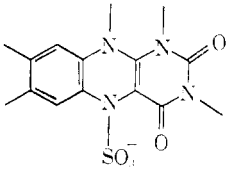
Reaction of Sulfite with Isoalloxazines†

Laszlo Hevesi‡ and Thomas C. Bruice*

ABSTRACT: The reaction of SO_3^{2-} (30° in H_2O, μ = 2.0) with isoalloxazines is kinetically a two-step process of the form A $\rightleftarrows$ B $\rightarrow$ C. Both steps are first order in SO_3^{2-} and B exists in reactive acid and base forms. A kinetically non-rate-determining conversion of C $\rightarrow$ D is also dependent upon $[SO_3^{2-}]$. Kinetic and thermodynamic parameters as well as the structures of the products and intermediates are summarized in Scheme I. From this investigation two observations are of particular interest. The first is that SO_3^{2-} carries out a nucleophilic addition to the 8 position of V and VI with expulsion of SO_3^{2-} from the 5 position. The second observation deals with nucleophilic addition of SO_3^{2-} to the 5 and 4a positions. When the 6 position is unoccupied, SO_3^{2-} adds to the 5 position (Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244*, 4007) of flavines, flavinium salts, and isoalloxazines (III $\rightleftarrows$ V, Scheme I). However, substitution of a bulky and charged sulfonic acid substituent at the 6 position facilitates formation of the 4a adduct (IX $\rightarrow$ X, Scheme I). The formation of X by addition of SO_3^{2-} to IX marks the first instance of the formation of a 4a adduct of an isoalloxazine (flavine) by overall nucleophilic addition in a dark reaction.

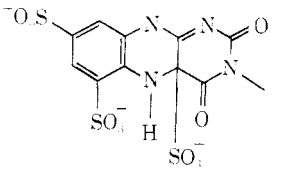
The reaction of sulfite with flavines (Müller and Massey, 1969) and flavoenzymes (Swoboda and Massey, 1966; Massey *et al.*, 1969) was initially reported by Massey and coworkers. These studies were not only of a most important biochemical nature but revealed an interesting aspect of the dark reactions of flavines. Among the many flavoenzymes investigated (oxidases, reductases, dehydrogenases, and hydroxylases) only the oxidases were found to yield sulfite flavine addition products. Thus, a correlation exists between the ability of fully reduced flavoenzymes to react with O_2 and the ability of their oxidized forms to react with sulfite. The flavine moiety of oxidases and dehydrogenases must, therefore, reside at very different types of enzyme sites. Further support for this contention arises from the observation that oxidases yield red (anionic) semiquinone while reductases and dehydrogenases yield blue (neutral) semiquinone forms. All the enzyme–sulfite complexes studied by Massey and coworkers were catalytically inactive and insensitive to oxygen. On the basis of spectral evidence, the general structure I was assigned to the isolable



I

sulfite adducts of the flavinium salts. Subsequently, Michaels *et al.* (1970, 1971) reported that adenyl sulfate reductase (apparently a flavoprotein dehydrogenase) reacts with sulfite to form a catalytically active adduct which may well be an intermediate in the transfer of sulfite to AMP at the level of sulfate.

Initial presentation of I seems odd since its formation entails nucleophilic attack at the nitrogen terminal of an azomethine bond. Though the –N(5)=C(4a)– bond forms a portion of a conjugated system, little precedent exists in the literature for this type of process which is somewhat akin to a nucleophilic attack at the oxygen of a conjugated carbonyl group. Surprise at this course of the reaction is somewhat alleviated by theoretical calculations (P.-S. Song, 1972)[1] which implicate the N(5) position of isoalloxazine rings as the most electrophilic. If for no other reason than the apparent oddity of the reaction, we have investigated the reaction of sulfite with isoalloxazines (anaerobic) and found, most surprisingly, that the final product (aerobic) is assignable to the structure II. In this paper we describe our studies of the



II

kinetics of formation, isolation, and structural assignment to product II.

Experimental Section

Determination of the pK_{a2} of Sulfurous Acid. This was done by titrating bisulfite solutions of different concentration with standard sodium hydroxide. The pH was measured with a Radiometer pH meter Type PHM 26 at 30°. In each pK_a determination a solution of a known amount of K_2SO_3 was acidified with HCl to convert all SO_3^{2-} to HSO_3^- and diluted to the desired volume. The titrations were carried out with a Radiometer autoburet Type ABU 12 under nitrogen flow. The pK_a values were obtained by comparison of the milliliters of NaOH added *vs.* pH plots to theoretical titration curves and by use of a program written for an Olivetti-Underwood Programma 101 computer. From $[S_T]$ = 0.01–0.5 M at μ = 1.0 (with KCl) the pK_a = 6.62–6.63.

† From the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received September 15, 1972.* This work was supported by grants from the National Science Foundation and the National Institutes of Health.

‡ Postdoctoral Fellow, Department of Chemistry, University of California at Santa Barbara, Santa Barbara, Calif. 93106.

[1] Song, P.-S. (1972), personal communication.

Kinetic Measurements. All kinetic measurements were carried out at 30° in aqueous solution at μ = 2.0 (with potassium chloride) under anaerobic conditions by using modified Thunberg cuvets. The buffer solutions were prepared from potassium sulfite and hydrochloric acid (air free); distilled water was boiled and cooled under nitrogen before use. No external buffer was used. The reaction mixtures were obtained as follows: the necessary amount of sulfite–bisulfite buffer solution was introduced in the nitrogen flushed Thunberg cell and 0.2 ml of a DMF[1] solution of the isoalloxazine was deposited in the side arm of the cell. After closing, the cell was put under vacuum (water aspirator) for a few seconds. Nitrogen was readmitted. This cycle was repeated 30 times. The final nitrogen pressure in the cell was 1 atm. After equilibration of the cell at 30°, the content of the side arm was rapidly mixed with the buffer and the reactions were monitored on a Cary-16 spectrophotometer. In Figure 1, there is presented a repetitive scan of the course of reaction between 310 and 550 nm. Examination of Figure 1 reveals that two reactions may be monitored: (a) rapid isoalloxazine disappearance at 435 nm followed by (b) slow appearance of a final product at 365 nm. Kinetically the overall reaction is of the form A → B → C. The values of the pseudo-first-order rate constant (k_{obsd}) were calculated using programs written for an Olivetti-Underwood Programma 101 computer. The rate constants for the first reaction (λ = 435 nm) were calculated by use of the Guggenheim treatment; those for the second reaction (λ = 365 nm) were calculated by both a first order and a Guggenheim program. In the conversion of the pseudo-first-order rate constants into second-order rate constants, the dimerization equilibrium $2HSO_3^- \rightleftarrows S_2O_5^{2-} + H_2O$ ($K = 7 \times 10^{-2}$ M^{-1} (Schroeter, 1966)) has been taken into account to calculate the total buffer concentration [S_T]. Since the volume of the buffer (or diluted buffer) was 4 ml in the cell, the DMF concentration of the reaction mixture did not exceed 5% (v/v). At the end of each kinetic measurement, the pH of the solution was checked.

Materials. Potassium chloride, potassium sulfite, and potassium metabisulfite were reagent grade and used without further purification.

3-Methyl-10-(2′,6′-dimethylphenyl)isoalloxazine. *N*-(2,6-Dimethylphenyl)-*o*-phenylenediamine was prepared in a way similar to that used before in this laboratory (Main *et al.*, 1972). To a hot glacial acetic acid solution containing about 2.4 g (0.01 mol) of the diamine were added 1.6 g (0.01 mol) of alloxan monohydrate and 0.8 g (0.013 mol) of boric acid, also dissolved in hot acetic acid. For a very short time the mixture was colored blue, then it turned dark green, while a green-brown precipitate was formed. Stirring was continued for 1 hr at 60°. The mixture was allowed to stand overnight at room temperature and it was then filtered (0.7 g of white solid), and the filtrate was evaporated to dryness *in vacuo*. The residue, 10-(2′,6′-dimethylphenyl)isoalloxazine, was then methylated at the 3 position in DMF solution at room temperature with methyl iodide and in the presence of powdered carbonate (Hemmerich, 1964). The degree of methylation was followed with thin-layer chromatography. After 36 hr of reaction, the solid components were filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was taken up with chloroform and chromatographed on a silica gel column using a mixture of chloroform–hexane (50% v/v) as eluent. The compound was recrystallized twice from a mixture of acetone–hexane (75–25% v/v); 1.4 g (42%) of yellow prisms, mp 298–300° (Main *et al.* (1972), 297–299°).

3,10-Dimethylisoalloxazine was prepared in the same manner, but in a lower yield (10%), mp 301–330° (Main *et al.* (1972), 302–304°).

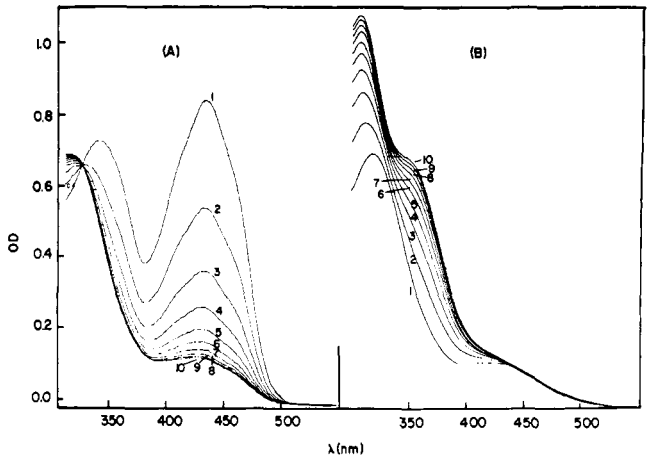


FIGURE 1: Time course of the reaction of sulfite with III ($[III]_0$ = 1.033×10^{-4} M^{-1}, $[S_T]$ = 0.5 M^{-1}, pH 6.1). (A) Scan speed ≃ 10 nm/sec: curve 1, 10 sec after mixing; curves 2–10, 50-sec interval. (B) Curve 1, 10 min after mixing; curves 2–10, 30-min interval.

7,8,10-Trimethylisoalloxazine (lumiflavin) was obtained in the following manner: 2-nitro-4,5-dimethylaniline was methylated (Johnstone *et al.*, 1969) to give the corresponding *N*-methylaniline, which was reduced with hydrazine over Raney nickel catalyst. The *o*-phenylenediamine obtained was then condensed with alloxan in hot acetic acid solution and in the presence of a slight excess of boric acid. Lumiflavin precipitated almost quantitatively. The crude product was recrystallized twice from formic acid. The yellow crystals obtained were dried 10 hr under vacuum at 130°, mp 321° dec (Hemmerich *et al.* (1956), mp 322° dec).

Product Analysis

(*1*) *First Reaction.* Due to its solubility and instability in the absence of a great excess of inorganic sulfite, the product of the first reaction could not be isolated. However, on the basis of ultraviolet–visible spectral evidence, we attribute to it the structure of a 5-sulfite adduct I. Indeed, in more favorable cases, Müller and Massey (1969) isolated and identified 5-sulfite adducts which showed ultraviolet (uv) absorption in the 300–320-nm region. In our case, the product of the first reaction with 3-methyl-10-(2′,6′-dimethylphenyl)-isoalloxazine has an absorption band which is similar to those observed by Müller and Massey (1969), both in its shape and its position (λ_{max} 307 nm; estimated ϵ_{max} ≃8700 M^{-1} cm^{-1} corrected for buffer absorption).

(*2*) *Second Reaction.* (a) ANAEROBIC REACTION WITH AEROBIC WORK-UP. A typical attempt to realize the second reaction on a preparative scale was the following: 30 g of $K_2S_2O_5$ (0.135 mol) and 12 g of K_2SO_3 (0.076 mol) were dissolved in 90 ml of deaerated water (pH 6.2); 0.33 g (0.001 mol) of 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine was suspended in the sulfite–bisulfite solution and the mixture was heated to 90° and stirred under nitrogen flow for ~16 hr. One drop of the resulting clear yellow solution was diluted anaerobically in water and the ultraviolet–visible spectrum recorded; λ_{max} at 304 nm and 380 nm. When crystals of K_2SO_5 and $K_2S_2O_5$ were added to the anaerobic solution, the longer wavelength absorption band shifted to shorter wavelengths and the spectrum resembled very closely the spectra obtained at the end of the kinetic runs (λ_{max} at 304 nm [ϵ 11,800 M^{-1} cm^{-1}]

and 355–365 nm [ϵ 7200 M^{-1} cm^{-1}] corrected for buffer absorption).

For the product isolation, the reaction mixture was then poured into a fritted Büchner funnel (jacketed with an ice–sodium chloride mixture) containing concentrated HCl through which N_2 was being passed by means of the fritted bottom. By this means sulfite was converted to SO_2 which was swept from the reaction mixture. Soon potassium chloride began to precipitate and the solution turned orange-red. When no more SO_2 was detectable (at this time much of the solvent water was also eliminated), 50 ml of methanol was added to precipitate most of the potassium chloride. The mixture was filtered and the orange-red solution evaporated to dryness *in vacuo*. From this point on, no care was taken to exclude air. The brown-red residue was recrystallized from hot water. On cooling, small yellow needles were formed which were filtered off and dried over P_2O_5 (yield, ~400 mg, 70%).

The product turns gradually brown and then black above 310° without melting. It is insoluble in chloroform, but soluble in alcohol and water. The λ_{max} values of 221, 270, 335, and 435 nm (ϵ 31,300, 30,000, 8900, and 10,600 M^{-1} cm^{-1}) are typical for isoalloxazine and similar to that of the starting material (λ_{max} 265, 345, and 435 nm; ϵ 37,000, 8500, and 10,400 M^{-1} cm^{-1}, respectively); infrared (ir) spectra 3420 (br), 3000–3100 (br), 1770, 1700, 1630, 1585, 1560, 1470, 1430, 1390, 1350, 1320, 1240 (br), 1210 (br), 1108, 1054, 1037, 1004, 890 (weak), 870 (weak), 790, 770, 750, 660, 630 and 600 cm^{-1}; nuclear magnetic resonance (nmr) spectra in Me_2SO-d_6 solution, singlet at 115 Hz from Me_4Si (6 H, from the 2,6-dimethylphenyl substituent), singlet at 197 Hz from Me_4Si (3 H, N(3)–CH_3), doublet at 420 Hz (J = 2 Hz, 1 H, aromatic ring of the isoalloxazine nucleus), singlet at 446 Hz (3 H, aromatic ring of the 10 substituent), doublet at 500 Hz (J = 2 Hz, 1 H, aromatic ring of the isoalloxazine nucleus); neutralization equiv, 2.05.

Anal. Calcd for $C_{19}H_{16}N_4O_5S_2 \cdot 4H_2O$ (mol wt = 564.5): C, 40.42; H, 4.28; N, 9.93; S, 11.36. Found: C, 40.69–40.10; H, 4.36–4.16; N, 9.93; S, 11.12.

(b) AEROBIC REACTION AND AEROBIC WORK-UP. The same procedure was employed as in (a) with the exception that air was not excluded and the reaction was not quenched in HCl under N_2. At the completion of reaction and prior to working up of product an aliquot of the reaction mixture was diluted with H_2O and its spectrum scanned as in (a). In this experiment a different product was obtained as attested to by the λ_{max} 365 and a shoulder at ~300 nm. Without destroying excess sulfite, the reaction mixture was evaporated to dryness and the residue extracted with absolute methanol. The methanol extract was then evaporated to dryness and the resulting residue once again extracted with a smaller amount of methanol, and so on, until the final residue contained only a small amount of sulfite–bisulfite; then it was recrystallized twice from water (small yellow crystals which fall apart on heating and do not melt by 360°).

The uv–visible spectrum of the product was identical with that obtained in (a); ir spectrum 3500 (br), 1720, 1660, 1615, 1585, 1560, 1460, 1420, 1385, 1350, 1310, 1225 (br) 1110, 1060, 1040, 1015, 885, 870, 804, 775, 752, 720, 675, 640, 630, and 607 cm^{-1}. The nmr spectra are very similar to those of (a) (same attribution of peaks): singlet at 116 Hz (6 H), singlet at 138 Hz (3 H), doublet at 423 Hz (1 H), singlet at 449 Hz (3 H), doublet at 503 Hz (1 H).

Anal. Calcd for $C_{19}H_{14}N_4O_8S_2K_2 \cdot 4H_2O$ (mol wt = 640.7): C, 35.62; H, 3.46; N, 8.74; S, 10.01; K, 12.2. Found: C, 34.97; H, 3.62; N, 8.95; S, 10.25; K, 11.66. (The per cent K was determined by atomic absorption analysis.)

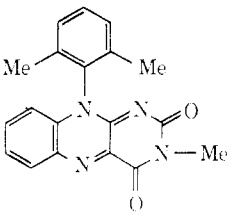
When the dipotassium salt, dissolved in a minimum volume of hot water, was acidified with concentrated HCl, the solution turned from yellow to red and on cooling deposited small brown-red needles. Collection of the crystals and recrystallization from hot methanol yielded a yellow crystalline product identical with the disulfonic acid of (a).

(c) ANAEROBIC REACTION IN D_2O. Three grams of $K_2S_2O_5$ and 4.3 g of K_2SO_3 were dissolved in 7 ml of deaerated D_2O to which was then added 0.33 g of 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine and the suspension was refluxed (105°) with stirring under an argon atmosphere for 100 hr. The mixture was then diluted twice with deaerated D_2O to give a dark yellow solution. The nmr spectrum of this solution was recorded: singlet (6 H) at 137 Hz, singlet (3 H) at 196 Hz, doublet (1 H, $J \simeq 2.3$ Hz) at 344 Hz, and a singlet (3–4 H) at 440 Hz. Small absorptions were observed (summed intensity corresponding to 1.5–2 H) in the 120–240-Hz region.

A portion of the dark yellow solution was oxidized by air bubbling for 2 hr. The resulting solution was clear yellow and had the following nmr spectrum: singlet (3 H) at 112 Hz, singlet (3 H) at 137 Hz, singlet (3 H) at 196 Hz, doublet (0.85 H, J = 2 Hz) at 402 Hz, narrow multiplet (3 H) at 440 Hz, and a doublet (1 H, J = 2 Hz) at 475 Hz. The small absorptions in the 120–240-Hz region observed before oxidation had all disappeared. The uv–visible spectrum of the oxidized solution was identical (λ_{max} 365 nm, shoulder at ~300 nm) with that of the aerobic reaction product.

Results

The reaction of isoalloxazines with sulfite buffers is kinetically a two-step process. A careful product and kinetic analysis of the reaction was carried out with 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine (III). Under the experimental conditions of total sulfite concentration ([S_T] ≫ [III]) both kinetically detectable processes are pseudo first order to at least 3 half-lives (pH 5.90–7.66 at total sulfite buffer concentration of ~0.05–0.8 M; 30° in H_2O, μ = 2.0 with KCl). Plots of the pseudo-first-order rate constants for the first kinetic step (k_{obsd_1}) *vs.* [S_T] at the various pH values employed are shown in Figure 2. The slopes of the plots of Figure 2 (k_2') are plotted *vs.* pH in Figure 3. Though the reaction of III is apparently first order in [SO_3^{2-}] to completion of the reaction, the per cent of the initial isoalloxazine consumed is a function of $[SO_3^{2-}]_{t=0}$. This observation establishes that the first reaction is an equilibrium process



III

$$k_{obsd_1} = k_{obsd_f} + k_{obsd_r} \quad (1)$$

$$\text{III} + SO_3^{2-} \xrightarrow{k_f} F^-SO_3^- \quad (2)$$

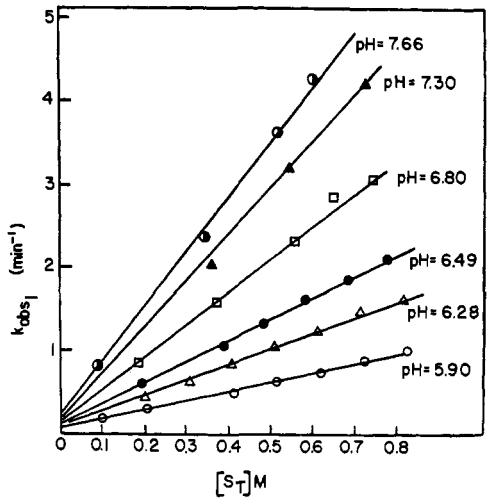


FIGURE 2: Effect of the total buffer concentration ([S_T]) on the pseudo-first-order rate constants (k_{obsd_1}) for the first kinetic step (λ 435 nm, T = 30°, solvent H_2O, μ = 2.0 with KCl, [S_T] has been corrected for the dimerization equilibrium of HSO_3^-).

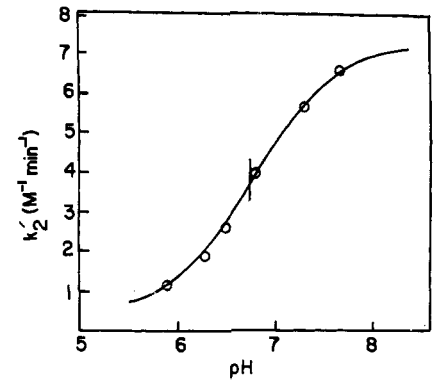


FIGURE 3: pH dependence of the apparent second-order rate constant (k_2') for the first kinetic step.

$$F^-SO_3^- \xrightarrow{k_r} III + SO_3^{2-} \quad (3)$$

$$F^-SO_3^- \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} HFSO_3^- \quad (4)$$

(K_{a_3})

so that

$$k_{obsd} = k_f[SO_3^{2-}] + k_r\left(\frac{K_{a_3}}{K_{a_3} + a_H}\right) \quad (5)$$

$$= k_2'[S_T] + k_r\left(\frac{K_{a_3}}{K_{a_3} + a_H}\right) \quad (6)$$

where

$$k_2' = k_2\left(\frac{K_{a_2}}{K_{a_2} + a_H}\right) \quad (7)$$

and

$$H_2SO_3 \underset{+H^+}{\overset{pK_{a_1}\ -H^+}{\rightleftharpoons}} HSO_3^- \underset{+H^+}{\overset{pK_{a_2} = 6.63\ -H^+}{\rightleftharpoons}} SO_3^{2-} \quad (8)$$

From eq 7 the value of k_2 ($\equiv k_f$) is calculated: 6.5 l. mol^{-1} min^{-1}. Defining

$$K_e = \frac{[F^-SO_3^-] + [HFSO_3^-]}{[F][SO_3^{2-}]} = \frac{[FS_T]}{[F][SO_3^{2-}]} \quad (9)$$

Then

$$\frac{k_f}{k_r} = \frac{K_e K_{a_3}}{K_{a_3} + a_H} \quad (10)$$

The value of K_e can be estimated (Table I) at each pH value from the residual absorption of III at 435 nm at $t = \infty$ for the first step and K_{a_3} is determinable (Figure 4) from the pH dependence of the second kinetic step (see following paragraph). This allows the calculation of k_f/k_r = 6.5 l. mol^{-1} min^{-1}/0.328 min^{-1} = 19.8 M^{-1}.

The pseudo-first-order rate constants for the second kinetically detectable process (k_{obsd_2}) were found to be linearly dependent on [S_T] at any constant pH. The experimental results for this process are summarized in Table II. Plots of the k_{obsd} values of Table II *vs.* pH at any constant value of [S_T] are found to be bell-shaped. This result is readily explained on the basis of the reaction of SO_3^{2-} with the undissociated product of the first reaction ($HFSO_3^-$) which exists in acid–base equilibria with its conjugate base form (eq 7)

$$v = [SO_3^{2-}](k_3[HFSO_3^-] + k_4[^-FSO_3^-]) \quad (11)$$

$$k_{obsd} = \frac{k_3 K_{a_2} a_H + k_4 K_{a_2} K_{a_3}}{(K_{a_2} + a_H)(K_{a_3} + a_H)}[S_T] \quad (12)$$

or

$$k_{obsd} = \left(\frac{k_3 a_H + k_4 K_{a_3}}{K_{a_3} + a_H}\right)[SO_3^{2-}] \quad (13)$$

The form of eq 12 is seen to provide the requisite bell shape (Bruice and Benkovic, 1966) for a plot of k_{obsd} *vs.* pH at constant [S_T]. From the kinetically equivalent expression of eq 13, a plot of $k_{obsd}/[SO_3^{2-}] = k_2''$ *vs.* pH should be of sigmoid shape with a inflection at pK_{a_3}. This is shown in Figure 4 where the points are experimental and the curve theoretical for an acid of pK_{a_3} = 6.31. The value of k_3 is obtained from the curve where $a_H \gg pK_{a_3}$ and the value of k_4 from the curve where $pK_{a_3} \gg a_H$. The requisite constants to generate the curve of Figure 2 are k_3 = 0.16 and k_4 = 0.014

TABLE I: Estimated Values of K_e for Reversible Addition of SO_3^{2-} to III.

pH	K_e
5.90	74.2
6.28	42.0
6.49	34.2
6.80	28.6
7.30	21.0
7.66	20.1

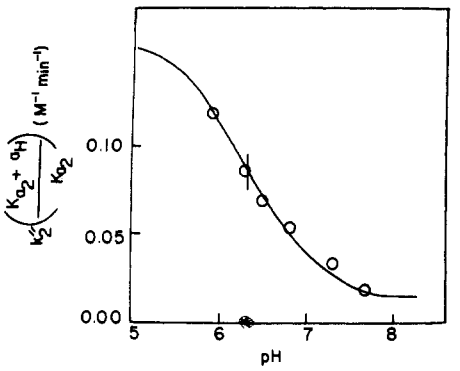
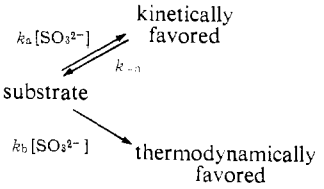


FIGURE 4: pH dependence of the apparent second-order rate constant $k_o''(1 + a_H/K_{a_2})$ for the second kinetic step.

M^{-1} min^{-1}. The reversibility of the second kinetic process was investigated by comparing the OD_∞ values at different buffer concentration and different pH values. They were found to be constant as far as the substrate concentration was constant showing irreversibility at least at the concentrations employed.

Discussion

It has previously been noted in sulfite addition reactions to nitrogen heterocycles that a two-step process may indicate the initial formation of a kinetically favored product prior to the formation of the thermodynamically more stable product (eq 14) (Pitman and Ziser, 1970; Pitman *et al.*, 1970; Albert



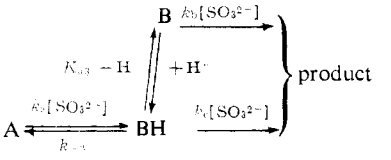
(14)

et al., 1961). The isoalloxazine III reacts with SO_3^{2-} in two kinetically discernible steps. The first is an equilibrium process which in the forward direction is first order in [III] and first order in $[SO_3^{2-}]$. The second kinetic step is apparently irreversible and represents two parallel processes first order in $[SO_3^{2-}]$. In one of the parallel reactions, the substrate is an acid of $pK_a = 6.31$ and in the second its conjugate base.

Since our first kinetic process goes to virtual completion at its equilibrium position prior to the onset of the second kinetically determinable step, it follows that if the scheme of eq 14 were in effect and $k_1 > k_2 > k_{-1}$ then III would be present at steady-state concentration during the formation of the thermodynamically favored product. The condition that $k_1 > k_2 > k_{-1}$ pertains in the region of pH 6.0. A steady-state derivation reveals that formation of the thermodynamically favored product would then be independent of $[SO_3^{2-}]$ (eq 15).

$$\frac{-d[\text{thermodynamically stable product}]}{dt} = \frac{k_a k_{-a}[\text{substrate}]}{k_a + k_b} \quad (15)$$

Since both kinetic steps are first order in $[SO_3^{2-}]$ the scheme of eq 14 is not possible and it is concluded that the correct sequence is of the type A $\rightleftarrows$ B $\rightarrow$ C. Taking into account the pH-dependent parallel nature of the second kinetic process, we arrive at eq 16.



(16)

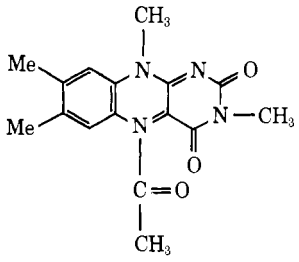
Müller and Massey (1969) assigned to the product from the reaction of SO_3^{2-} with flavines and flavinium salts the general structure of I. This assignment was based on spectral properties and the similarity of the spectra of their products to that of IV whose absolute structure was determined by X-ray (Kierkegard *et al.*, 1971). The structure of the initial sulfite

TABLE II: Effect of pH and Buffer Concentration on the Rate of the Second Reaction.[a]

pH	$[S_T]$ (M)	$10^3 \times k_{obsd}$ (min^{-1})	$10^3 \times k_2'$
5.90	0.8183	14.89	
	0.7160	12.91	
	0.6137	11.22	
	0.5114	10.11	
	0.4091	7.91	
	0.3069	6.48	
	0.2046	4.10	
	0.1023	2.01	18.6
6.28	0.8060	20.32	
	0.7052	19.18	
	0.6045	16.56	
	0.5037	13.35	
	0.4030	11.07	
	0.3022	8.27	
	0.2015	5.13	27.3
6.49	0.7690	21.87	
	0.6729	19.62	
	0.4806	14.79	
	0.3845	11.37	
	0.1922	5.50	29.2

pH	$[S_T]$ (M)	$10^3 \times k_{obsd}$ (min^{-1})	$10^3 \times k_2''$
6.80	0.7352	23.02	
	0.6433	19.41	
	0.5514	17.41	
	0.3678	11.78	
	0.1838	5.48	31.5
7.30	0.7170	18.13	
	0.5377	15.20	
	0.3585	9.89	27.2
7.66	0.6783	11.31	
	0.5935	8.82	
	0.5087	8.83	
	0.3391	5.28	
	0.1696	2.60	
	0.0848	0.93	16.3

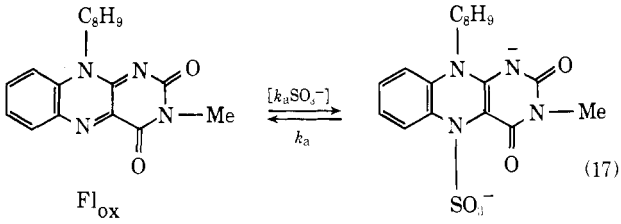
[a] Temperature 30°; solvent, H_2O; $\mu = 2.0$.



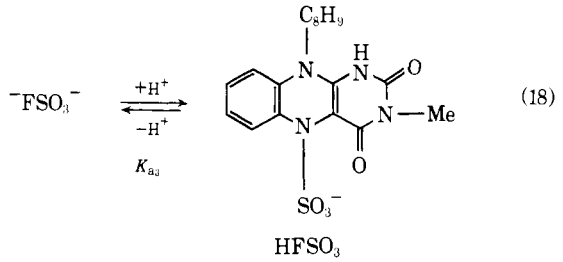
IV

adduct of III (B + BH of eq 16) may be assigned in like manner (λ_{max} 307 nm; ϵ 8700 M^{-1} cm^{-1} at pH 7.1).

The kinetics for the formation of the adduct and its dissociation to starting material must take into account the equilibrium steps of eq 17 and 18 (see Results). The reaction



(17)



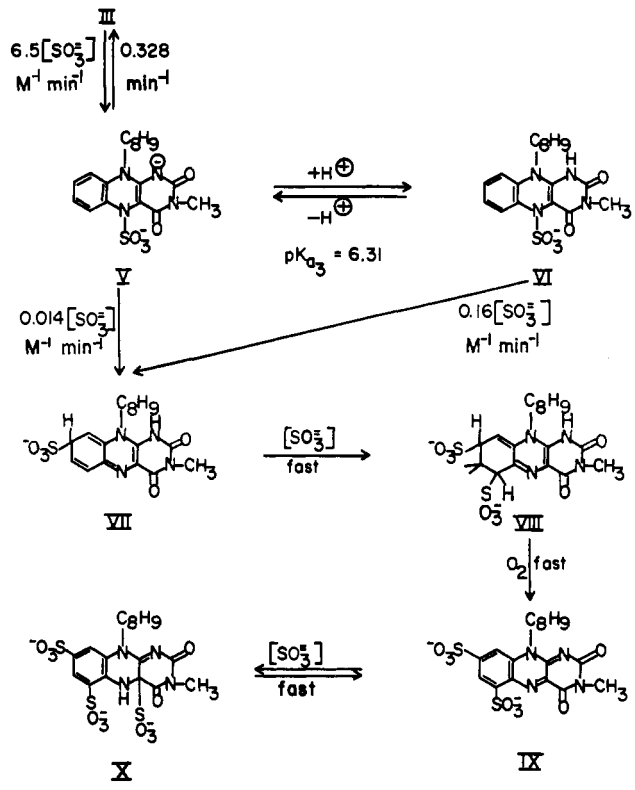
(18)

of SO_3^{2-} with $F^-SO_3^-$ and $HFSO_3^-$ comprises the second kinetically detectable reaction (the reaction proceeding through k_b and k_c of eq 16). Under aerobic conditions, the product of this reaction is assigned the structure of X formed through the reactions of Scheme I. Reactions following III → (V + VI) → VII are not rate determining and kinetically nondiscernible. In what follows the rationale for Scheme I and for its structures is presented.

As already discussed, structures V and VI have been assigned on the basis of uv spectral data. VII is a postulated intermediate in the formation of VIII whose structure is strongly suggested by uv and nmr spectra, as well as by the synthetic experiments. The uv band positions (304 and 355–365 nm) and extinction coefficients (11,800 and 7,200 M^{-1} cm^{-1}, respectively) are consistent with a reduced structure but different from the 1,5-dihydro form. Stronger indication is provided by the nmr spectrum of the anaerobic reaction product in D_2O: disappearance of all the aromatic hydrogens (except those of the 10-dimethylphenyl substituent), appearance of an olefinic doublet at 344 Hz, and of new absorptions in the aliphatic region (120–240 Hz). The new absorptions disappear upon air oxidation and a small amount (~15%) of deuterium exchange is observed. This result is expected if stereospecific DSO_3^- trans addition occurs to the 6,7 double bond of VII (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970), followed by air oxidation which also proceeds with high stereospecificity (~85% cis).

For the 1,5-dihydro form of 7,8-dimethylisoalloxazines,

SCHEME I



molecular orbital calculations (P.-S. Song, 1972[2]) predict *via* superdelocalizability the electrophilicity position series of 10a > 5a = 9a > 8 > 9 ≃ 6 while frontier orbital density gives 10a > 9a > 4a > 5a > 8 > 9 > 6. Assuming the similarity for V and VI to 1,5-dihydroisoalloxazine these calculations support, when the stereochemistries of V and VI are taken into account, the 8 position as that which should react with the second SO_3^{2-} species. Thus, the SO_3^{2-} moiety at the 5 position is expected to hinder nucleophilic attack of an also negatively charged species at the adjacent 4a and 5a positions, while the 2,6-dimethylphenyl substituent at the 10 position sterically hinders attack at the 9a and 10a positions.

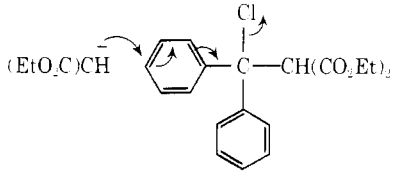
Synthesis a (see Experimental Section) also provides evidence for structure VIII. Both sulfonate groups are incorporated at the end of the anaerobic reaction, since quenching with HCl followed by air oxidation gives the 6,8-disulfonic acid derivative IX (product analysis). The structure of IX has been elucidated by elemental analysis (of acid and K salt), neutralization equivalent of acid, and by uv, nmr, and ir spectroscopies (Experimental Section). The acidic form of IX contains 2 acid equiv per molecule; the uv–visible spectrum is of the isoalloxazine type; the ir spectrum contains characteristic absorptions of the sulfonate groups at 1225, 1040, and 750 cm^{-1} (Hayatsu *et al.*, 1970; Shapiro *et al.*, 1970); the nmr spectra of both the potassium disulfonate and the disulfonic acid forms are well interpreted in terms of the 6,8-disubstituted derivatives: altogether, five aromatic hydrogens [three for the 10-(2′,6′-dimethylphenyl) substituent and two from the isoalloxazine nucleus]; the observed coupling constants (2 Hz) are characteristic of meta hydrogens. On the basis of deuterium incorporation we can assign the doublet at 420 (423) Hz to H_7 and the doublet at 500 (503) Hz to H_9.

In favor of structure X are its uv and nmr spectra and the fact that addition of sulfite to the 1,5 position of IX would

result in an adduct having two bulky sulfonate groups in an adjacent position. The nmr spectrum of X (air-oxidized portion of the anaerobic reaction mixture in D_2O; see Experimental Section) reveals bending of the molecule along the N(5)–N(10) axis giving rise to distinct signals (112 and 137 Hz) of the 10-(2′,6′-dimethylphenyl) methyl groups as compared to IX. The splitting of methyl absorptions arising from the 2,6-dimethylphenyl substituent (112 and 137 Hz) can be assigned to hindered inversion at N(10), but probably the main contribution arises from the differentiated magnetic environment provided by an asymmetrically substituted 4a-carbon atom in the case of a 4a adduct. Moreover, the uv–visible spectrum of X resembles very closely that of other 4a derivatives which have λ_{max} at 360–365 nm and, importantly, a shoulder at ~300 nm with comparable extinctions (Walker *et al.*, 1967; Hemmerich *et al.*, 1971; Brüstlein, 1971; Knappe, 1971).

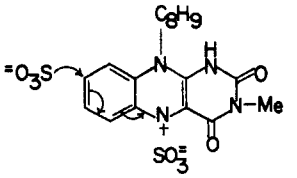
That addition of SO_3^{2-} to III occurs at the 5 position, whereas with IX the product is a 4a product, is of considerable interest. Nucleophilic addition has been strongly favored (Hemmerich, 1970; Hamilton, 1971) as the first step in the oxidation of substrates by flavine cofactors. Various arguments have been put forth favoring the 4a, 5, and 10a positions. By employment of III the 10a position has been eliminated as the essential electrophilic center for oxidation of dihydronicotinamides, mercaptans, and dimethyl dihydrophthalate (Bruice *et al.*, 1971; Main *et al.*, 1972). The present study establishes that both the 5 and 4a adducts may be formed through a nucleophilic addition and that in an enzymatic reaction one or the other could be favored depending upon the stereochemistry of the active site.

For compounds of general structure C_6H_3NRX, nucleophilic addition to the benzene ring with displacement of –X has not been established. Nucleophilic attack of SO_3^{2-} at the 8 position to displace SO_2^{2-} from the 5 position is possible as in the formation of VIII from V and VI (Bruice and Bradbury, 1963), eq 19. A more likely mechanism would involve



(19)

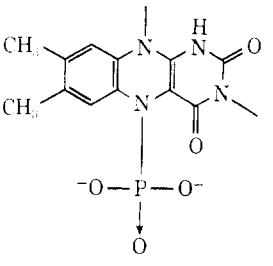
SO_3^{2-} attack upon a nitrenium ion pair (Gassman and Campbell, 1972), eq 20.



(20)

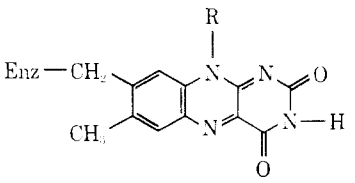
Nitrenium Ion Pair

It is conceivable that the displacement of a good leaving group from the 5 position of a 1,5-dihydroflavine is an important biochemical reaction. A nucleophilic addition reaction at the 5 position brings about reduction of the flavine to the fully reduced 1,5-dihydro level. At the same time, the nucleophile is oxidized. Thus, the sulfite adduct may be considered as a sulfamic acid of 1,5-dihydroflavine. This particular adduct may well be important in converting sulfite to an active sulfate (Michaels *et al.*, 1970, 1971). Possibly, other enzymic reactions of this nature will be found which do not involve sulfur. The possible biochemical importance of structure XI has occurred to us. However, the addition of



XI

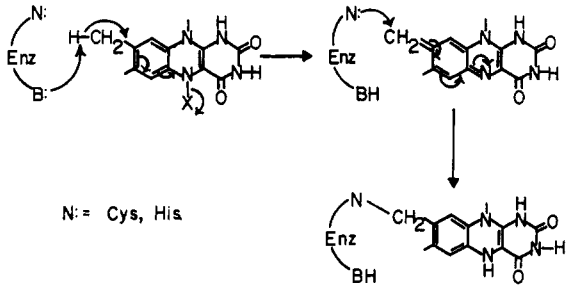
phosphite to III was found not to occur under conditions in which sulfite readily adds. Another possibility arises in the synthesis of enzymes which contain flavine mononucleotide or flavine adenine dinucleotide attached in covalent linkage through the 8-methyl group (XII). Flavine cofactors have



XII

been established to be attached *via* covalent linkage to apoenzyme in succinate dehydrogenase (Singer *et al.*, 1971; Salach *et al.*, 1972; Walker *et al.*, 1972), in monoamine oxidase (Walker *et al.*, 1971; Kearney *et al.*, 1971), and in chromatium cytochrome C-552 (Hendriks *et al.*, 1972). A means which might be considered for the establishment of the covalent linkage of cofactor to apoenzyme is provided in Scheme II.

SCHEME II



In order to obtain experimental support of this scheme, we have tried to effect a reaction of 7,8-dimethylisoalloxazines (riboflavin and lumiflavin) with sulfite–bisulfite in great excess. In the reaction of sulfite with 7,8-dimethylisoalloxazines, we hoped to see incorporation of a sulfonate group in the 8-CH_3; however, the reaction did not proceed beyond the 5 adduct (7 days) as originally reported by Müller and Massey (1969). In spite of the failure of these experiments, we do not conclude by rejecting Scheme II; rather we attribute it to the possibility that sulfite or bisulfite anions are not strong enough bases to extract one of the 8-CH_3 protons. This might be more successfully achieved by the appropriate enzyme.

References

Albert, A., Armarego, W. L. F., and Spinner, E. (1961), *J. Chem. Soc.*, 2689.

Bruice, T. C., and Benkovic, S. (1966), "Bioorganic Mechanisms," Vol. I, New York, N. Y., W. A. Benjamin.

Bruice, T. C., and Bradbury, W. C. (1963) *J. Org. Chem. 28*, 3403.

Bruice, T. C., Main, L., Smith, S., and Bruice, P. Y. (1971), *J. Amer. Chem. Soc. 93*, 7327.

Brüstlein, M. (1971), Ph.D. Thesis, University of Konstanz, Germany.

Gassman, P., and Campbell, G. A. (1972), *J. Amer. Chem. Soc. 94*, 3891.

Hamilton, G. (1971), *Progr. Bioorg. Chem. 1*, 83.

Hayatsu, H., Wataya, Y., and Kai, K. (1970), *J. Amer. Chem. Soc. 92*, 725.

Hemmerich, P. (1964), *Helv. Chim. Acta 47*, 473.

Hemmerich, P. (1970), *Vitam. Horm. (New York) 28*, 467.

Hemmerich, P., Fallab, S., and Erlenmeyer, H. (1956), *Helv. Chim. Acta 39*, 1242.

Hemmerich, P., Ghisla, S., Hartman, U., and Müller, F. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press.

Hendriks, R., Cronin, J. R., Walker, W. H., and Singer, T. P. (1972), *Biochem. Biophys. Res. Commun. 46*, 1262.

Johnstone, R. A. W., Payling, D. W., and Thomas, C. (1969), *J. Chem. Soc. C*, 2223.

Kearney, E. B., Salach, J. I., Walker, W. H., Seng, R., and Singer, T. P. (1971), *Biochem. Biophys. Res. Commun. 42*, 490.

Kierkegaard, P., Norrestam, R., Werner, P.-E., Csöregh, I., von Glehn, M., Karlsson, R., Leijonmarck, M., Rönnquist, O., Stensland, B., Tillberg, O., and Torbjörnsson, L. (1971), *in* "Flavins and Favoproteins," Kamin, H., Ed., Baltimore, Md., University Park Press.

Knappe, W. R. (1971), Ph.D. Thesis, University of Konstanz, Germany.

Main, L., Kasperek, G. J., and Bruice, T. C. (1972), *Biochemistry 11*, 3991.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P. (1969), *J. Biol. Chem. 244*, 3999.

Michaels, G. B., Davidson, J. T., and Peck, H. D., Jr. (1970), *Biochem. Biophys. Res. Commun. 39*, 321.

Michaels, G. B., Davidson, J. T, and Peck, H. D., Jr. (1971), *in* "Flavins and Flavoproteins," Kamin, H., Ed., Baltimore, Md., University Park Press.

Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244*, 4007.

Pitman, I. H., Shefter, E., and Ziser, M. A. (1970), *J. Amer. Chem. Soc. 92*, 3413.

Pitman, I. H., and Ziser, M. A. (1970), *J. Pharm. Sci. 59*, 1295.

Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., and Hartmann, U. (1972), *Eur. J. Biochem. 26*, 267.

Schroeter, L. C. (1966), "Sulfur Dioxide," Oxford, Pergamon Press.

Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Amer. Chem. Soc. 92*, 422.

Singer, T. P., Salach, J., Walker, W. H., Gutman, M., Hemmerich, P., and Ehrenberg, A. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press.

Swoboda, B. E. P., and Massey, V. (1966), *J. Biol. Chem. 241*, 3409.

Walker, W. H., Hemmerich, P., and Massey, V. (1967), *Helv. Chim. Acta 50*, 2269.

Walker, W. H., Kearney, E. B., Seng, R., and Singer, T. P. (1971), *Biochem. Biophys. Res. Commun. 44*, 287.

Walker, W. H., Singer, T. P., Ghisla, S., and Hemmerich, P. (1972), *Eur. J. Biochem. 26*, 279.

Preparation and Characterization of Free Cell Suspensions from the Immature Rat Uterus†

David Williams‡ and Jack Gorski*

ABSTRACT: This report describes a procedure for the preparation of free cell suspensions from the immature rat uterus. Characterization of the cell suspensions indicates that 40–50% of the cells present in the intact uterus can be obtained as free cells in suspension. These cells exhibit viabilities in excess in 95% and carry out a number of metabolic processes at constant rates for up to 10 hr in suspension. The estradiol binding properties of the uterus are quantitatively recovered in the dispersed cells. The cell suspensions appear to provide a useful experimental system with which to study the interaction of estradiol with the uterine binding proteins in the intact cell at physiological temperatures.

Although some properties of the estrogen binding proteins of the rat uterus have been examined in crude and partially purified extracts (Ellis and Ringold, 1971; Giannopoulos and Gorski, 1971a,b; Puca *et al.*, 1971), we have very little information about the behavior of these proteins in the intact cell at physiological temperatures. Information about the equilibrium and kinetic binding behavior of these pro-

† From the Departments of Physiology and Biophysics and Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received August 18, 1972.* Supported by National Institutes of Health Grant HD 4828 and Ford Foundation Training Grant 700-0333.

‡ National Institutes of Health Predoctoral Fellow 5 F01 GM 43656-03. Present address: Department of Biochemistry, University of California Medical Center, San Francisco, Calif. 94122.