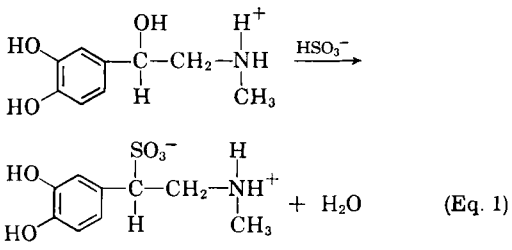
Stabilization of Epinephrine against Sulfite Attack

By SIDNEY RIEGELMAN and ELIEZER Z. FISCHER†

Sulfite and bisulfite ions are known to attack the optically active side chain of epinephrine in the absence of oxygen by an S_N2 mechanism. Evidence is presented whereby chelation of the catechol nucleus with boric acid results in marked stabilization of epinephrine from this type of degradation. The effects of variation of the boric acid and hydrogen ion concentration are investigated. The equilibrium reaction of boric acid and epinephrine are reported in detail and the stability constant for the chelate is evaluated.

In 1958 Schroeter and Higuchi (1, 2, 3) reported on a reaction of epinephrine with bisulfite in an oxygen-free atmosphere. The mechanism of this reaction appears to involve nucleophilic attack of sulfite at the partially positive carbon of the epinephrine side chain to yield a carbon-sulfur bond. There is a loss of optical activity simultaneous to epinephrine and bisulfite loss; the result is a zwitterionic sulfonate



(Eq. 1)

The latter compound had been prepared by Tomino (4) and by Schroeter (5), although the former worker proposes that the sulfonate group is attached at the β-carbon atom.

Studies of the kinetics of the reaction by Higuchi and Schroeter definitely confirm the reaction to be a second-order reaction, dependent on both the concentration of the epinephrine and of the bisulfite. Epinephrine analogs and model compounds were examined by these workers (2) who pointed to the general nature of this type of nucleophilic attack on *p*-substituted benzyl alcohol derivatives. *Meta*-substituted derivatives did not react. The results led to the suggestion that the *p*-quinoid structure of the benzyl alcohol derivatives is the reactive form.

Based on this background information, the present authors postulated that chelate formation between boric acid and the catechol grouping of epinephrine might affect the overall rate of sulfite attack on the compound. Chelate formation of boric acid with polyhydroxy compounds is well known (6, 7). Boric acid chelates of catechol derivatives were first studied by Boeseken and Mijs (8) whose main interest was directed toward preparation of the optically active forms of the chelates. Recently Näsänen (9) has studied the chelate formation of catechol-3,5-disulfonic acid with boric acid by a potentiometric method. The titration of dilute mixed solutions of boric and catechol disulfonic acids with sodium hydroxide solution revealed the formation of only a one-to-one chelate. Since boric acid was found to stabilize epinephrine against sulfite attack, it was deemed important to determine the stability constant for the formation of boro-epinephrine chelate in order to elucidate the quantitative aspects of the stabilization. The results of the degradation and chelate formation studies are the basis of this report.

EXPERIMENTAL

Kinetic Studies.—Epinephrine solutions were prepared at varying concentrations in phosphate or boric acid buffers at varying pH. The pH was determined at 25°. These solutions were filled into hard-glass ampuls, flushed with nitrogen, degassed in a vacuum and once again filled with nitrogen, and then sealed. Identical solutions were prepared containing sodium bisulfite. As mentioned earlier, the reaction of epinephrine with bisulfite had been shown to be a bimolecular reaction with simultaneous loss of optical activity and sulfite. In order to accelerate the rate of reaction, a swamping excess of sodium bisulfite was used in the reaction system converting the system to a pseudo first-order reaction. Thus it was possible to make the observations on samples equilibrated in a constant temperature bath at 25°. Individual ampuls were removed periodically and the optical activity of the solution at 25° was determined on a Rudolph research polarimeter using filtered sodium light (589 mμ) and a 2.0-dm. microtube. The chemical activity of the epinephrine was verified in a number of the solutions by isolation of the triacetyl derivative according to the method reported by Higuchi, Sokoloski, and Schroeter (10). The bisulfite concentration of the ampuls was also determined by iodometric titration.

Chelate Formation Studies.—Solutions of epinephrine, phenylephrine, and boric acid were prepared

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using exact equivalents of hydrochloric acid to prepare the salts from the free bases. Sodium chloride was added to adjust the ionic strength of the solutions. The solutions were transferred to a four-necked flask and degassed with nitrogen purified with a vanadous sulfate solution to remove residual traces of oxygen (11). The potentiometric measurements were made using the Beckman standard combination electrode (No. 39142) and the Beckman model G pH meter. Standard sodium hydroxide solution was delivered from a 5-ml. microburet.

Chelate formation was studied spectrophotometrically using 0.4% epinephrine solutions in the presence of an excess of boric acid. The solutions were adjusted to varying pH's by the addition of 10 *N* sodium hydroxide. The pH's of the solutions were measured as noted above. The ultraviolet absorption spectrum was recorded using a Cary model 11 spectrophotometer.

RESULTS AND DISCUSSION

Typical degradative attack of sulfite on epinephrine in the absence of boric acid is shown in Fig. 1. In these solutions, the concentration of epinephrine was held at 0.082 *M* and the concentration of sodium bisulfite was 0.96 *M*. It is evident from Fig. 1 that the reaction follows first-order kinetics under these conditions. The amount of sulfite consumed was completely accounted for by the simultaneous loss in epinephrine, as measured chemically, or by the change in specific rotation of the solutions. It is noted that in the absence of a complexing agent the rate of sulfite attack increases with decrease in hydrogen-ion concentration. This is in agreement with the observations of Higuchi and Schroeter (2) at elevated temperatures and different concentration conditions.

When identical solutions in respect to epinephrine and sulfite concentrations are prepared containing 0.318 *M* boric acid adjusted to the same pH, an interesting effect takes place relative to the rate of sulfite attack. Figure 2 presents the data for the deterioration of epinephrine at the same pH values as shown in Fig. 1. In the presence of boric acid, the rate of sulfite attack is reduced as the hydrogen ion concentration decreases. The half-life for epinephrine under the reaction conditions at pH 6.0 in the absence of boric acid was 195 hours, while in the presence of boric acid the half-life was found to be 267 hours. At pH 7.5, however, the half-life of epinephrine was found to be 74 hours in the absence, and 1,270 hours in the presence of boric acid. It might be postulated that epinephrine is increasingly chelated by the boric acid molecules as the pH is made more alkaline, and that the chelated epinephrine is far less susceptible to sulfite attack than free epinephrine. This is analogous to the findings of Higuchi and Lachman in their studies on the effect of complexation of benzocaine with caffeine (12). In that latter study it was concluded that the complexed form of the drug does not undergo any perceptible cleavage at the ester linkage of the benzocaine. In order to ascertain more facts relative to the stability of the boro-epinephrine chelate to sulfite attack, it was necessary to determine the stability constant for the reaction.

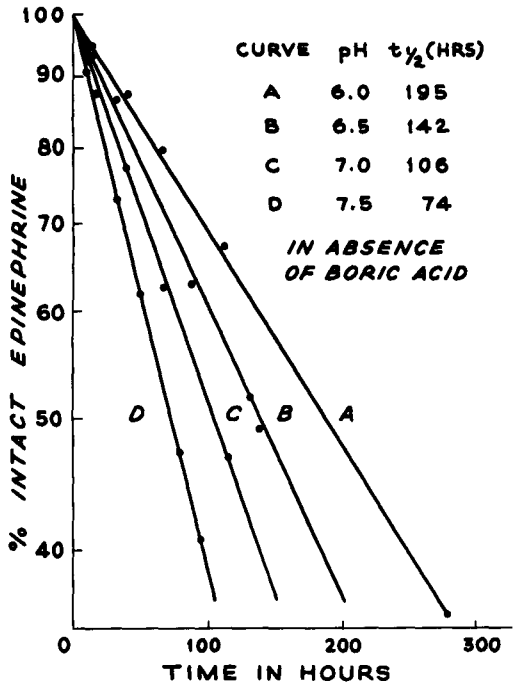


Fig. 1.—The per cent epinephrine remaining in a 1.5% (0.082 *M*) solution containing 10% (0.96 *M*) sodium bisulfite. Solutions were stored at 25° in sealed ampuls in a nitrogen atmosphere in the absence of oxygen. The solutions contained no boric acid.

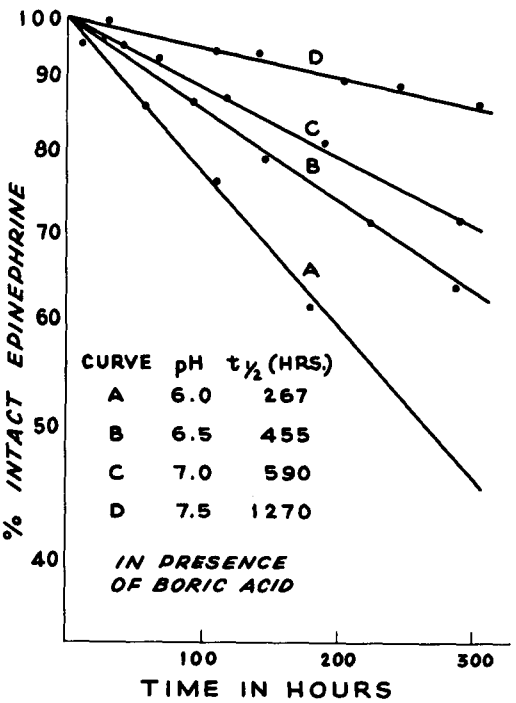


Fig. 2.—The per cent epinephrine remaining in a 1.5% (0.082 *M*) solution containing 10% (0.96 *M*) sodium bisulfite and 2.0% (0.318 *M*) boric acid. The solutions were stored at 25° in sealed ampuls in a nitrogen atmosphere in the absence of oxygen.

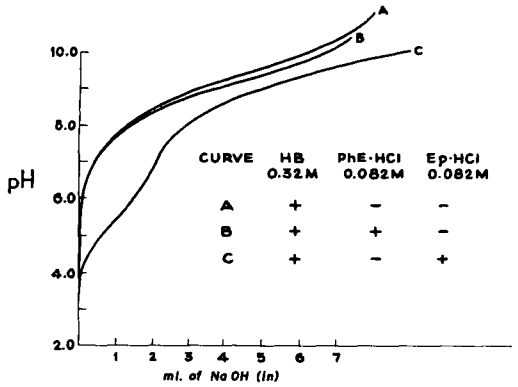


Fig. 3.—Titration of 0.318 *M* boric acid alone (*A*), in the presence of 0.082 *M* phenylephrine hydrochloride (*B*), and in the presence of 0.082 *M* epinephrine hydrochloride (*C*).

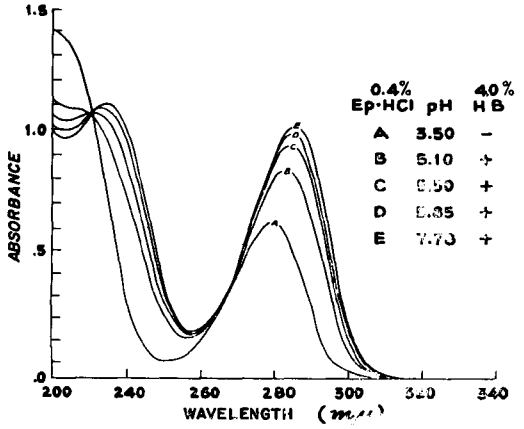
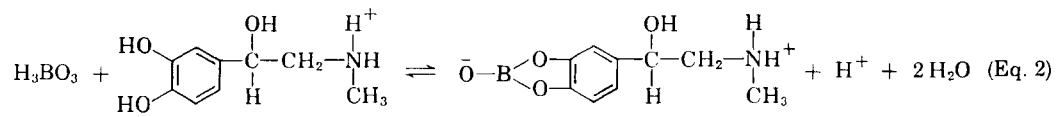


Fig. 4.—Ultraviolet absorption spectra of 0.022 *M* epinephrine alone and in the presence of 4% (0.65 *M*) boric acid at various hydrogen ion concentrations, path length 0.1 mm.

Figure 3 presents the titration curves of 0.32 *M* boric acid alone (*A*), in the presence of 0.082 *M* phenylephrine hydrochloride (*B*), and in the presence of 0.082 *M* epinephrine hydrochloride (*C*). Phenylephrine, of course, differs from epinephrine in that it is a *m*-cresol amine while epinephrine is a catechol amine derivative. Thus epinephrine is able to form a chelate through its dihydroxy structure and the absence of any chelate formation with phenylephrine indicates that the site of binding does not involve the side chain. Curve *B* is slightly shifted relative to the pure boric acid titration curve (*A*) due to the simultaneous neutralization of the first acid function of phenylephrine hydrochloride. Similar titration data for the 1:1 ratio of boric acid to epinephrine have been reported by Riegelman, *et al.* (13). These data may be represented by the reaction



(Eq. 2)

The equilibrium constant for the reaction represented by Eq. 2 may be represented as follows

$$K = \frac{[H^+][EpB^-]}{[Ep][Hb]} \qquad \text{(Eq. 3)}$$

where [HB], [Ep], and [EpB^-] represent the equilibrium concentrations of the boric acid, epinephrine, and boro-epinephrine chelate, respectively. Activity coefficients are ignored. In analogy to the results of Näsänen (9) who found only the 1:1 chelate, one might expect only a 1:1 chelate under the present conditions where the concentration of boric acid is equal to or exceeds the concentration of the epinephrine. This is also verified by the reaction of only one mole of base per mole of epinephrine added and the existence of an isosbestic in the absorption spectral curves (Fig. 4).

The stability constant for the reaction can best be calculated in the pH region where base is not consumed by the excess of boric acid present. Under these conditions the amount of base consumed is equal to the amount of chelate formed, and each of the equilibrium concentrations represented in Eq. 3 are thereby easily calculated from simple stoichiometric relationships. The data in Table I list the results from the calculations at two different ratios of boric acid to epinephrine. Since the ionic strength of the two systems differ, the difference in the calculated stability constants may be due to ionic strength effect or to other experimental considerations. Näsänen found a pK of 4.0 for catechol disulfonic acid with relatively large ionic strength effects.

Figure 4 presents the ultraviolet absorption spectra of epinephrine 0.022 *M* in the presence of 0.65 *M* boric acid at varying hydrogen ion concentrations. Since the extinction coefficient of boro-epinephrine chelate is not known with certainty, a modification of the method of Rosenblatt (14) must be used to calculate the stability constant from the absorption data. Equation 3 may be rewritten as

$$K' = [HB]K = \frac{[H^+][EpB^-]}{[Ep]} \qquad \text{(Eq. 4)}$$

since under the condition of the spectrophotometric analysis, [HB] $\sim$ constant. Substituting the appropriate extinction coefficients, we have

$$\frac{1}{K'} = \frac{1}{[HB]K} = \frac{\epsilon_{(EpB^-)} - \epsilon_n}{\epsilon_n - \epsilon_{(Ep)}} \frac{1}{[H^+]_n} \qquad \text{(Eq. 5)}$$

where ϵ_{Ep} = specific extinction coefficient of Ep; $\epsilon_{(EpB^-)}$ = specific extinction coefficient of EpB^-, and ϵ_n = specific extinction coefficient of a mixture of each component at the hydrogen ion concentration, $[H^+]_n$, or

$$-[H^+]_n\,\epsilon_n\left(\frac{1}{K'}\right) + [H^+]_n\left(\frac{\epsilon_{Ep}}{K'}\right) + \epsilon_{(EpB^-)} = \epsilon_n \qquad \text{(Eq. 6)}$$

This is a linear equation of four terms and three

unknowns since $[H^+]_n$ and ϵ_n are measurable quantities. This may be solved by use of a third-order determinant, or

$$[HB]K = K' = \frac{\begin{vmatrix} [H^+]_1\ \epsilon_1 & [H^+]_1\ 1 \\ [H^+]_2\ \epsilon_2 & [H^+]_2\ 1 \\ [H^+]_3\ \epsilon_3 & [H^+]_3\ 1 \end{vmatrix}}{\begin{vmatrix} \epsilon_1 & [H^+]_1\ 1 \\ \epsilon_2 & [H^+]_2\ 1 \\ \epsilon_3 & [H^+]_3\ 1 \end{vmatrix}}$$

The results from the analysis at two wavelengths are given in Table II. The calculated stability constants from the spectrophotometric studies compare surprisingly well with those determined by the titration method.

TABLE I.—CALCULATION OF THE STABILITY CONSTANT FOR THE BORO-EPINEPHRINE CHELATE FROM THE TITRATION DATA

Composition	pH	pK
Ep = 0.082*M* HB = 0.082*M*	4.66	4.65
	4.78	4.63
	5.07	4.66
	5.18	4.65
	5.45	4.69
	5.72	4.69
		4.66 ± 0.04[a]
Ep = 0.082*M* HB = 0.32*M*	3.88	4.76
	4.30	4.71
	4.68	4.70
	4.88	4.71
	5.00	4.72
		4.71 ± 0.01[a]

[a] Mean ±95% confidence interval.

TABLE II.—CALCULATION OF STABILITY CONSTANT FOR THE BORO-EPINEPHRINE CHELATE FROM U. V. SPECTROPHOTOMETRIC DATA

Composition	pH	A_{285} mμ	A_{240} mμ
Ep = 0.022 *M* HB = 0.648 *M*	5.10	0.835	0.725
	5.50	0.932	0.880
	5.83	0.950	0.960
	$pK_{285\ m\mu}$ = 4.66		
	$pK_{240\ m\mu}$ = 4.71		
[HB] equil. was assumed to be 0.625 *M*			

Closer examination of the sulfite attack on epinephrine in the presence of boric acid may now be made. The stability constant calculated above indicates that the reaction illustrated by Eq. 2 is predominantly shifted to the left at low pH. The ratio of the boric acid to the epinephrine and the hydrogen ion concentration both affect the concentration of free epinephrine in the system. Since there is a practical limit to the concentration to which we can increase the concentration of boric acid, the most important variable in the reaction is the hydrogen ion concentration. As the hydrogen ion concentration is decreased, the boro-epinephrine chelate concentration is increased, and the preparation is thereby stabilized to sulfite attack.

At pH's above 6.0 and at relatively high concentrations of boric acid, it becomes increasingly difficult to estimate the concentration of free epinephrine directly from Eq. 3. This is due to the fact that boric acid forms autocomplexes in the concentration ranges utilized which, in turn, affect the apparent dissociation constant of the boric acid (15, 16). In the pH range where the sulfite attack was studied, a portion of the base was utilized to titrate boric acid and polyboric acids.

The amount of free and chelated epinephrine can be evaluated by an alternative approach. Using the same ratio of boric acid to epinephrine as utilized in the deterioration studies (3.9/1), a series of solutions were made at 0.022 *M* epinephrine and 0.084 *M* boric acid and adjusted to the exact pH values used in the deterioration studies. The ultraviolet absorption spectra of the solutions were examined in a 0.1-mm. path length. Since two isosbestics were observed, solutions contained only two absorbing species. The spectrum of a solution of epinephrine hydrochloride in the absence of boric acid and a mixture of boric acid and epinephrine at pH 9.0 was also recorded. By analysis of the system at two wavelengths, the concentration of free epinephrine was determined for each solution. The data are recorded in Table III. Column 2 lists the fraction of free epinephrine as determined spectrophotometrically. In analogy to the approach of Higuchi and Lachman, the overall rate of reaction of epinephrine with sulfite is dependent on the reaction rate for the free and chelated epinephrine, or

$$k_{obs} = k_f F_f + k_c F_c \qquad \text{(Eq. 8)}$$

where the subscripts f and c stand for the free and chelated epinephrine, respectively, and F = the fraction of each form. If we assume that the rate of degradation of the chelated epinephrine is extremely small compared with the free, and substituting reciprocal half-lives for the respective rate constants we have, $F_f = (t_{1/2})_f/(t_{1/2})_{obs}$.

TABLE III.—COMPARISON OF CALCULATED AND EXPERIMENTAL ESTIMATION OF FREE EPINEPHRINE CONCENTRATION

pH	Calculated Free Epinephrine	Observed Free Epinephrine
6.0	0.45	0.45
6.5	0.22	0.315
7.0	0.069	0.15
7.5	0.031	0.058

Column 3, Table III lists the calculated fraction free from the data given in Figs. 1 and 2. It should be recalled that the solutions used in the degradation studies included 0.96 *M* sodium bisulfite, which produced an ionic strength far different from that used in the spectrophotometric evaluations. In addition, the latter measurements utilized solutions of identical ratio but diluted 3.75-fold in order to measure their absorbancies. While the deviation in columns 2 and 3 may be due to the changes in experimental conditions, an assumption that $k_c \leq 0.05\ k_f$ can approximate the values observed.

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Effect of Boric Acid and Bisulfite on the Rate of Oxidation of Epinephrine

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An investigation of epinephrine and sulfite oxidation in the presence and absence of light was undertaken to determine what effect complexation may have on the rate of oxidation of epinephrine under controlled conditions. The initial oxidative step is shown to be the oxidation of the sulfite ion. At a critical sulfite ion concentration, adrenochrome commences to form; however, the residual sulfite apparently reacts with the adrenochrome, resulting in a colorless adrenochrome sulfonate. It continues to form until all the sulfite is consumed, whereupon the solution deteriorates with visible discoloration. In the dark, epinephrine will remain stable to oxidation until light catalyzed, even after the destruction of all the sulfite and the formation of the adrenochrome sulfonate.

IT HAS BEEN reported recently by the authors (1) that boric acid stabilizes epinephrine against sulfite attack in an oxygen-free atmosphere. Epinephrine reacts with boric acid forming a boro-epinephrine chelate in accordance with the reaction given previously (1). The stability constant for the reaction was found to be 2×10^{-5}; however, use of an excess of boric acid and neutral or slightly alkaline pH conditions converts the vast majority of the epinephrine into the chelate form. The chelate form was found to be stable to sulfite attack. Since these conditions maintain stability of the epinephrine in the package until opened, it remains to investigate the effect of these additives on the stability of the epinephrine under oxidative attack.

The oxidation of epinephrine has been studied by many workers and has recently been reviewed by Heacock (2). At intermediate pH, epinephrine in aqueous buffered solution is oxidized to red substances; the speed of the reaction and the nature of the final products are dependent on the catalysts and buffers employed. In general, oxygen uptake begins only after a considerable induction period. Trace metals, particularly copper, manganese, and nickel, have been shown to initiate the reaction, possibly by the formation of an autoöxidizable epinephrine-metal chelate (3–5). Once the reaction is initiated, adrenochrome becomes a significant factor in the overall reaction rate (6, 7). Iron and copper chelating agents such as EDTA have been shown to increase the rate of oxidation, and other chelating agents also have been shown to increase the oxidation rate (8, 9). The catalytic action of adrenochrome has been postulated as being due to a chelated form of the compound (6). Trautner and Messer (10) reported on the effect of boric acid on the oxygen uptake of epinephrine and reported a stabilizing effect on epinephrine in alkaline solution.

Reducing agents such as sodium bisulfite (11), ascorbic acid, cysteine, and other reducing and

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