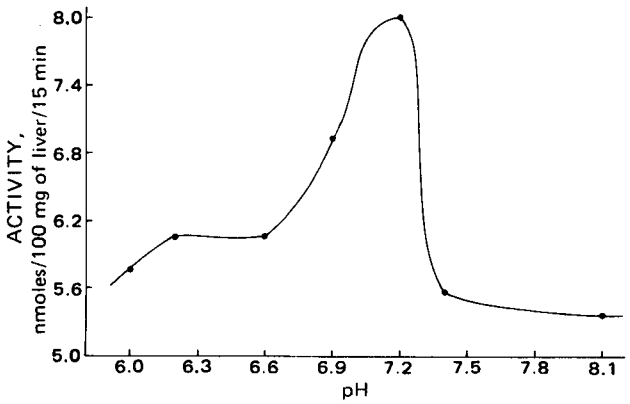

Figure 2—*Hepatic microsomal oxime-amide rearrangement enzyme activity toward fluorenone oxime determined as a function of pH. Each point is the average of six determinations.*

II was not stimulated by the addition of either inorganic sulfate (6.5×10^{-4} *M*) or adenosine triphosphate plus sulfate. Furthermore, the reaction was not inhibited by *p*-nitrophenol (1×10^{-4} *M*), a known consumer of sulfate (14) (and, therefore, a potential competitive inhibitor of the oxime-amide rearrangement system), or 3′,5′-adenosine diphosphate, another known sulfation inhibitor (14). These results suggest that enzymatic conversion of I to II does not involve oxime substrate esterification prior to rearrangement. The reaction mechanism remains a mystery but is being studied.

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Stabilization of 5-Azacytidine by Nucleophilic Addition of Bisulfite Ion

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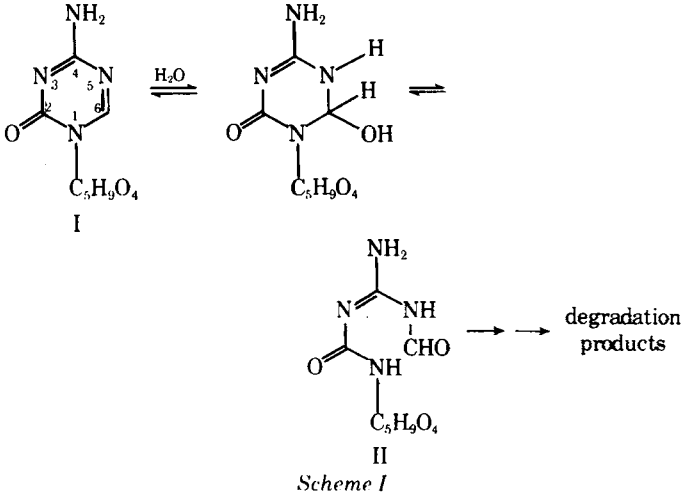
Abstract □ 5-Azacytidine (I) stability was increased approximately 10-fold over its stability in water or lactated Ringer injection by the addition of excess sodium bisulfite and the maintenance of pH ~2.5. The increased stability in the presence of bisulfite at pH 2.5 was attributed to the addition of bisulfite across the 5–6 protonated imine bond of I, which prevented the hydrolytic attack on this labile double bond. However, above pH 4, bisulfite increased I degradation. At higher pH, the compound was no longer protonated and bisulfite did not form the stable addition product. The addition compound quickly decomposed above pH 6 to give back the parent compound and, thus, acted as a I prodrug. The intact drug remaining was assayed by high-pressure liquid chromatography (HPLC), and the reversibility of the bisulfite–I addition product above pH 6 was demonstrated by UV spectrophotometry and HPLC. The potential utility of the bisulfite–I addition product as a I prodrug in parenteral and possible oral dosage forms is discussed.

Keyphrases □ Azacytidine—prodrugs, sodium bisulfite, stabilization in aqueous solutions, effect of pH □ Sodium bisulfite—stabilization of azacytidine in aqueous solutions, effect of pH, prodrugs □ Prodrugs—azacytidine, stabilization by sodium bisulfite □ Antineoplastic agents—azacytidine, prodrugs, sodium bisulfite complex

The use of 5-azacytidine (I) in acute myelogenous leukemia (1–3) is often limited by severe and sometimes dose-limiting nausea and vomiting (4, 5). Although the GI toxicity can be controlled effectively by administering the drug as a slow infusion (6, 7), extreme drug instability (8–10) poses a serious problem. Even when I is infused in lactated Ringer injection, which provides optimum stability conditions for the drug at pH 6.4, ~10% of I is lost in 2 hr at room temperature (8).

BACKGROUND

Systematic kinetic studies on the I hydrolysis (8–10) revealed that hydrolysis proceeds by the classical acid-catalyzed hydration of the 5–6 imine double bond (Scheme I), followed by deamination to yield the formyl derivative, *N*-(formylamidino)-*N*-β-D-ribofuranosylurea (II).



Scheme I

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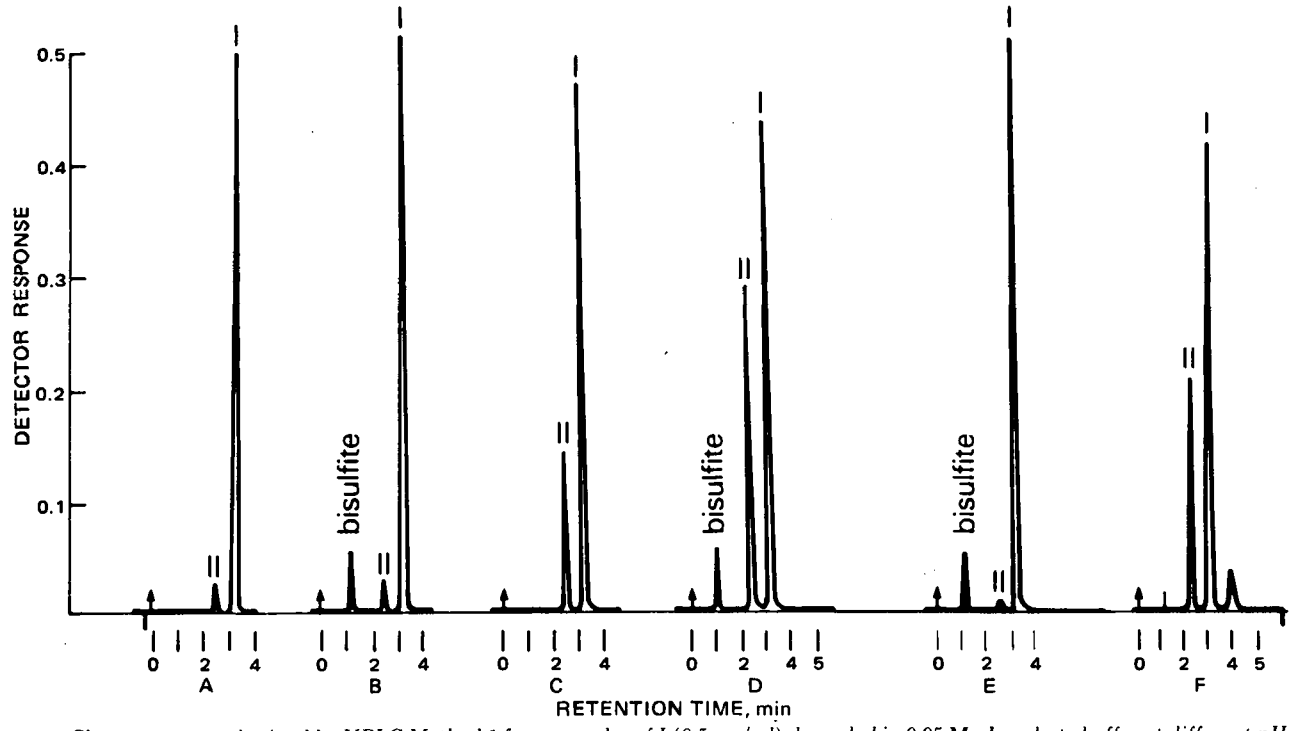


Figure 1—*Chromatograms obtained by HPLC Method 1 from samples of I (0.5 mg/ml) degraded in 0.05 M phosphate buffer at different pH values and room temperatures. Key: A, freshly prepared solutions of I in pH 7.0 phosphate; B, freshly prepared solution of I in pH 7.0 or 2.5 buffer containing 5 mg of sodium bisulfite/ml; C, sample in 1A after 70 min; D, sample in 1B with pH 7.0 buffer after 70 min; E, sample in 1B with pH 2.5 buffer after 70 min; and F, solution of I in pH 2.5 buffer in the absence of bisulfite after 70 min.*

The formyl derivative, II, can either reverse back to I or degrade subsequently (8–10).

The I hydrolysis rate, which is dictated by the pH dependence of hydration and deamination steps, is slowest around pH 6.5–7.0. However, even at this pH of maximum stability, drug degradation is too fast. Therefore, a different approach to improve I stability was needed.

The key to the instability of I is its 5–6 double bond, and saturating this double bond could improve drug stability. Synthesis of one such stable compound, 5,6-dihydro-5-azacytidine, was reported (11). Although the stability of this dihydro compound is excellent, approximately 20 times the dose of I is needed to yield an equivalent antitumor activity in experimental animals (11, 12).

Another approach to render the 5–6 double bond of I stable to water attack would be to add a stronger nucleophile capable of attacking the 5–6 double bond preferentially over water. Such a nucleophile should assure that the addition product does not undergo subsequent deamination; it should be stable in the infusion solution and, most important, it should quickly convert back to I under physiological conditions. The nucleophile should also be physiologically compatible.

Sodium bisulfite is a strong nucleophile and a common antioxidant additive in various pharmaceutical preparations. The nucleophilic addition of the bisulfite ion across the 5–6 double bond of I was demonstrated (9), but no attempt was made to study the results of this reaction or the stabilizing effect of sodium bisulfite on I. This paper reports the effect of sodium bisulfite on I stability in aqueous solutions.

EXPERIMENTAL

Materials—5-Azacytidine for injection (100 mg) was used as received[1]. Water was double distilled in an all-glass apparatus. All other chemicals were reagent grade.

Equipment—A high-pressure liquid chromatograph[2] equipped with a fixed-volume loop-type injection valve[3] and a variable-wavelength detector with a wavelength drive[4] attached to a computing integrator[5] were used. The columns used were a 250-mm × 4.6-mm i.d. reversed-phase column[6] and a 250-mm × 3.2-mm i.d. strong cation-exchange column[7]. Spectrophotometric analyses were carried out on a recording spectrophotometer[8]. All pH measurements[9] were taken at room temperature.

Degradation Studies—Solutions of I (0.5 mg/ml unless otherwise specified) were made by accurately weighing ~10 mg of I in a 20-ml screw-capped vial and adding 20.0 ml of the required buffer. Sodium bisulfite, when required, was incorporated into the buffer before adjustment of its final pH. The solutions were stored at room temperature (22–26°) and at refrigeration temperature (2–6°). The amount of intact I remaining at various time intervals was assayed by high-pressure liquid chromatography (HPLC) as described under *HPLC Method 1*. The time for 10% I degradation was obtained by extrapolation from the amount of intact I remaining at various time intervals.

UV Spectrum—The bisulfite–I mixture[10] was diluted 25-fold with pH 2.5 and 6.5 buffers (0.05 *M* phosphate), and their respective UV spectra using appropriate blanks were recorded[8]. UV spectra of I alone in pH 2.5 and 6.5 buffers were also recorded after appropriate dilution.

Assay and Identification Methods—*HPLC Method 1*—A reversed-phase column[6] with the mobile phase (0.02 *M* phosphate buffer, pH 6.5) at a flow rate of 2.0 ml/min was used to assay I. A 10-μl full loop sample volume was injected quantitatively, and the recorder was set at 0.64 full scale (254-nm detector). The area of the peak due to I was used to calculate the amount of intact I remaining in the sample. Typical run times were less than 6 min. The UV spectrum of the peaks were recorded when needed for identification by stopping the flow at the maximum of the peak and scanning them from 300 to 220 nm using the wavelength drive of the detector[4].

HPLC Method 2—A strong cation-exchange column[7] was used for separation of I from the I–sodium bisulfite addition product. The mobile phase consisted of 0.01 *M* NaCl with 0.01 *M* HCl in water (pH 2), and it was pumped at 1.25 ml/min. A 10-μl full loop sample volume was injected, and the recorder was set at 0.32 full scale (254-nm detector).

[1] Lot BV-77-270, Ben Venue Laboratories, Bedford, Ohio.
[2] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[3] Velco type valve, Spectra-Physics, Santa Clara, Calif.
[4] Model SF770 spectroflow monitor with SFA339 wavelength drive, Schoeffel Instrument Corp., Westwood, N.J.
[5] Autolab System I, Spectra-Physics, Santa Clara, Calif.
[6] Zorbax C-8 column, 6-μm average particle size, DuPont Instruments, Wilmington, Del.
[7] Partisil 10 SCX, 10-μm average particle size, Altex Scientific, Berkeley, Calif.
[8] Cary 17, Varian Instrument Division, Palo Alto, Calif.
[9] Expandomatic SS-2 pH meter, Beckman Instruments, Fullerton, Calif.
[10] Unless otherwise specified, the bisulfite–I mixture was 0.5 mg of I and 5 mg of sodium bisulfite/ml of 0.05 *M* phosphate with the pH adjusted to 2.5.

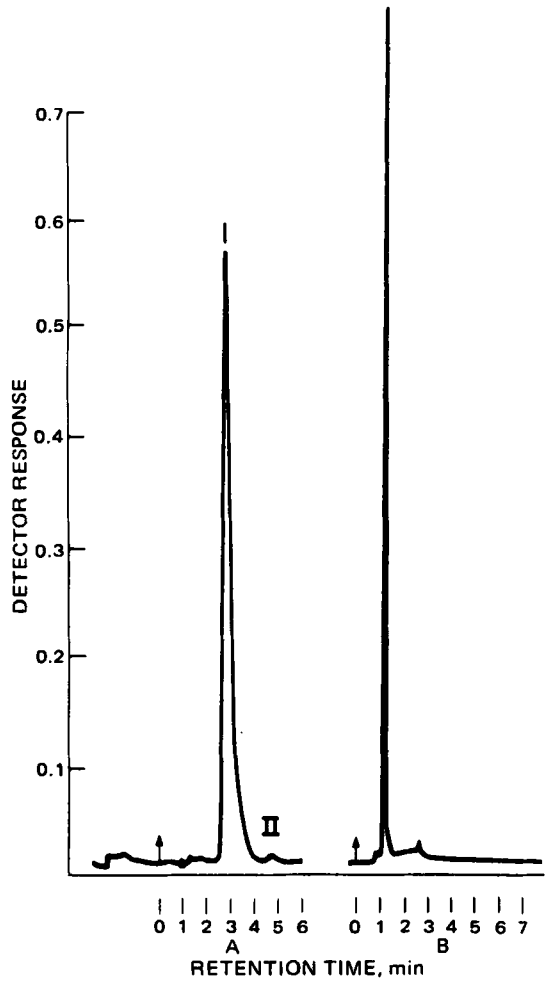


Figure 2—*Chromatograms obtained by HPLC Method 2 from samples of I (0.5 mg/ml) in 0.05 M phosphate buffers at different pH values and room temperature. Key: A, freshly prepared solution of I in pH 7.0 buffer; and B, freshly prepared solution of I in pH 2.5 buffer containing 5 mg of sodium bisulfite/ml.*

RESULTS AND DISCUSSION

HPLC Method 1 was similar in principle to the reversed-phase HPLC method used previously (8) to separate I and II. However, a different reversed-phase column[6] and mobile phase were used. A pH 6.5 buffer (0.02 *M* phosphate) was chosen as the mobile phase to prevent the retention time of I from changing when samples in concentrated buffers at low pH (2.5) were analyzed. Also, as discussed later, the maintenance of a high pH (>6) of the mobile phase was essential for the instant decomposition of the bisulfite–I addition product to yield I during the HPLC analysis.

As seen in Fig. 1F, the column[6] clearly separated the major degradation product, II (retention time of 150 sec), and an unidentified reaction product (retention time of 245 sec) from the peak due to I (retention time of 190 sec). Peak areas of I increased linearly with concentration from 0.3 to 0.5 mg/ml and were used for the calculation of intact I present in the samples.

Figure 1A shows the chromatogram of a freshly prepared solution of I in pH 7.0 buffer using HPLC Method 1. In addition to the major peak (I), a small peak (II) was present. Figure 1C is a chromatogram of the sample in Fig. 1A after 70 min at room temperature. Loss of I (~7%) was accompanied by an increase in peak II. Figure 1F shows the chromatogram of a I solution at pH 2.5 after standing for 70 min at room temperature. As is evident, I was less stable at pH 2.5 than at pH 7.0.

The chromatograms of freshly prepared solutions of I with bisulfite in either pH 2.5 or 7.0 buffer were identical (Fig. 1B). Peak I had the same retention time in all samples shown in Fig. 1. However, after 70 min at room temperature, the sample kept at pH 7.0 degraded almost 15% (Fig. 1D) whereas the same sample (Fig. 1B) kept at pH 2.5 showed little degradation (Fig. 1E). This finding indicates that bisulfite has a strong stabilizing effect on I hydrolysis at pH 2.5 (compare Figs. 1C–1E). This effect is more evident when the chromatogram of the I sample at pH 2.5 in the absence of bisulfite (Fig. 1F) is compared with that in Fig. 1E.

Table I—Time for I to Reach 90% of Its Original Concentration (0.5 mg/ml) in Buffer Solutions at Room Temperature (23–26°)

pH	Buffer	Time to Reach 90%, hr With Bisulfite[a]	Time to Reach 90%, hr Without Bisulfite
6.3	Lactated Ringer injection	—	2.3
7.0	0.05 *M* phosphate	0.8	2.6
6.5	0.05 *M* phosphate	1.2	2.3
6.0	0.05 *M* phosphate	1.0	1.5
4.0	0.05 *M* acetate	1.3	1.4
3.2	0.05 *M* phosphate	8.7	1.2
2.5	0.05 *M* phosphate	20	1.1
2.5	0.05 *M* phosphate	18[b]	—
2.5	0.05 *M* phosphate	6[c]	—
2.5	0.05 *M* phosphate	96[d]	—

[a] Sodium bisulfite, 5 mg/ml, unless specified otherwise. [b] Sodium bisulfite, 3 mg/ml. [c] Sodium bisulfite, 1 mg/ml. [d] Refrigeration temperature (2–6°).

Table I summarizes the effect of bisulfite on I stability at various pH values. Bisulfite increased the I degradation rate when the solution pH was maintained above 4.0. However, below pH 4.0, bisulfite slowed I degradation; the stabilization was more pronounced as the solution pH approached 2.5. Furthermore, at pH 2.5, increasing the amount of bisulfite increased the stabilization. At pH 2.5 in the presence of a 10-fold excess of bisulfite (5 mg of sodium bisulfite/ml with 0.5 mg of I/ml), ~20 hr was required for 10% I degradation at room temperature. This represents almost one order of magnitude improvement over the stability of I in the presently used clinical formulation of drug in lactated Ringer injection.

The large increase in I stability by bisulfite at acidic pH cannot be attributed to the effects of ionic strength but must be attributed to the protection of I by some direct interaction. To confirm that the peaks at 190 sec (Figs. 1B and 1E) (pH 2.5 sample) were due to I and not to the bisulfite–I product (III), the UV spectra of all peaks at 190 sec (Figs. 1A–1E) were recorded and found to be identical to those obtained from pure I. This finding demonstrates that whatever the stabilization mechanism, the addition product (III) eluted as I during HPLC Method 1. However, when the mobile phase had a pH of 2 (HPLC Method 2), the peak corresponding to I was missing in the chromatogram of the bisulfite–I mixture (Figs. 2A and 2B). The chromatogram in Fig. 2A was obtained from the sample represented in Fig. 1A; the chromatogram in Fig. 2B, which shows the absence of peak I, was obtained from the pH 2.5 sample represented in Fig. 1B. Therefore, in spite of high dilution during the HPLC procedure, when the mobile phase of low pH (pH 2) was used, III remained intact and did not elute as a peak corresponding to I. However, at pH 6.5, III rapidly reverted back to I; therefore, the peak resulting from III corresponds to I alone.

Another indication of the reversibility of III to I at pH 6.5 was obtained when equimolar (based on molar concentration of I) concentrations of I and the bisulfite–I mixture gave similar UV spectra (Fig. 3) at pH 6.5 (both I and the bisulfite–I mixture had λ_{max} of 239 nm, ϵ = 6800). However, at pH 2.5, the spectrum (Fig. 3) obtained from the bisulfite–I mixture was much less intense and different from that obtained with I (at pH 2.5, I had λ_{max} at 241, ϵ = 3700, and the bisulfite–I mixture had λ_{max} at 243, ϵ = 1900). Since the loss of absorption at 240 nm reflects the loss of the 5–6 double bond (8, 9), it is reasonable to suggest that the lower ϵ value at pH 2.5 of the bisulfite–I mixture was due to addition of bisulfite across this bond.

Stabilization Mechanism—The results of the present study indicate that bisulfite forms an addition product with I across the 5–6 double bond that is stable at low pH (*i.e.*, 2.5) and thus prevents the hydrolytic attack on the 5-azacytosine ring. The addition reaction at pH 2.5 is reversible and requires excess bisulfite to maintain the equilibrium in favor of the addition product. As seen in Table I, at pH 2.5, when the bisulfite concentration decreased, I stability also decreased. Based on these observations and in accordance with the hydrolysis mechanism proposed for I (9), it appears that the major reaction responsible for stabilization of I is as shown in Scheme II.

The formation of III proceeds through the protonation of N-5, similar to the protonation that usually precedes the nucleophilic attack on imine bonds (13) and as suggested for I hydrolysis (9). Therefore, formation of III (and, hence, stabilization of I against hydrolysis) is expected to increase as the pH is lowered and more I is protonated. If the pKa of I is assumed to be of the order of 2–3 from the reported pKa of the related

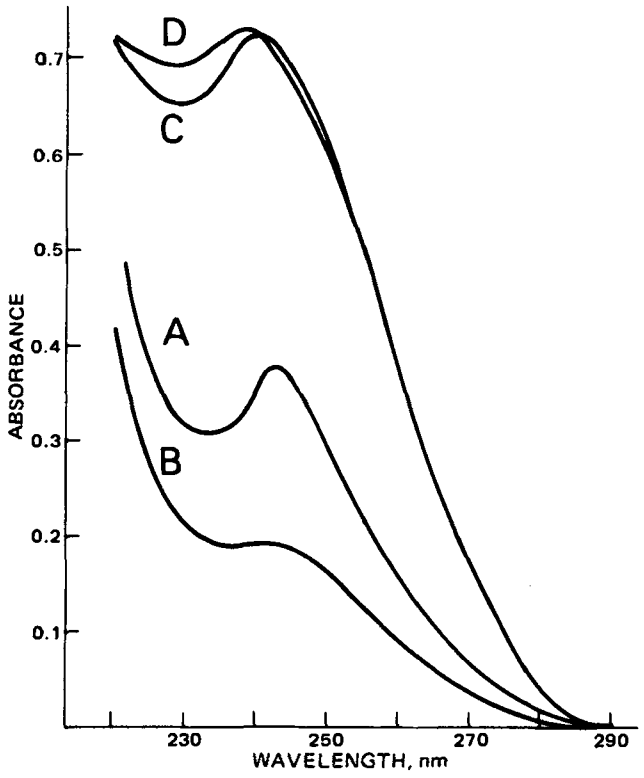
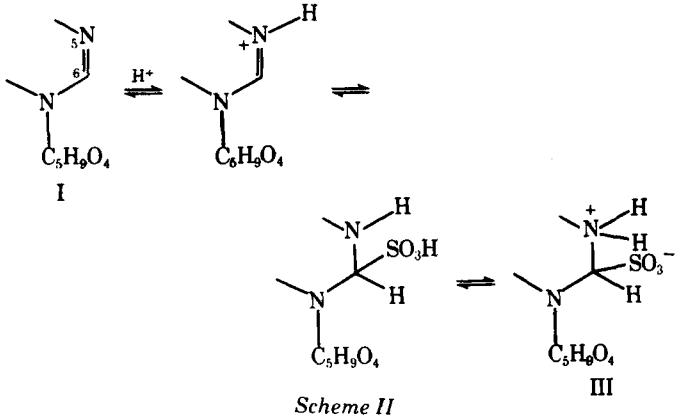


Figure 3—*UV spectrum of I and the bisulfite–I mixture in 0.05* M *phosphate buffer at different pH values. Concentration of I (or its equivalent) in the spectrophotometer cell was 30 μg/ml. Key: A, I in pH 2.5 buffer; B, bisulfite–I mixture in pH 2.5 buffer; C, I in pH 6.5 buffer; and D, bisulfite–I mixture in pH 6.5 buffer.*



Scheme II

compound 5-azacytosine (9), the stabilization would be expected to increase as the pH is lowered below 4, provided the concentration of reactive nucleophile, bisulfite anion (HSO_3^-), is constant.

A substantial portion of sulfurous acid species would exist as the bisulfite anion up to pH 1.5 (pK_1 of sulfurous acid is 1.7), and increased I stability was observed up to pH 1.5. However, in strongly acidic solutions (*i.e.*, 1 *N* HCl), although I is expected to be completely protonated, the fraction of reactive nucleophile, bisulfite anion, is extremely small. Therefore, at very low pH, the addition reaction is expected to be slower. This was the case when a mixture of I and sodium bisulfite in 1 *N* HCl showed almost 90% free I after 1 min and 40% free I after 8 min (HPLC Method 2) whereas a mixture of the same concentrations of I and sodium bisulfite adjusted to pH 2 showed no free I (HPLC Method 2) even after 30 sec. This finding shows that the III formation rate is very fast at pH 2 but relatively slower in 1 *N* HCl.

To demonstrate that this slower rate is not due to an increase in the rate of reverse (desulfonation) reaction at this low pH, the bisulfite–I mixture[10] (where almost all I existed as III, Fig. 2B) was diluted with an equal volume of 2 *N* HCl and the resulting solution was analyzed by HPLC Method 2. The peak height due to I was 3, 20, and 40% of the theoretical amount of I after 0.5, 8, and 20 min, respectively. This finding

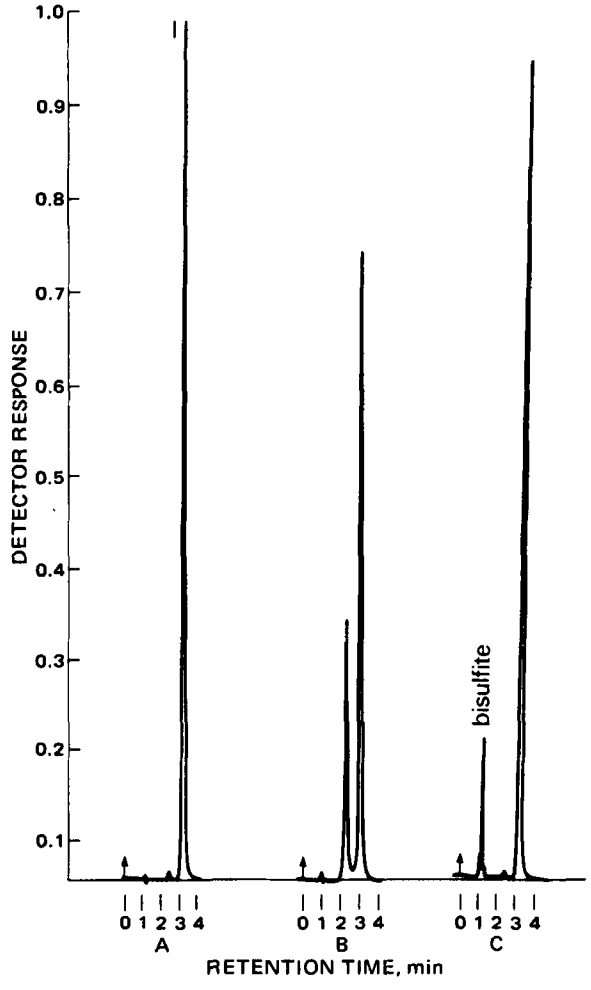


Figure 4—*Chromatograms obtained by HPLC Method 1 from samples of I (0.5 mg/ml). Key: A, freshly prepared solution of I in water; B, freshly prepared solution of I in 0.01* N *HCl; and C, sample in 4B after addition of sodium bisulfite (5.0 mg/ml).*

demonstrates that III does not decompose to I instantly in acidic solutions.

The degree of stabilization would also be influenced by the reverse reaction rate. The data presented in this paper indicate that the desulfonation rate of III to I is relatively slow at acidic pH whereas desulfonation is instantaneous at pH 6.5. It is unlikely that this fast reaction above pH 6 would be due to a direct replacement of bisulfite by hydroxyl ion followed by dehydration to yield I because (*a*) the hydroxyl-ion concentration is too small at pH 6.5 to compete effectively with bisulfite ion and (*b*) the product formed by the attack of hydroxyl ion would be expected to deaminate very fast at pH 6.5 (13). A more likely explanation is that, above pH 6, the cationic nitrogen of III would lose the proton and the resulting lone pair of electrons in N-5 would help in the desulfonation of III to I. At low pH (*i.e.*, pH ~2), the N-5 still would be protonated and desulfonation would be relatively slow. No further studies of this reaction were performed.

Catalysis of I Hydrolysis by Bisulfite at Neutral pH—The described stabilization mechanism of I by bisulfite does not explain why the I degradation rate above pH 4 is increased in the presence of bisulfite compared to buffer alone (Table I). As seen in Figs. 1C and 1D, the degradation product in the presence or absence of bisulfite at pH 7.0 is the same, *i.e.*, II. Therefore, the sulfurous acid species present at these pH values (pH 4–7) apparently does not form any other reaction product but catalyses I hydrolysis. Furthermore, the sulfite dianion appears to be a stronger catalyst than the bisulfite anion because bisulfite decreased I stability by a factor of 3 at pH 7 whereas the stability decrease was only 15% at pH 6, even though the total sulfurous acid concentration was the same (Table I). Since the pK_2 of sulfurous acid is ~6.7, it is reasonable to assume that faster degradation of I at pH 7.0 in bisulfite compared to that at pH 6.0 is due to the presence of a higher fraction of sulfurous acid species in the sulfite dianion form. No attempts were made to elucidate

further the mechanism of bisulfite-ion catalysis of I degradation.

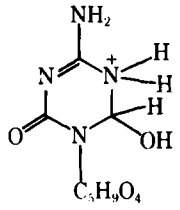
Pharmaceutical Significance—*Parenteral Route*—The results of this study indicate that if the solution in I is maintained at pH 2.5 and if a five- to 10-fold excess of sodium bisulfite (relative to the concentration of I) is provided, I is stable (<10% degradation) for 20 hr at room temperature and for ~4 days at refrigeration temperature. This conclusion represents a substantial improvement in the stability of I in solutions. Further, the ready reversibility of the bisulfite–I addition product (III) to yield I at neutral pH values indicates that III will quickly convert to I in the body and thus act as a I prodrug. Such increased stability will assure better control over the preparation of the I infusion solutions and over the dose being administered.

The results of this study also indicate that 1–2 g of sodium bisulfite would be necessary to provide a stable solution of 200 mg of I[11] to be infused over 24 hr. Infusions of similar amounts of sodium bisulfite in commercial amino acid solution are used routinely, so it appears unlikely that addition of bisulfite would add significantly to the drug toxicity.

Oral Route—The mixture of I with bisulfite also holds promise as an oral dosage form. The administration of I alone is not practicable because of its relatively short stability at 37° over the pH range of the stomach and intestine. Furthermore, at the highly acidic stomach pH, a substantial portion of I would be in the hydrated form (IV) and, as suggested (9), the bioreversibility of IV to I at physiological pH may not be complete. Above pH 6.0, a significant portion of IV probably would deaminate to II and subsequent degradation products, and conversion of IV to I may not be quantitative.

This argument is supported by comparing the chromatogram of a solution of I in 0.01 *N* HCl (Fig. 4B) with that of the same concentrations of I in water (Fig. 4A) using HPLC Method 1. There was a 25% decrease in peak I and an increase in peak II immediately after dissolving I in 0.01 *N* HCl. After further storage, there was a more gradual decrease in peak I in Fig. 4B (an additional 10% decrease in peak I after 20 min at room temperature). Based on the stability studies of I at acidic pH (9), it is unlikely that a 25% loss of I would occur instantly at pH 2 (0.01 *N* HCl); therefore, it must be attributed to the decomposition of IV during HPLC with pH 6.5 buffer as the mobile phase. This conclusion is substantiated by the fact that when bisulfite was added to the solution of I in 0.01 *N* HCl (Fig. 4B), the chromatogram showed complete loss of peak II and an increase in peak I to approximately the original height of peak I in Fig. 4A (Fig. 4C). If II were already present in the sample of Fig. 4B in 0.01 *N* HCl, it is unlikely that it would cyclize immediately and yield I upon addition of the bisulfite. Therefore, in the presence of bisulfite in acidic pH, IV apparently is converted to III, which then elutes as I in HPLC Method 1 as discussed earlier.

This discussion indicates that a significant portion of IV resulting from dissolving I in acidic pH in the absence of bisulfite does not reverse back to I on reaching physiological pH. However, a mixture of I and sodium bisulfite would quickly form a relatively stable compound (III) in the acidic pH of the stomach and then rapidly revert back to I above pH 6. Thus, drug absorbed in the stomach probably would quickly release I on reaching the higher pH (7.4) of blood. Although the stability of the bisulfite–I mixture at the higher pH of the ileum is uncertain, a mixture of I with bisulfite as an oral preparation holds promise as a stabilized dosage form during its passage through the stomach and warrants further investigation by the oral route. The toxicity and efficacy of III and its application in developing suitable dosage forms of I are presently being investigated.



IV

[11] Usual daily dose of I is 100–200 mg/ml.

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