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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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chelating agents (12) have been shown to inhibit the oxidation of epinephrine by molecular oxygen. Among these agents, sodium bisulfite appears to be the most commonly used antioxidant.

The oxidation of sulfite ions by oxygen has been shown to be a chain reaction, very sensitive to both positive and negative catalysts. One of the classical papers on this subject is the work of Fuller and Chryst in 1941 (13). These workers found that extreme precautions had to be taken to prevent metal contamination of their system. Double distilled water stored in hard-glass bottles was found to introduce spurious results after storage for longer than 3 hours. The rate of oxidation was affected by the rate of stirring and an adequate rate of oxygen absorption could not be maintained in solutions containing more than 0.015 *M* sulfite. The rate of cupric ion catalysis was found to be 10^9-fold the uncatalyzed rate under their conditions. Mannitol acted as a negative catalyst. The rate of oxidation is dependent primarily on the sulfite ion concentration, and bisulfite was much less easily oxidized. Later workers have confirmed the basic facts of the studies of Fuller. A detailed mechanism for the autoxidation of sulfite ions has been proposed by Abel (14, 15).

EXPERIMENTAL

Materials and Solution Preparation.—No special purification of distilled water, glassware, or of the reagent grade chemicals was undertaken. A single source of each chemical was used throughout. Efforts were taken to standardize the procedures used in the preparation of the solution to avoid the accidental incorporation of chance contaminants. Therefore, it is possible that the solutions were reproducible in respect to contamination and reflect best commercial manufacturing techniques.

Oxidative Method A.—Solutions containing 0.082 *M* epinephrine hydrochloride and varying amounts of sodium bisulfite and boric acid were adjusted to pH 6.5. A 25-ml. quantity of solution was placed in 200-ml. multiple-dose vials and attached to a reciprocating shaking device in a constant temperature bath at 25°. The solutions were open to air and exposed to light during the day from overhead fluorescent light, from a distance of approximately 10 feet. Samples were taken at the time of appearance of the visible red coloration of the solutions and at appropriate intervals thereafter. The solutions were examined for their absorbance at 440, 480, 550, and 600 mμ.

Oxidative Method B.—A fluorescent desk lamp containing two 20-inch fluorescent G. E. daylight tubes was inverted so that the light was reflected upward. Two polymethacrylate plates were separately spaced above the lights to allow air circulation. Glass beakers were thereby placed 2.5 inches from the light source. A Weston photometer was used to select that portion of the fluorescent tube which yielded uniform light intensity. Approximately the middle two-thirds was used.

Solutions containing sodium bisulfite and epinephrine, with or without boric acid, were studied alone and in combination. Air was bubbled through a coarse sintered-glass gas dispersion tube after water saturation and in a manner that the gas flow was essentially equal in all tubes. The rate of gas flow was as rapid as possible without dispelling a portion of the contents from the containers. In several determinations, the solutions were also stirred at 1750 r.p.m. by a two-bladed propeller. Certain solutions were covered with aluminum foil to exclude light, yet allowing other conditions of the experiment to remain unchanged.

The solutions were assayed for their sulfite content by iodometric titration and for their epinephrine deterioration products by spectrophotometric methods.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical determination utilizing oxidative method B. These solutions contained 0.1% sodium bisulfite and were adjusted to pH with standard sodium hydroxide. Since the conditions for the reaction were not as ideal as used by Fuller, the curves were not exactly duplicated on repetition. However, the variation was of a minor qualitative nature. Curve A illustrates the rate of oxidation observed with the sodium bisulfite solutions buffered to pH 7.0 with boric acid and sodium hydroxide. The half-life of the reaction was observed to be 8 to 9 minutes in a series of five duplicate determinations. Curve B illustrates the results in the absence of boric

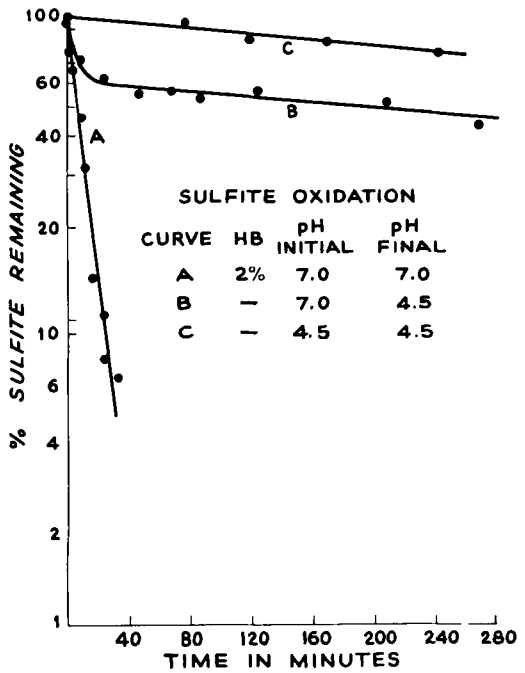

Fig. 1.—Oxidation of 0.1% sodium bisulfite solution at various initial hydrogen ion concentrations in the presence and absence of boric acid (HB). Data were obtained by oxidative method B.

acid buffer. Under these conditions, the oxidation of sulfite to sulfate ion increases the acidity of the solution. At pH 4.5, virtually all the sulfite ion vanishes and the oxidation rate is dependent on the remaining bisulfite ion concentration. This is verified by the data represented in curve C where the initial pH is adjusted to 4.5 and the rate of oxidation follows the linear portion of curve B.

As is well recognized, the first visible product of the oxidation of epinephrine is the formation of the red adrenochrome, which has a broad absorption maximum at 480 mμ. The onset and relative rate of oxidation can, therefore, be detected spectrophotometrically. The effect of sulfite and boric acid concentration on the rate of oxidation of epinephrine (method A) buffered at pH 6.5 is shown in Fig. 2. Two groups of curves are presented, all containing 1.5% (0.082 *M*) epinephrine. A comparison of the 0.2% to the 0.4% sodium bisulfite series indicates that the induction period is not doubled by a doubling of the concentration of the bisulfite. A solution containing 2.0% boric acid and 0.2% bisulfite has an induction period of 51 hours *vs.* 68 hours at the 0.4% level. An increase in concentration of boric acid at a fixed pH of 6.5 appears to accelerate the rate of oxidation of the bisulfite and therefore influences the length of the induction period. This may be due to trace impurities contained in the boric acid. Within experimental error, the slopes of the curves are identical, indicating that the rate of oxidation of the epinephrine is constant after the exhaustion of the antioxidant. An increase in concentration of boric acid will increase proportionately the concentration of the boro-epinephrine chelate, as is apparent from the reaction given by Eq. 1. This chelate does not influence the oxidative process under the present experimental conditions, possibly due to the presence of trace metals which form a much stronger chelate.

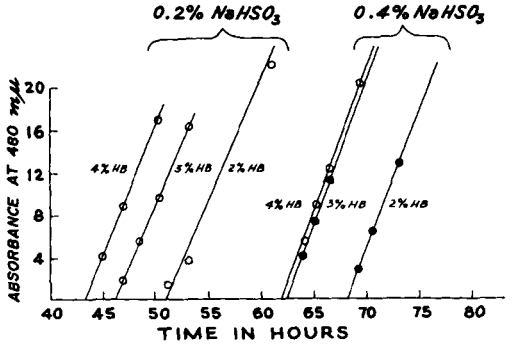

Fig. 2.—Oxidative studies on 1.5% epinephrine solutions at pH 6.5 by oxidative method A.

In the course of the assay of epinephrine solutions exposed to oxygen in the presence of bisulfite and boric acid buffer, a secondary ultraviolet absorption peak was observed at 350 mμ, increasing in intensity just prior to the development of the visible adrenochrome discoloration. In a recent study, van Espen (16), reported the formation of an adrenochrome bisulfite addition product, and presented detailed evidence indicating that this product is an adrenochrome sulfonate with the sulfonate group attached at position 4 or 7. The absorption spectrum of the

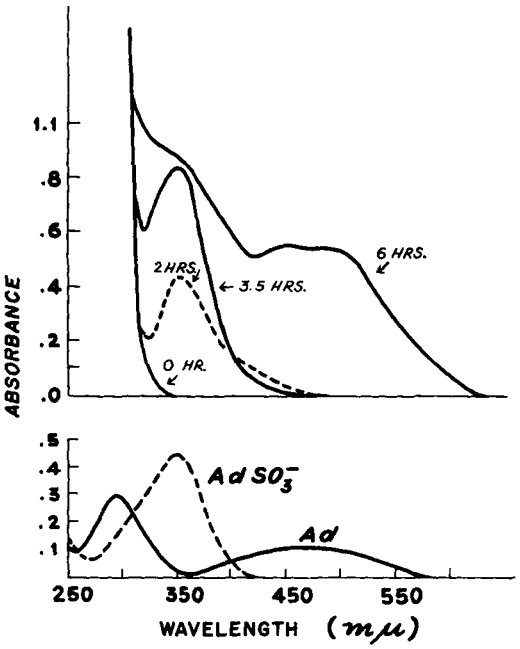

Fig. 3.—Upper curves: absorption spectra of epinephrine-bisulfite-boric acid solution at various intervals during the oxidation by method B. The curves were obtained from solution in a 3.0-mm. path length. Lower curves: absorption spectra of adrenochrome sulfonate ($AdSO_3^-$) and adrenochrome (Ad), redrawn from van Espen (16). Concentration = 2.8×10^{-5} *M*/L.; 1.0-cm. path length.

isolated compound was reported, and is redrawn in Fig. 3. The upper curves of Fig. 3 indicate the typical results obtained in spectrophotometric analysis of the epinephrine solutions. Prior to the exposure to oxygen by method B, the undiluted solution shows very strong absorption below 315 mμ due to the high concentration of epinephrine. Figure 3 includes the absorption curves for the same solution after 2, 3.5, and 6 hours. At the earlier periods, the solution contains bisulfite ions and is colorless to the eye; however, the absorption peak at 350 mμ indicates the presence of an oxidative product of epinephrine, probably the adrenochrome sulfonate, reported by van Espen. The 6-hour curves illustrates the results obtained after the oxidative reaction has exhausted all of the antioxidant. The curve indicates the presence of the adrenochrome sulfonate peak at 350 mμ, a considerable portion of adrenochrome since there is a peak with a maximum at approximately 480 mμ, and also the presence of higher oxidation products due to the secondary maximum at approximately 435 mμ.

Figure 4 presents a detailed experiment conducted by oxidative method B, utilizing a 1.5% epinephrine solution buffered at pH 7.0 including 2% boric acid and 1.0% sodium bisulfite. The rate of oxidation of the sulfite was followed iodometrically and the results are reported as the curve drawn through the closed circles. Under the conditions, the rate of oxidation is first order in respect to the bisulfite concentration. Even though the experimental conditions duplicated those used in the data presented in Fig. 1, the half-life for the initial portion of the reac-

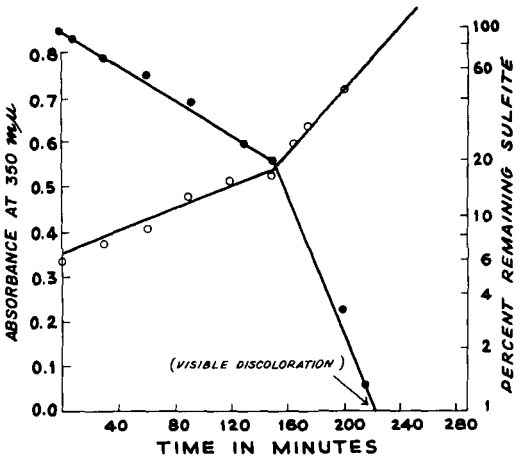

Fig. 4.—Illustrating the rate of oxidation of sulfite ions in the presence of 1.5% epinephrine and 2.0% boric acid at pH 7.0 (closed circles), and the rate of formation of adrenochrome sulfonate as measured by the absorbance at 350 mμ (open circles).

tion is approximately 65 minutes, while the identical solution in the absence of epinephrine possessed a half-life of 8 to 9 minutes. Catechol derivatives are known to be strong chelating agents (17, 18) for cupric and many other metallic ions, and it might be proposed that the epinephrine is a stabilizing agent for the sulfite by chelation of the trace metals.

It was interesting to note the marked change in rate of oxidation of the sulfite near the end of the reaction, even though the pH of the solution remained essentially constant. This second curve was duplicated repeatedly and could be followed to the last traces of sulfite in the solution. The half-life for the second reaction was roughly approximated to be 16 minutes. In a number of the experiments, the second portion was nonlinear. Simultaneous with the analysis of the sulfite content of the solutions, samples were examined spectrophotometrically at 350 mμ to obtain a more definitive understanding of the rate of appearance of the postulated adrenochrome sulfonate compound. The results are reported in Fig. 4 (open circles), and give a clear indication of the events taking place. Apparently there is a small progressive oxidation of a small number of the epinephrine molecules to adrenochrome sulfonate throughout the early part of the reaction, even though the sulfite is present in the solution. When the concentration of the bisulfite falls to approximately 20% of the original value, the rate of appearance of the adrenochrome sulfonate increases rapidly and the sulfite ions are withdrawn from the solution by two processes: (*a*) oxidation of sulfite, and (*b*) reaction with adrenochrome with the formation of adrenochrome sulfonate. The data of van Espen allows one to estimate the molar extinction coefficient of the adrenochrome bisulfite to be approximately 15,000. The increased rate of disappearance of sulfite in the second portion of the reaction cannot be accounted for completely on the basis of reaction with the adrenochrome. Therefore, an increase in the direct oxidative reaction must be presumed.

It must be emphasized that all of the oxidative studies of epinephrine reported above have been conducted under direct (method B) and diffuse (method A) exposure to fluorescent light. When identical solutions were run in the dark by either method, the rate of oxidation of the sulfite was represented by a constant rate of oxidation similar to the first portion of the bisulfite curve in Fig. 4. However, when all of the bisulfite is oxidized, epinephrine in the solution does not immediately oxidize. A long induction period follows which may be continued for days. Immediately after exposure to light, these solutions commence to oxidize and turn red.

It seems apparent, therefore, that some component in the solution is photochemically active in initiating the reaction. Since the 2-hour and 3.5-hour curves of Fig. 3 indicate the presence of an absorbing compound, believed to be adrenochrome sulfonate, it is postulated that this compound is the photochemically active molecule which catalyzes the initial stage in the epinephrine deterioration in the presence of sodium bisulfite. When the last traces of sodium bisulfite disappear, adrenochrome is no longer transformed to the colorless, but photochemically active sulfonate. Since adrenochrome absorbs in the visible, it can be postulated that higher light intensity in this region favors the catalysis via the adrenochrome.

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