield **5**$^-$ with spontaneous conversion of **5**$^-$ → I. An alternate scheme that would satisfy both the thermodynamic and kinetic findings would be that of Scheme II. Here I is converted to **5H** *via* general acid (by $HSO_3^-$) catalyzed attack of $SO_3^{2-}$ and to **5**$^-$ by unassisted attack of $SO_3^{2-}$, **4a** arising from rearrangement of **5H**. Arguments are presented which favor the mechanism of Scheme I.

This study deals with the reaction of $SO_3^{2-}$ with 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate (I) to yield both 5 (**5**$^-$ + **5H**) and 4a (**4a**) adducts (eq 1). In the previous paper (Hevesi and Bruice, 1973) it was established that **4a** was the predominant product, at neutrality, in the reaction of I with $SO_3^{2-}$. This result may be compared to the reaction of eq 2 (Hevesi and Bruice, 1973) which typifies the normal mode of addition of $SO_3^{2-}$ to flavines and flavinium salts (Müller and Massey, 1970). Inspection of the structures of I and II suggests that the formation of **4a** from I is due to steric and possibly electrostatic hindrance of approach of $SO_3^{2-}$ to the N-5 of I. Also possible is the initial formation of a steric and electrostatically destabilized 5 adduct followed by migration of the $SO_3^{2-}$ moiety to the 4a position (eq 3).

The object of this investigation has been to determine the difference in the free energies of **5H** and **4a** and to establish the mechanisms of their formation from I. Though the mechanisms of oxidation–reduction reactions involving flavines have been speculated to proceed *via* covalent adducts (Hamilton, 1971; Hemmerich, 1972), the only nucleophilic additions at the 5 position involve sulfite (Müller and Massey, 1970; Bruice *et al.*, 1971; Hevesi and Bruice, 1973) and phosphines (Müller, 1972), while the only 4a adduct arising *via* nucleophilic addition (dark) is **4a** (Hevesi and Bruice, 1973).

## Experimental Section

Kinetic studies were carried out at the $\lambda_{max}$ (435 nm) of I as previously described (Hevesi and Bruice, 1973). The concentration range of total sulfite buffer ($[S]_T$, where $[S]_T$ =

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[†] From the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received December 11, 1972.* This work was supported by grants from the National Institutes of Health and the National Science Foundation.

[‡] Postdoctoral Fellows at the University of California.

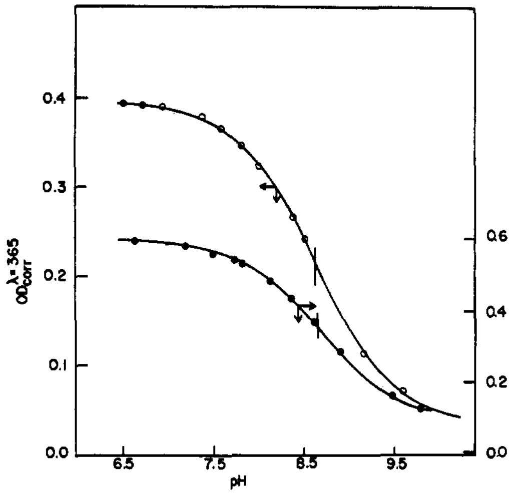
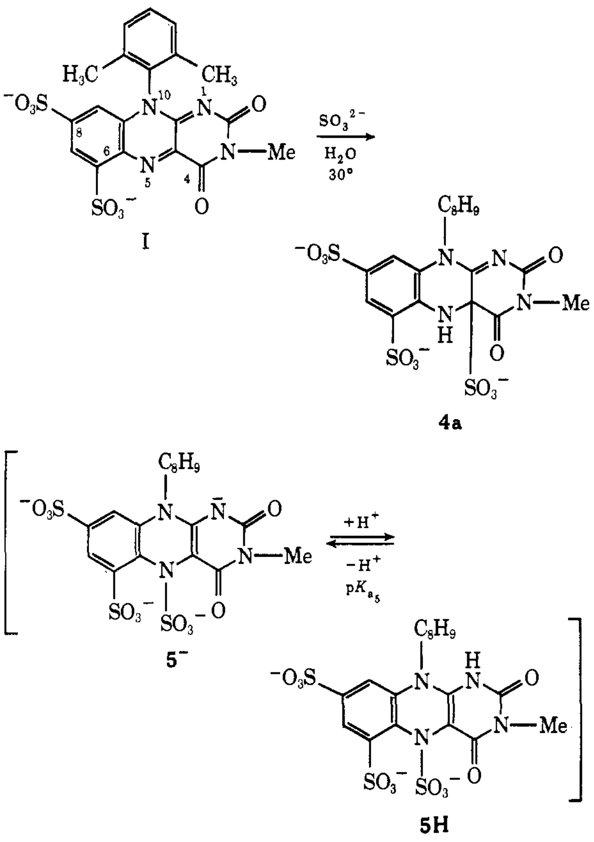
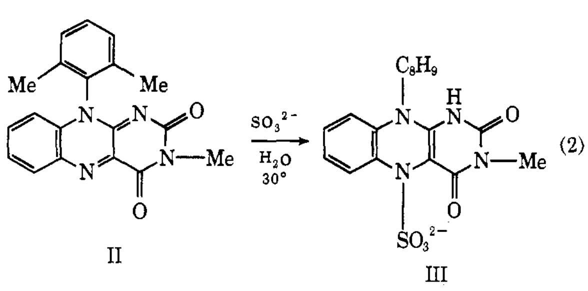


FIGURE 1: Spectrophotometric titration determination of the $pK_{app}$ which controls the ratio of products (*i.e.*, [**4a**]/[**5** + **5**$^-$]) in the reaction of I with $HSO_3^-$ + $SO_3^{2-}$: open circles, $[S]_T$ = 0.745 M; full circles, $[S]_T$ = 0.149 M ($\mu$ = 2.0; $T$ = 30°).



(1)



(2)

$$\text{I} \longrightarrow (\mathbf{5}^- + \mathbf{5H}) \longrightarrow \mathbf{4a} \quad (3)$$

$[SO_3^{2-}]$ + $[HSO_3^-]$) employed was 0.005–0.049 M. In this concentration range dimerization of the $HSO_3^-$ species (Schroeter, 1966) need not be taken into account. Buffer dilutions carried out under anaerobic and aerobic conditions were found to be indistinguishable.

Materials employed were those of the preceding study (Hevesi and Bruice, 1973).

The $pK_a'$ of $HSO_3^-$ was determined titrimetrically as previously described (Hevesi and Bruice, 1973). The conditions employed were those of the kinetic measurements (30°, 2.0 M KCl, and 4 vol % dimethylformamide). A value of $pK_{a_2}$ = 6.74 ± 0.02 was obtained.

Equilibrium distribution of products as a function of pH was determined at two different sulfite buffer concentrations ($[S]_T$ = 0.745 and 0.149 M) in the pH range of 6.5–9.8. The dimerization equilibria of $HSO_3^-$ (Schroeter, 1966) were taken into account by adjustment of $[S]_T$. At completion of reaction, accurate optical densities were measured on a Cary 16 spectrophotometer at 365 and 435 nm. In addition, the spectra of each of these reaction solutions were scanned on a Cary 15 recording spectrophotometer. Because the equilibrium mixtures always contain variable concentrations of I, the index for concentration of **4a** (absorbance at the $\lambda_{max}$ of **4a**, *i.e.*, 365 nm) required correction. This was done by assuming the adducts not to absorb at the $\lambda_{max}$ of I (435 nm). This is a most reasonable assumption since it is known that the $\epsilon$ values for 4a and 5 adducts of flavines are insignificant at this wavelength (Brüstlein and Hemmerich, 1968). Knowing $[I]_0$ plus its $\epsilon$ at 435 nm, the concentration of I could be calculated from the absorbance at $t_\infty$ at 435 nm. The total concentration of adducts ($[A]_T$) is then $[I]_0 - [I]_{t=\infty}$. However, since I absorbs at 365 nm, the absorbance at this wavelength ($OD_{app}^{365}$) is

$$OD_{app}^{365} = OD_{[A]_T}^{365} + OD_{[I]_{t=\infty}}^{365} \quad (4)$$

The value of $OD_{[A]_T}^{365}$ was then determined from the known values of $OD_{app}^{365}{}_{[I]_{t=\infty}}$ and the $\epsilon$ of I at 365 nm. In the pH range employed only the 5 adduct is ionizable (eq 5). Unlike

$$[A]_T = [\mathbf{5}^-] + [\mathbf{5H}] + [\mathbf{4a}] \quad (5)$$

usual spectrophotometric titrations, $[A]_T$ is not constant (*i.e.*, the observed difference in absorption as a function of pH is not due solely to interconversion of species **5**$^-$, **5H**, and **4a** but also to changing $[A]_T$), so that the OD observed at a given pH has to be adjusted ($OD_{cor}^{365}$) so that at each pH it reflects a constant $[A]_T$. Equation 6 has been employed for both

$$OD_{cor}^{365} = OD_{[A]_T}^{365}\left[1 + \frac{[I]_{t=\infty}}{[I]_{t=0} - [I]_{t=\infty}}\right] \quad (6)$$

titrations (Figure 1) at each pH value. The $pK_{app}$ values obtained for the adduct at the two values of $[S]_T$ are 8.62 and 8.64.

## Results

*Kinetic Studies.* The disappearance of I in sulfite buffers ($[S]_T$ = 0.005–0.049 M) between pH 6.00 and 7.49 was found to be pseudo first order. Plots of $k_{obsd}$ *vs.* $[S]_T$ at constant pH values are provided in Figure 2. From inspection of Figure 2, one may arrive at two important conclusions. (a) Since the buffer dilution plots exhibit an upward curvature the reaction

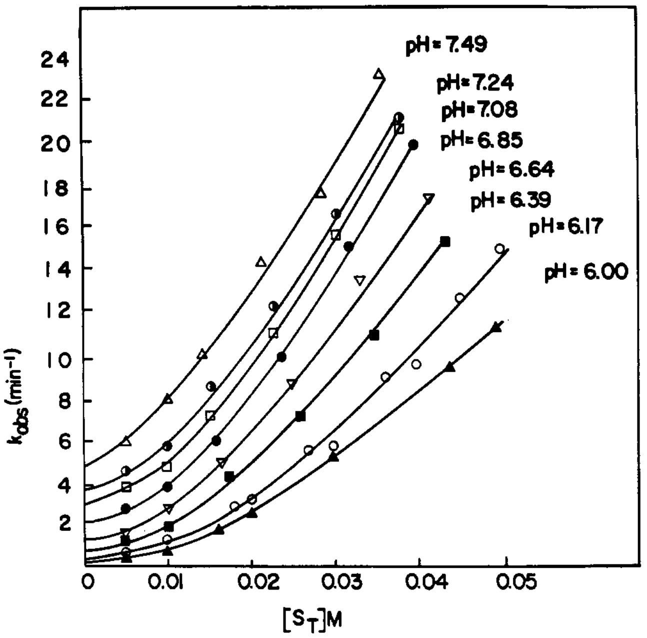


FIGURE 2: Dependence of the observed pseudo-first-order rate constants ($k_{obsd}$) on total buffer concentration ($[S]_T$) in the reaction of I with $S_T( = HSO_3^- + SO_3^{2-})$.

is dependent upon $[S]_T$ to more than the first power. (b) Extrapolation of the buffer dilution plots to $[S]_T = 0$ provides positive intercepts which is in accord with a reaction proceeding to an equilibrium position. At any constant pH the data of Figure 3 may be correlated by empirical eq 7 and 8.

$$k_{obsd} = A([S]_T)^2 + B[S]_T + C \tag{7}$$

$$\frac{k_{obsd} - C}{[S]_T} = A[S]_T + B \tag{8}$$

By iteration, values of $C$ were chosen which provided linear plots of $(k_{obsd} - C)/[S]_T$ *vs.* $[S]_T$. From these plots (Figure 3) the values of $A$ and $B$ were obtained as slopes and intercepts, respectively. The best values of $A$, $B$, and $C$ are collected in Table I.

Since the $C$ values represent intercepts to the buffer dilution plots at $[S]_T = 0$, $C$ must relate to the mole fraction of a product which is reverting to the starting material I in a back reaction. From the hydrogen ion dependence of $C$, eq 9 follows

TABLE I: Values of the Empirical Constants $A$, $B$, and $C$ of Equation 8.

| pH | $A$ ($M^{-2}$ min⁻¹) | $B$ ($M^{-1}$ min⁻¹) | $C$ (min⁻¹) |
|---|---|---|---|
| 6.00 | 4120 | 27.4 | 0.34 |
| 6.17 | 5210 | 38.6 | 0.39 |
| 6.39 | 6210 | 59.7 | 0.66 |
| 6.64 | 8310 | 88.2 | 0.95 |
| 6.85 | 9540 | 100 | 1.95 |
| 7.08 | 9990 | 114 | 2.94 |
| 7.24 | 8160 | 183 | 3.40 |
| 7.49 | 7900 | 255 | 4.60 |

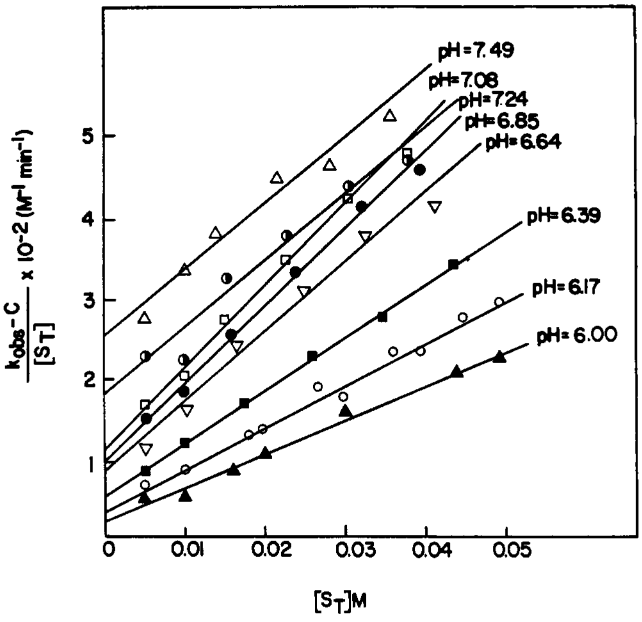


FIGURE 3: $(k_{obsd} - C)/[S_T]$ *vs.* $[S]_T$ after iteration for $C$ to give the best straight line.

$$C = k_c K_{a_c}/(K_{a_c} + a_H) \tag{9}$$

$$\frac{1}{C} = \frac{1}{k_c} + \frac{a_H}{k_c K_{a_c}} \tag{10}$$

From a plot of $1/C$ *vs.* $a_H$ (Figure 4) $1/k_c$ is obtained as the intercept and $1/k_c K_{a_c}$ as the slope ($k_c \cong 5.43$ min⁻¹; $pK_{a_c} \cong 7.21$). In considering the term $B[S]_T$ it is safe to assume that the most nucleophilic species of $[S]_T$ is $SO_3^{2-}$ represented as the mole fraction $K_{a_2}/(K_{a_2} + a_H)$ where $K_{a_2}$ is the dissociation constant of $HSO_3^-$. A plot of $B(K_{a_2} + a_H)/K_{a_2}$ *vs.* pH is found to be independent of pH (Figure 5) so that the relationship of eq 11 pertains, where $k_B = 183$ $M^{-1}$ min⁻¹. The positive de-

$$B[S]_T = k_B\left(\frac{K_{a_2}}{K_{a_2} + a_H}\right)[S]_T \tag{11}$$

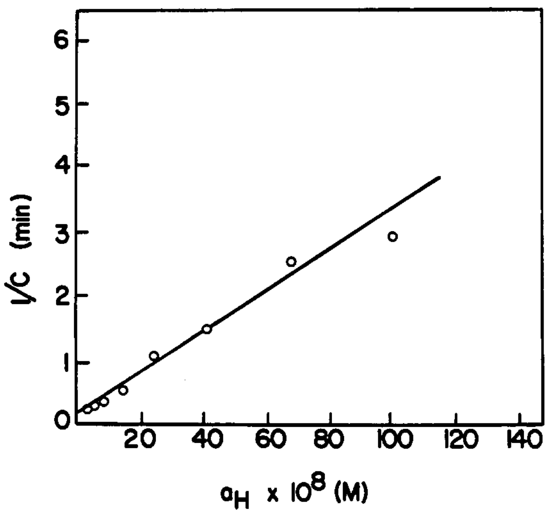


FIGURE 4: $1/C$ *vs.* $a_H$.

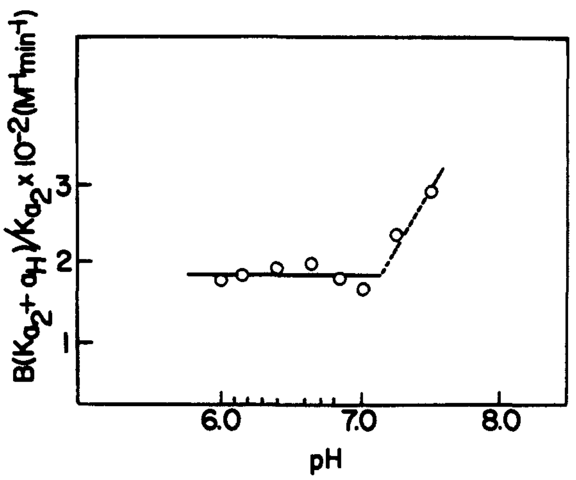


FIGURE 5: pH dependence of $B(K_{a_2} + a_H)/K_{a_2}$.

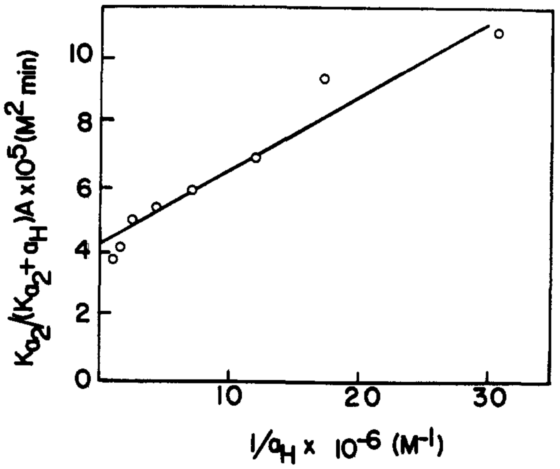


FIGURE 6: Dependence of $K_{a_2}/(K_{a_2} + a_H)A$ on $1/a_H$.

viation of the two values of $B$ above pH 7.24 may be attributed to intervention of an [HO$^-$]-dependent term at high pH. For the $A$ term, as in the case of the $B$ term, we may assume $SO_3^{-2}$ to be the attacking nucleophile. In this case, $[S]_T^2$ may pertain to either $[HSO_3^-][SO_3^{2-}]$ or to $[SO_3^{2-}]^2$. For either, $A$ must contain $K_{a_2}/(K_{a_2} + a_H)$ (eq 12). Equation 12

$$A[S]_T^2 = \frac{k_A K_{a_2} a_H}{(K_{a_2} + a_H)^2}[S]_T^2 \qquad (12)$$

can be rewritten as eq 13 which provides the linear relationship of Figure 6 from which we may derive the constants

$$\frac{K_{a_2}}{(K_{a_2} + a_H)A} = \frac{1}{k_A} + \frac{K_{a_2}}{k_A}\frac{1}{a_H} \qquad (13)$$

ship of Figure 6 from which we may derive the constants $k_A$ = 24,300 M$^{-2}$ min$^{-1}$ and p$K_{a_2}$ = 7.24. The kinetically derived value of p$K_{a_2}$ may be noted to exceed p$K_{a_2}$ obtained by titration (6.74) by 0.5 unit.

Since any conjugate acid of I would not possess a p$K_a$ value above zero, we may assume that p$K_{a_c}$ pertains to an acid dissociation constant of one of the products. This cannot be **4a** and comparison of the p$K_{a_c}$ value to that of III (p$K_{a_5}$ = 6.12), eq 2 (Hevesi and Bruice, 1973), suggests that acid dissociation of a 5 adduct (**5H**) accounts for the $C$ term. With this in mind, we can combine eq 9, 11, and 12 to arrive at eq 14 to describe the kinetics for reaction of I with sulfite buffer.

$$k_{obsd} = 24,300[SO_3^{2-}][HSO_3^-] + 183[SO_3^{2-}] + \frac{5.43K_{a_5}}{K_{a_5} + a_H} \qquad (14)$$

*pH Dependence of Product Distribution.* Figure 7 shows the effect of pH on the ultraviolet–visible spectra of the equilibrated reaction mixtures at constant buffer concentration ($[S]_T$ = 0.149 M). At low pH little I is present and the spectrum is mostly due to the 4a adduct ($\lambda_{max}$ 365 nm and a smooth shoulder around 300 nm). As the pH is raised the equilibrium mixture contains more and more of the starting material I as can be seen from the increasing absorbances at 435 nm. Also, another even more important change takes place. The decrease in 4a adduct is not only due to a smaller amount of I being transformed, but also to the appearance of the 5 adduct as shown by the increase in absorption at 305 nm and appearance of a maximum at this wavelength. At the highest pH values the 4a adduct has almost disappeared, the spectra being due to the anionic form (**5**$^-$) of the 5 adduct and starting material. The intersections of the spectra define three good isosbestic points at 298, 349, and 396 nm. A plot of the corrected absorbance of **4a** (Experimental Section, $OD_{cor}^{365}$) *vs.* pH at $[S]_T$ values of 0.745 and 0.149 M is provided in Figure 1. The points of Figure 1 are experimental and the lines theoretical titration curves for an acid of p$K_{app}$ = 8.62 and 8.64.

An equilibrium constant expressing the ratio of **5H**/**4a** ($K_e$) can be determined directly from the thermodynamic studies of the product distribution. For any mechanistic scheme the equilibrium of eq 15 pertains. From a material balance

$$\mathbf{4a} \underset{}{\overset{K_e}{\rightleftarrows}} \mathbf{5H} \underset{+H^+}{\overset{\overset{K_{a_5}}{-H^+}}{\rightleftarrows}} \mathbf{5}^- \qquad (15)$$

$$[\text{adduct}]_T = [\mathbf{4a}] + [\mathbf{5H}] + [\mathbf{5}^-] \qquad (16)$$

it follows that

$$[\mathbf{4a}] = \frac{[\text{adduct}]_T}{K_e + 1}\left[\frac{a_H}{a_H + K_{a_5}K_e/(K_e + 1)}\right] \qquad (17)$$

and

$$K_{app} = \frac{K_{a_5}K_e}{K_e + 1} \qquad (18)$$

From titration of $[\text{adduct}]_T$ (Figure 1) p$K_{app}$ was determined to be 8.62, while from the kinetics of the $SO_3^{2-}$ reaction with I (Figure 4) the p$K_{a_5}$ value has been determined as 7.21. Employing eq 18 with p$K_{a_5}$ = 7.21 and p$K_{app}$ = 8.62 it follows that $K_e \cong 4.12 \times 10^{-2}$.

From eq 17 the equilibrium constant $K_x$ = [**4a**]/[I] at $t_\infty$ may be expressed as in eq 19 and for the equilibrium constant $K_y$ = $[\mathbf{5}^-]/[I]_{t=\infty}$ eq 20 pertains. The measurement of $[I]_{t=\infty}$

$$K_x = \frac{[\text{adduct}]_T}{[I]_{t=\infty}}\left[\frac{a_H}{K_{a_5}K_e + K_e a_H + a_H}\right] \qquad (19)$$

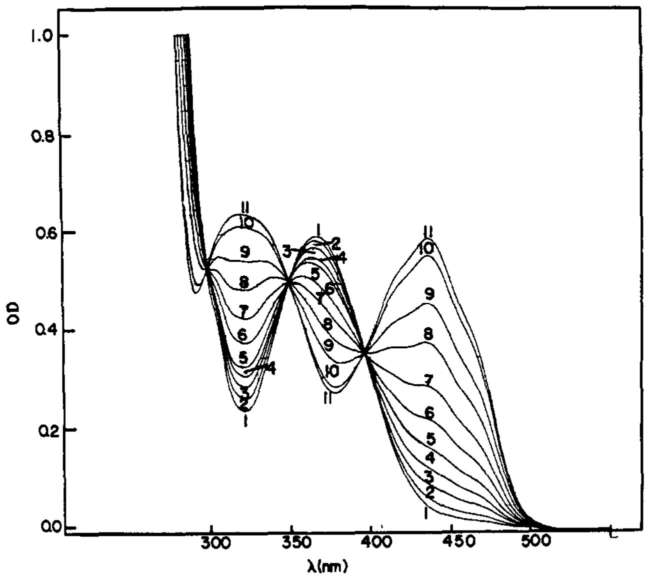


FIGURE 7: Effect of pH on ultraviolet–visible spectra of equilibrated reaction mixtures ($t_\infty$) $[S_T] = 0.149$ M ($\mu = 2.0$; $T = 30°$). By increasing numbering pH 6.62, 7.19, 7.48, 7.72, 7.81, 8.12, 8.35, 8.60, 8.90, 9.45, and 9.79.

$$K_y = \frac{[\text{adduct}]_T}{[\text{I}]_{t=\infty}}\left[\frac{K_{a_5}K_e}{K_{a_5}K_e + K_e a_H + a_H}\right] \qquad (20)$$

and $[\text{adduct}]_T$ (*i.e.*, $[\text{I}]_0 - [\text{I}]_{t=\infty}$, Experimental Section) at two different $[S]_T$ values provided $K_x$ and $K_y$ values as a function of pH (Figure 8). The shapes of the plots of Figure 8 are those of titration curves. At the higher pH values where dimerization of buffer is not significant, the values of $K_x$ and $K_y$ are proportional to $[S]_T$; the values of $K_x$ and $K_y$ being five times greater at the $[S]_T = 0.745$ M than at $[S]_T = 0.149$ M. This establishes that $K_x$ and $K_y$ are dependent upon $[HSO_3^-]^{1.0}$ and $[SO_3^{2-}]^{1.0}$, respectively.

## Discussion

The pH dependences of the concentration of isoalloxazine and 4a and 5 adducts (*i.e.*, [I + **4a** + **5H** + **5**$^-$]) at $t_\infty$ have been determined at two concentrations of $[S]_T$ (when $[S]_T \gg [\text{I}]_{t=0}$) as a function of pH. The results of this thermodynamic study dictate that: (a) at low pH **4a** predominates while at high pH (**5H** + **5**$^-$) predominates; (b) the concentration of **4a** relative to (**5H** + **5**$^-$) is a function of pH, being controlled by an apparent $pK_{app}$ of 8.62; (c) most importantly, $pK_{app}$ is not a function of $[S]_T$; and (d) the ratio of total adducts to starting material {[**4a** + **5H** + **5**$^-$]/[I]} is decreased with an increase in pH. The kinetics of the reaction of $SO_3^{2-}$ with I were studied under the conditions of $[S]_T \gg [\text{I}]_{t=0}$ and found to be pseudo first order ($k_{obsd}$) at any value of $[S]_T$ and pH. From the dependence of $k_{obsd}$ upon $[S]_T$ and pH the expression of eq 14 was arrived at. Because we are following the rates of reactions to an equilibrium position $k_{obsd}$ is the sum of the pseudo-first-order terms for forward and reverse reactions (eq 21). What remains is the assignment of the various

$$k_{obsd} = k_{obsd_f} + k_{obsd_r} \qquad (21)$$

terms for $k_{obsd}$ (eq 14) to either specific forward or reverse reaction steps with the restriction that any reaction sequence must be in accord with the findings that: (i) [**4a**]/[I] at $t_\infty$ increases proportionately to $[HSO_3^-]$; (ii) [**5**$^-$]/[I] at $t_\infty$ increases proportionately to $[SO_3^{2-}]$; and (iii) the [**5H**]/[**4a**] is independent of sulfite buffer concentration and pH.

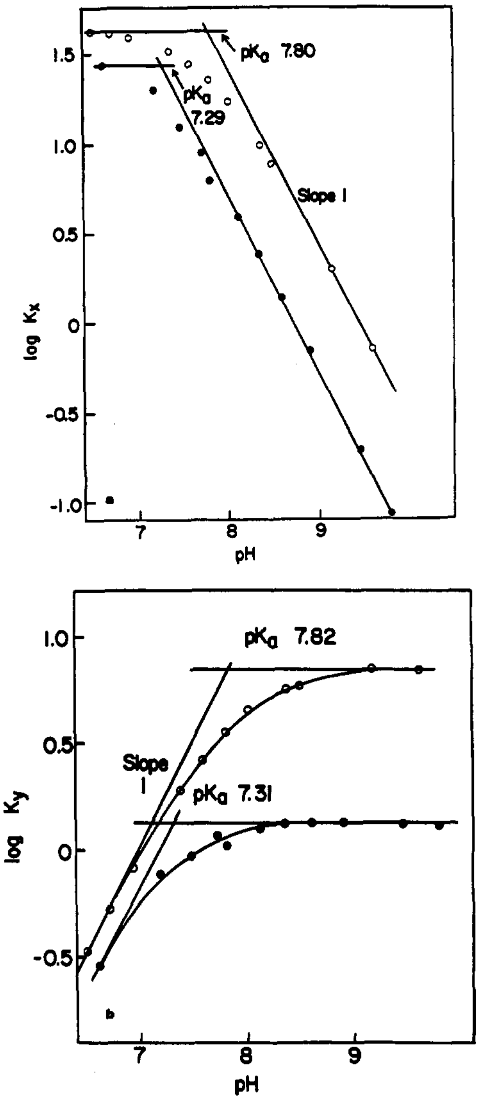
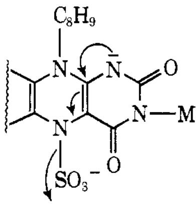


FIGURE 8: (a) pH dependence of equilibrium I ⇆ **4a**: (○) $[S_T] = 0.745$ M; (●) $[S_T] = 0.149$ M; (b) pH dependence of equilibrium I ⇆ **5**$^-$: (○) $[S_T] = 0.754$ M; (●) $[S_T] = 0.149$ M. At the $[S_T]$ values required, dimerization of $HSO_3^-$ is quite serious below pH ~8. This is reflected in the shift of apparent $pK_a$ values for $HSO_3^-$ and the fact that the effective concentration of $SO_3^{2-}$ does not increase in parallel to increase in $[S_T]$ below pH 8.

Since the $5.43K_{a_5}/(K_{a_5} + a_H)$ term (eq 14) arises from intercepts at $[S]_T = 0$ of plots of $[S]_T$ *vs.* $k_{obsd}$ at constant pH values, *it must* pertain to a reaction involving product reverting to starting material. Because the contribution of this term to $k_{obsd}$ is dependent upon the mole fraction of the basic species of an acid–base pair whose $pK_a$ (7.21) can only be associated with the deprotonation of N-1 of a 1,5-dihydroisoalloxazine, this term must pertain to IV. The $24{,}300[SO_3^{2-}] \times [HSO_3^-]$ term is assignable to either a forward or reverse process. Since it is not dependent upon the mole fraction of **5**$^-$ or **5H** present, it must pertain to a reaction proceeding from either starting flavine (I) or 4a adduct. The formation of sulfite-5 adducts of isoalloxazines and flavines is simply first order in $[SO_3^{2-}]$. Therefore, I → **5H** is not likely to depend

SCHEME I

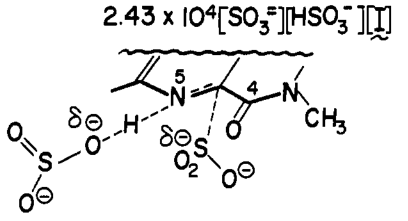
$$\begin{tikzcd}[column sep=huge]
\mathrm{I} \arrow[r, shift left, "{k_{\mathrm{ga}_1}[\mathrm{HSO_3}^-][\mathrm{SO_3}^{2-}]}"] \arrow[d, shift right, swap, "{k_n[\mathrm{SO_3}^{2-}]}"] & \mathbf{4a} \arrow[l, shift left, "{k_{\mathrm{gb}_1}[\mathrm{SO_3}^{2-}]}"] \arrow[d, dashed, shift left, "K_{\mathrm{e}}"] \\
\mathbf{5}^- \arrow[u, shift right, swap, "k_{-n}"] \arrow[r, shift left, "+\mathrm{H}^+"] & \mathbf{5H} \arrow[l, shift left, "-\mathrm{H}^+ \quad K_{\mathrm{a}_5}"] \arrow[u, dashed, shift left]
\end{tikzcd}$$



$$\frac{5.43K_{\mathrm{a}_5}}{K_{\mathrm{a}_5} + a_{\mathrm{H}}}$$

IV

upon $[\mathrm{HSO_3}^-][\mathrm{SO_3}^{2-}]$. It is most difficult to imagine how **4a** could revert to I in a reaction involving the product $[\mathrm{SO_3}^{2-}] \times [\mathrm{HSO_3}^-]$. On the other hand, conversion of I → **4a** through a transition state involving I, $\mathrm{SO_3}^{2-}$, and $\mathrm{HSO_3}^-$ (*e.g.*, V) is easy to envision. The general acid (by $\mathrm{HSO_3}^-$) catalyzed attack of a nucleophile ($\mathrm{SO_3}^{2-}$) upon the 4a position is to be anticipated. Thus, the basicity of N-5 of I is far less than that of the catalyst ($\mathrm{HSO_3}^-$), but in the product the p$K_\mathrm{a}$ of N-5–H greatly exceeds that of $\mathrm{H_2O}$ and $\mathrm{HSO_3}^-$. Since the transition state for nucleophilic attack at the 4a position must have a structure somewhere intermediate between that of I and the N-5 anion of **4a**, protonation of N-5 in the transition state V should greatly reduce its free-energy content. Since $\mathrm{HSO_3}^-$ has a p$K_\mathrm{a}$ between that of I and **4a** it should serve admirably as a general acid catalyst. Conversion of I → **5H** *via* $\mathrm{HSO_3}^-$ general acid catalyzed attack of $\mathrm{SO_3}^{2-}$ is not expected. Formation of 5 adducts on reaction of $\mathrm{SO_3}^{2-}$ with isoalloxazines has not been previously noted to involve general acid catalysis. Furthermore, the p$K_{\mathrm{a}_5}$ of **5H** is 7.21; $\mathbf{5}^-$ is a stable species so that the p$K_{\mathrm{a}_2}$ value of $\mathrm{HSO_3}^-$ should exceed that for the conjugate acid of the transition state. The $183[\mathrm{SO_3}^{2-}]$ term of eq 14 may pertain to a sum of or one of the following reactions: (i) I → **4a** by nucleophilic attack; (ii) I → $\mathbf{5}^-$ by nucleophilic attack; and/or (iii) conversion of **4a** → I by general base abstraction of a proton from N-5. Reaction i may be discarded, for by microscopic reversibility it would require spontaneous conversion of **4a** → I and no term in the rate eq 14 can be assigned to this process. Reaction ii is most reasonable and iii is required by microscopic reversibility V if we are to have attack of $\mathrm{SO_3}^{2-}$ upon I to yield **4a** *via* $\mathrm{HSO_3}^-$ general acid catalysis.



V

From the foregoing discussion we arrive at the following possible assignments to the rate terms of eq 14. From the assignments of eq 22, it is required that equilibrium constants

TABLE II: Calculated Values of $k_n$ and $k_{\mathrm{gb}_1}$ Employing Thermodynamic and Kinetic Values for Dissociation of $\mathrm{HSO_3}^-$ ($K_{\mathrm{a}_2}$).

| Equation | p$K_{\mathrm{a}_2}$ = 6.74 (Titrimetric) | p$K_{\mathrm{a}_2}$ = 7.24 (Kinetic) |
|---|---|---|
| 26 | $k_n = 11$ | 41 |
| | $k_{\mathrm{gb}_1} = 172$ | 142 |
| 23 and 24 | $k_n = 49$ | 50 |
| | $k_{\mathrm{gb}_1} = 38$ | 109 |

$$\mathrm{I} \underset{k_{\mathrm{gb}_1}[\mathrm{SO_3}^{2-}]}{\overset{k_{\mathrm{ga}_1}[\mathrm{HSO_3}^-][\mathrm{SO_3}^{2-}]}{\rightleftarrows}} \mathbf{4a} \qquad \mathrm{I} \underset{k_{-n}}{\overset{k_n[\mathrm{SO_3}^{2-}]}{\rightleftarrows}} \mathbf{5}^- \tag{22}$$

$K_x$ (= [**4a**]/[I]) and $K_y$ (= [$\mathbf{5}^-$]/[I]) are expressed as in eq 23 and 24. The correctness of the assignments of kinetic terms

$$K_x = \frac{k_{\mathrm{ga}_1}[\mathrm{HSO_3}^-]}{k_{\mathrm{gb}_1}} = \frac{k_{\mathrm{ga}_1}a_{\mathrm{H}}[\mathrm{S}]_{\mathrm{T}}}{k_{\mathrm{gb}_1}(K_{\mathrm{a}_2} + a_{\mathrm{H}})} \tag{23}$$

$$K_y = \frac{k_n[\mathrm{SO_3}^{2-}]}{k_{-n}} = \frac{k_nK_{\mathrm{a}_2}[\mathrm{S}]_{\mathrm{T}}}{k_{-n}(K_{\mathrm{a}_2} + a_{\mathrm{H}})} \tag{24}$$

(eq 22) is attested to by the fact that equilibrium determinations establish (Results) that the value of $K_x$ is dependent upon $[\mathrm{HSO_3}^-]^{1.0}$ and $K_y$ upon $[\mathrm{SO_3}^{2-}]^{1.0}$.

The summation of the assigned terms of eq 22 provides eq 25, which is seen to be of the form of the experimentally

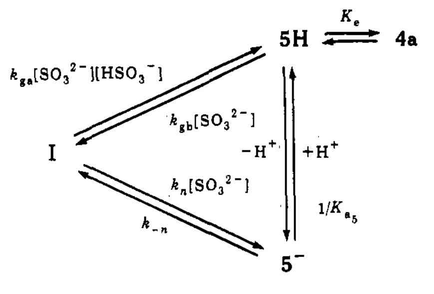
$$k_{\mathrm{obsd}} = k_{\mathrm{ga}_1}[\mathrm{SO_3}^{2-}][\mathrm{HSO_3}^-] + (k_n + k_{\mathrm{gb}_1})[\mathrm{SO_3}^{2-}] + \frac{k_{-n}K_{\mathrm{a}_5}}{K_{\mathrm{a}_5} + a_{\mathrm{H}}} \tag{25}$$

derived rate equation (14) where $k_{\mathrm{ga}_1} = 2.43 \times 10^4\ \mathrm{M}^{-2}\ \mathrm{min}^{-1}$, $k_n + k_{\mathrm{gb}_1} = 1.83 \times 10^2\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$, $k_{-n} = 5.43\ \mathrm{min}^{-1}$, and p$K_{\mathrm{a}_5}$ = 7.21. From eq 22 we arrive at Scheme I. The value of $K_\mathrm{e}$ is known from equilibrium measurements (Results) to be independent of pH and $[\mathrm{S}]_\mathrm{T}$, and the validity of Scheme I can be tested since the same value of $K_\mathrm{e}$ should pertain to the kinetic results. Mass balance of Scheme I provides eq 26. In eq 26, $K_\mathrm{e}$ is independent of $[\mathrm{S}]_\mathrm{T}$ and pH as shown experimentally.

$$K_{\mathrm{e}} = \left(\frac{k_{\mathrm{gb}_1}}{k_{\mathrm{ga}_1}}\right)\left(\frac{k_n}{k_{-n}}\right)\left(\frac{K_{\mathrm{a}_2}}{K_{\mathrm{a}_5}}\right) = \frac{(183 - k_n)k_n \times K_{\mathrm{a}_2}}{(2.43 \times 10^4)(5.43)(6.077 \times 10^{-8})} \tag{26}$$

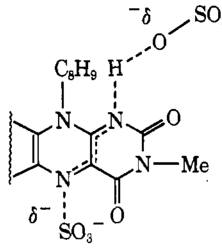
The independent values of $k_n$ and $k_{\mathrm{gb}_1}$ may now be determined from eq 26 or from eq 23 and 24 employing either the titrimetric or kinetically apparent value of p$K_{\mathrm{a}_2}$ (dissociation constant of $\mathrm{HSO_3}^-$). The results of these computations are provided in Table II. Examination of Table II reveals consistency in the values of $k_n$ and $k_{\mathrm{gb}_1}$ when the kinetically apparent values of p$K_{\mathrm{a}_2}$ are employed. It is a most common phenomena to find the thermodynamic p$K_\mathrm{a}$ to be either

Scheme II



larger or smaller than the kinetically apparent p$K_a$ (Bruice and Benkovic, 1966). This phenomena, which is so general and not completely understood, may be due among others to complexation by one or the other of the acid–base pair with species in the kinetic solution or any equilibrium process preceding the rate determining step which either follows or comes before the acid dissociation step (Bruice and Schmir, 1959).

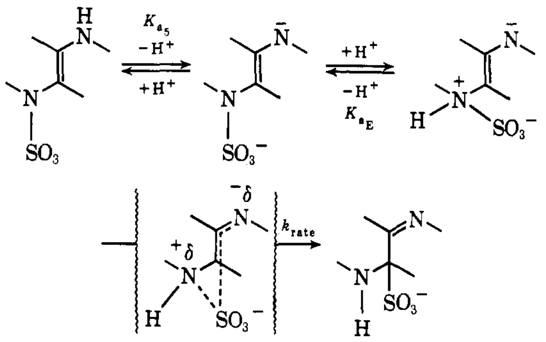
The only other reaction scheme which would be in accord with the thermodynamic and kinetic results is one in which **4a** arises directly from **5H** (Scheme II). Reasons have been provided for disfavoring general catalysis of the attack and its retrograde of $SO_3^{2-}$ upon I → **5** *via* VI, thus favoring Scheme I. A mechanism can be written for an isomerization of **5H** → **4a** (Scheme III). The rate determining step in Scheme III is an intramolecular nucleophilic attack by an ionic enamine species. Enamines like vinyl ethers are nucleophilic species (Coward and Bruice, 1969; Sollenberger and Martin, 1970) and an anionic enamine should be a powerful nucleophile. The kinetic expression for Scheme III is provided in eq 27. Since $K_{a_E} > K_{a_5}$ the reaction rate would be dependent on the mole fraction of **5H** as observed.



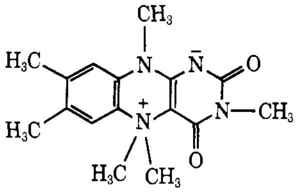
VI

$$k_{\text{obsd}} = k_{\text{rate}}\left[\frac{K_{a_5}a_H/(K_{a_5}+K_{a_E})}{\dfrac{K_{a_E}K_{a_5}}{(K_{a_5}+K_{a_E})}+a_H}\right] = \frac{k_{\text{rate}}K_{a_5}}{K_{a_E}}\left[\frac{a_H}{K_{a_5}+a_H}\right] \qquad (27)$$

Scheme III



The possibility of realizing an intramolecular **5** → **4a** group transfer reaction has been explored with 3,5,5-trimethyl-1,5-dihydrolumiflavine (VII). Compound VII was found to be stable at 60° in aqueous or ethanolic 1.0 N NaOH or HCl for 3 hr (Brüstlein and Bruice, 1972).



VII

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