formulating amine salt solutions of concentration greater than the free base solubility is to adjust the pH of the system so that it is at least a half pH unit from the solubility-limiting line.

Because amine solutions are poor self buffers in the pH range where they are totally in solution (as the protonated species), drifting of pH, particularly to more acidic values, is a real formulation problem. In the case of I in unbuffered systems, significant downward pH drifts were experienced in containers in which the solutions had contact with rubber components, particularly in disposable syringes. This was disastrous for this compound in terms of its chemical stability. In the general situation, pH shifts to more alkaline values are also possible. The latter could lead to oiling out of free base in sensitive systems. Both possibilities should be avoided. Thus, a buffer should be considered for each formulation. However, as evidenced in Fig. 2, the selection of a buffer and its concentration must be done with an eye to the buffer's influence on the amine solubility. The data in Fig. 2 are only for succinate buffers; data were also obtained for citrate and acetate buffers, each at a 0.05 *M* concentration, and the effect was qualitatively and quantitatively similar. A 0.05 *M* tromethamine buffer, on the other hand, did not produce as pronounced a depression of the solubility.

The influence of the succinate buffer (Fig. 2) is not interpretable unambiguously with the limited data, but salt formation cannot be ruled out. This raises another point about these systems: salts other than hydrochlorides will have different pH–solubility profiles. One method of improving the solubility picture in the acid pH range is to find a more soluble salt. However, since the dissociation depicted in Eq. 1 will be little affected by the salt anion chosen, solubility in the region of free base control will be negligibly influenced.

Since the dissociations of carboxylic acids and other acidic organic species parallel those discussed here for organic hydrochlorides, it is expected that their pH–solubility profiles can be characterized theoretically using the same treatment. In the special case of carboxylates the roles of the two species will be reversed, the free acid being much less soluble than the corresponding anionic base. Qualitatively, mirror image profiles with respect to the amine hydrochlorides will be obtained and the insolubility of the free acid will determine the minimum pH at which total solution is possible at a given concentration in excess of the free acid solubility. Thus, in carefully characterizing the amine hydrochloride system, the foundations for treatment of other weak electrolytes with respect to relative species solubilities have been laid.

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▲ To whom inquiries should be directed.

Inhibition of Platelet Aggregation by Bisulfite–Sulfite

KIYOMI KIKUGAWA▲ and KAZUHIRO IIZUKA

Abstract □ Blood platelets aggregate in hemostasis and thrombosis. The effect of bisulfite–sulfite, which is frequently added as an antioxidant in injections, on platelet aggregation was examined according to the method of Born and Cross. It was found that the agent (10^{-3}–10^{-2} *M*) inhibited adenosine diphosphate- and collagen-induced aggregation of rabbit platelets in platelet-rich plasma. The platelets that had almost lost their aggregating capacity by treatment with the agent recovered the capacity when they were treated with plasma for the long period. The inhibitory profile of the agent was characteristic, and the agent progressively deaggregated the platelet aggregates. The presence of other blood cells such as erythrocytes had little influence on the effects of the agent on platelets.

Keyphrases □ Platelet aggregation—inhibition by saline bisulfite–sulfite □ Inhibition of platelet aggregation—saline bisulfite–sulfite □ Bisulfite–sulfite—inhibition of platelet aggregation

Bisulfite or sulfite has been frequently added as an antioxidant in injections and used as an antiseptic. Halaby and Mattocks (1) and Wilkins *et al.* (2) demonstrated that the agent possesses great affinity for blood and nonspecific toxicity for tissues. Recent studies on the biopolymers such as nucleic acid (3–6), proteins (7, 8), and lipids (9) showed that the agent readily modified these biopolymers to cause mutation or damage to the functions of the biopolymers. However, little is known about the actions of bisulfite or sulfite on mammalian blood cells.

During this investigation of platelet aggregation, it was found that the functions of platelets were greatly altered by a bisulfite–sulfite mixture. Blood platelets are known to aggregate in hemostasis and thrombosis, and compounds that inhibit platelet aggregation have been extensively investigated (10). The authors now wish to report the strong inhibitory activity of bisulfite–sulfite on platelet aggregation.

MATERIALS AND METHODS

Materials and methods were partly described in the previous papers (11, 12). A mixture of sodium bisulfite and sulfite was ob-

Table I—Effect of Bisulfite-Sulfite on Adenosine Diphosphate- and Collagen-Induced Platelet Aggregation

Test Sample	Percent Inhibition[a] of —Aggregation Induced by— Adenosine Diphosphate	Collagen	Percent Deaggregation
Saline (control)	0	0	13
Bisulfite–sulfite:			
1.0×10^{-3} *M*	9	—	—
2.5×10^{-3} *M*	35	—	—
5.0×10^{-3} *M*	66	—	—
7.5×10^{-3} *M*	69	84	69
10.0×10^{-3} *M*	69	—	—
Adenosine: 10^{-4} *M*	69	59	37

[a] Buffered platelet-rich citrated plasma was incubated with the test chemical at 37° for 3 min. and challenged with adenosine diphosphate (10^{-5} *M*) or collagen at 37°.

tained by neutralizing sodium bisulfite[1] with sodium hydroxide at pH 7, and the bisulfite–sulfite was estimated by titrating with 1 *N* iodine. Platelet-rich citrated plasma obtained from male rabbits was immediately buffered with an equal volume of barbital buffer (pH 7.3) (13), stored at 20°, and used within 5 hr. The pH of the buffered plasma was 7.5–7.7 during the platelet aggregation assays. Platelet-poor plasma was obtained from buffered platelet-rich citrated plasma by centrifugation at 3000 r.p.m. for 10 min.

Platelet Aggregation—Platelet aggregation was measured according to the optical density method of Born and Cross (14) in an aggregometer[2]. One-milliliter samples of buffered platelet-rich citrated plasma were placed in polyethylene cells, preincubated exactly for 5 min., and mixed with a 10-μl. solution of saline (for control) or of test samples in saline. After treatment of the mixture at 37 or 20° for the indicated period, it was then challenged with a 10-μl. solution of adenosine diphosphate or a 100-μl. solution of collagen (0.8 mg.) in saline. Concentrations of adenosine diphosphate were 10^{-5} *M* at 37° and 10^{-6} *M* at 20° unless otherwise mentioned, since strong inhibitory effects of adenosine were observed under the

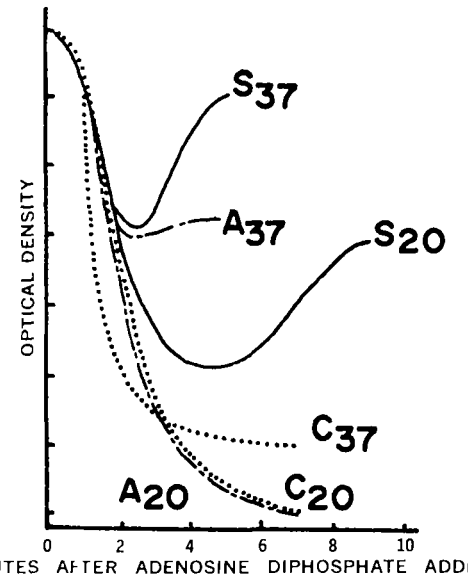

Figure 1—*Inhibition of adenosine diphosphate-induced platelet aggregation by bisulfite–sulfite and adenosine. Buffered platelet-rich citrated plasma, which was treated with the test sample at 20° for 15 min., was challenged with 10^{-5} M adenosine diphosphate at 20°. Key: C_{20}, saline; S_{20}, 7.5×10^{-3} M bisulfite–sulfite; and A_{20}, 10^{-4} M adenosine. Buffered platelet-rich citrated plasma, which was incubated with the test sample at 37° for 3 min., was challenged with 10^{-5} M adenosine diphosphate at 37°. Key: C_{37}, saline; S_{37}, 7.5×10^{-3} M bisulfite–sulfite; and A_{37}, 10^{-4} M adenosine.*

[1] Wako Pure Chemical Industries, Ltd.
[2] Evans EEL 169.

Table II—Effect of Treatment of Adenosine Diphosphate with Bisulfite–Sulfite on Platelet Aggregation

Adenosine Diphosphate	Incubation Time, min.	Aggregation, %
Adenosine diphosphate in platelet-poor plasma	0	100
	50	100
	160	79
Adenosine diphosphate + bisulfite–sulfite in platelet-poor plasma	0	94
	50	92
	160	92

conditions. A decrease in optical density of the stirred plasma was recorded, and the maximum deflection of the optical density curve was read within 15 min. The percent aggregation of a test sample and the percent inhibition of a test chemical were expressed by $100 \times T_{max}/C_{max}$ and $100(1 - T_{max}/C_{max})$, respectively, where T_{max} means the maximum deflection of the test sample and C_{max} means that of the control.

Deaggregation—One-milliliter samples of buffered platelet-rich citrated plasma placed in the cells were challenged with a 10-μl. solution of adenosine diphosphate (10^{-5} *M*) at 37°. After 6 min., maximum aggregation was observed; at this point, a 10-μl. solution of the test samples in saline was added. Percent deaggregation (recovery of the absorbance) after 14 min. was calculated against the maximum aggregation (Fig. 2 and Table I).

Effect of Treatment of Adenosine Diphosphate with Bisulfite–Sulfite on Platelet Aggregation—A saline mixture (0.5 ml.) which contained adenosine diphosphate, bisulfite–sulfite, and platelet-poor plasma (0.2 ml.) was incubated at 20° for the indicated period. A 1-ml. sample of buffered platelet-rich citrated plasma was challenged with 10 μl. of the saline mixture at 20°. The concentrations of adenosine diphosphate and bisulfite sulfite used in the saline mixture were 10^{-6} and 7.5×10^{-3} *M* (final concentration), respectively (Table II).

Effect of Treatment of Platelet-Poor Plasma with Bisulfite–Sulfite on Platelet Aggregation—A saline mixture (0.5 ml.) which contained bisulfite–sulfite, and/or platelet-poor plasma (0.2 ml.) was incubated at 20° for the indicated period. After treatment of 1-ml. samples of buffered platelet-rich citrated plasma with 10 μl. of the above mixture at 20° for the indicated period, it was challenged with 10^{-6} *M* adenosine diphosphate at 20°. The concentration of bisulfite–sulfite used in the saline mixture was 7.5×10^{-3} *M* when added to the buffered platelet-rich citrated plasma (Table III).

Effect of Bisulfite–Sulfite on Platelet Aggregation in Presence of Other Blood Cells—Rabbit citrated whole blood (3 ml.) was buffered with an equal volume of barbital buffer and treated with 60 μl. of the test samples at 20°. Buffered platelet-rich citrated plasma collected from the above mixture by centrifugation (1000 r.p.m., 5 min.) was challenged with 10^{-6} *M* adenosine diphosphate at 20°. For control, buffered platelet-rich citrated plasma (1 ml.) collected as already described from the buffered citrated whole blood (the

Table III—Effect of Treatment of Platelet-Poor Plasma with Bisulfite–Sulfite on Adenosine Diphosphate-Induced Platelet Aggregation

Mixture	Incubation Time, min.	Treatment of Platelet-Rich Citrated Plasma with the Mixture, min.	Inhibition of Adenosine Diphosphate-Induced Aggregation, %
Platelet-poor plasma (control)	0	3	0
	160	3	5
Bisulfite–sulfite	—	3	7
	—	60	29[a]
Platelet-poor plasma + bisulfite–sulfite	0	3	3
	50	3	10
	160	3	10
	160	60	18[a]

[a] Characteristic deaggregation was observed.

Table IV—Effect of Bisulfite–Sulfite on Platelet Aggregation in the Presence of Other Blood Cells

Platelets in	Treatment with	min.	Adenosine Diphosphate-Induced Aggregation—Maximum Aggregation[a]	Inhibition, %
Whole blood	Saline	90	20, 22	
	7.5×10^{-3} *M* Bisulfite–sulfite	90	14, 14	36
Platelet-rich citrated plasma	Saline	90	25	
	7.5×10^{-3} *M* Bisulfite–sulfite	90	13	48

[a] Maximum aggregation was expressed by a relative decrease of optical density.

same preparation as used in the last experiment) was treated with 10 μl. of the test samples at 20° and challenged with 10^{-6} *M* adenosine diphosphate at 20° (Table IV).

RESULTS AND DISCUSSION

Bisulfite–sulfite was found to inhibit strongly the adenosine diphosphate-induced aggregation of platelets in rabbit platelet-rich citrated plasma. The effects of bisulfite–sulfite on 10^{-5} *M* adenosine diphosphate-induced platelet aggregation at 37 and 20° are shown in Fig. 1. The inhibition of aggregation by 7.5×10^{-3} *M* bisulfite–sulfite at 37° was identical in terms of maximum deflection with that of 10^{-4} *M* adenosine, a known powerful inhibitor (14, 15). Adenosine showed no inhibition at 20°, whereas the agent showed activity under the similar conditions. In Table I, the percent inhibitions of adenosine diphosphate-induced platelet aggregation by bisulfite–sulfite compared with those by adenosine are shown. The inhibition was observed above 10^{-3} *M*, and the degree of inhibition was dependent on the concentration of the agent.

In the inhibitions by bisulfite–sulfite, characteristic strong deaggregations after the maximum deflections were observed, and they strengthened greatly the inhibitory activity of the agent (Fig. 1). Platelet aggregation mediated by adenosine diphosphate is characterized by subsequent deaggregation, which has been considered to be caused by breakdown of adenosine diphosphate to adenosine 5′-monophosphate and adenosine (14, 15) or by other unclear mechanisms (16, 17). Deaggregation induced by the agent at 37° was also much stronger than that of adenosine when compared by adding them separately to platelet-rich citrated plasma which had reached the maximum aggregation by adenosine diphosphate. The profiles and percent deaggregation are shown in Fig. 2 and Table I. The acceleration of deaggregation at 20° induced by bisulfite–sulfite (Fig. 1) could not be explained by the induced decomposition of adenosine diphosphate to adenosine 5′-monophosphate or adenosine, since adenosine diphosphate mixed with the agent in plasma and kept at 20° for 160 min. had the same aggregation potency as that kept for 0 min. (Table II). Similar characteristic strong deaggregation also has been observed in the inhibitions by hydroxylamine (11), 6-hydroxyaminopurine riboside, and 2-amino-6-hydroxyaminopurine riboside (12). Platelet deaggregation has been an interesting phenomenon with regard to the aggregation mechanisms (10, 14–17), and compounds that accelerate deaggregation would be useful tools for elucidating platelet aggregation mechanisms.

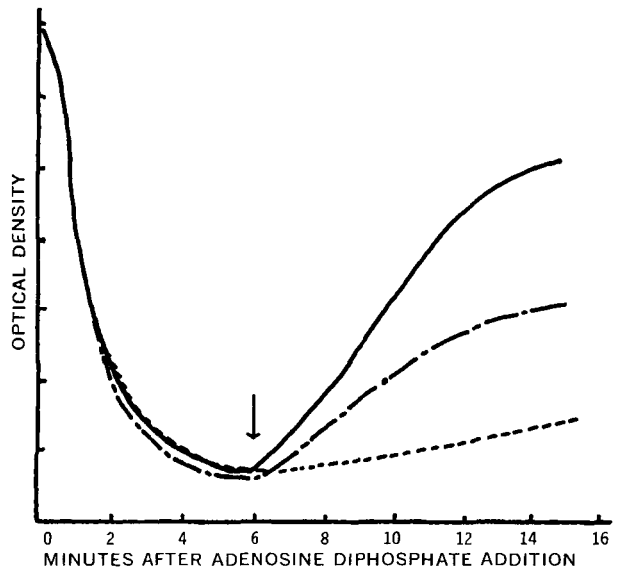

Figure 2—*Deaggregation by bisulfite–sulfite. Buffered platelet-rich citrated plasma was challenged with 10^{-5} M adenosine diphosphate at 37°, and after 6 min. (indicated by an arrow) the test sample was added. Key: - - -, saline; ——, 7.5×10^{-3} M bisulfite–sulfite; and – - –, 10^{-4} M adenosine.*

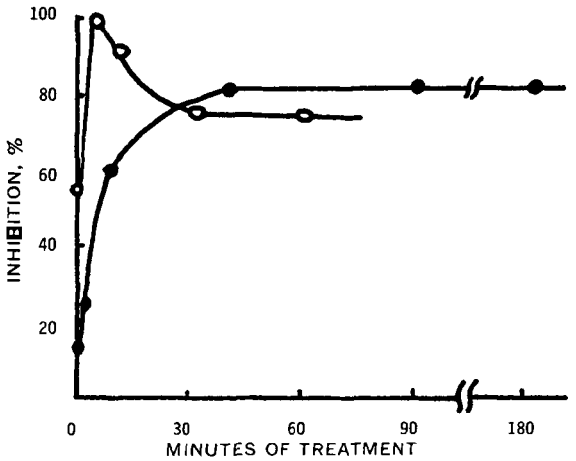

Figure 3—*Effect of treatment of platelet-rich citrated plasma with bisulfite–sulfite on adenosine diphosphate-induced platelet aggregation. Buffered platelet-rich citrated plasma was treated with 7.5×10^{-3} M bisulfite–sulfite for different intervals and was challenged with adenosine diphosphate. Key: ●, 20°; and ○, 37°.*

Treatment of platelet-rich citrated plasma with the agent at 20° for different periods before the addition of adenosine diphosphate showed that the inhibition gradually increased during 40 min. until it reached the maximum inhibition, 83% (Fig. 3). In the treatments longer than the 40-min. interval, not only the percent inhibitions but the rates of deaggregation were constant. This indicated that the bisulfite–sulfite-induced deaggregation observed during 10 min. after adenosine diphosphate addition (Figs. 1 and 2) was not due to the merely increasing action of the agent on platelets. Treatment of platelet-rich citrated plasma with bisulfite–sulfite at 37° for different periods before the addition of adenosine diphosphate showed that the inhibition was highest (100%) at 5 min. and gradually decreased thereafter (Fig. 3). This result indicated that the inhibition by the agent was reversible, as was the case with the inhibition by adenosine (18). The recovery of the aggregating capacity of platelets was considered due to the loss of the agent by some plasma proteins during the longer incubation period, since bisulfite has been demonstrated to react with plasma proteins (8).

Collagen-induced platelet aggregation was also inhibited by bisulfite–sulfite (7.5×10^{-3} *M*); the profile and the extent of inhibition compared with those by adenosine are shown in Fig. 4 and Table I.

With regard to the inhibitions of bisulfite–sulfite on platelet aggregation in platelet-rich citrated plasma, there was a possibility that the substances produced by the agent by reacting with plasma components were the actual inhibitors. Thus, the inhibitory activity of bisulfite–sulfite treated with platelet-poor plasma was tested against adenosine diphosphate-induced platelet aggregation (Table III). When the mixture of bisulfite–sulfite and platelet-poor plasma was kept at 20° for 160 min., the inhibitory activity of the agent was not largely increased unless the mixture was treated with

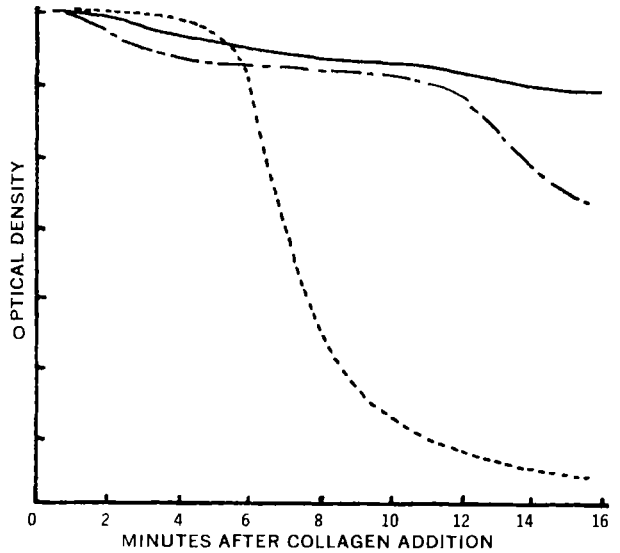

Figure 4—*Inhibition of collagen-induced platelet aggregation by bisulfite-sulfite. Buffered platelet-rich citrated plasma was treated with the test sample at 37° for 3 min. and challenged with collagen at 37°. Key: - - -, saline; -- --, 7.5 × 10^{-3} M bisulfite–sulfite; and — - —, 10^{-4} M adenosine.*

platelet-rich citrated plasma for an adequate period (*e.g.*, 60 min.). Furthermore, the inhibitory activity of the agent was decreased by incubation with platelet-poor plasma. These results indicated that the agent acted directly on platelets and that there was little possibility of the existence of other inhibitors derived from plasma.

All of the experiments described in this report were done with platelet-rich plasma, which was almost free from other blood cells such as erythrocytes. To examine the effect of the agent on platelets in whole blood, citrated whole blood was treated with the agent and the platelet-rich citrated plasma collected from the whole blood was challenged with adenosine diphosphate. In this case too, platelet aggregation was inhibited. Thus, the effects of the agent on platelets were independent of the presence of other blood cells (Table IV). With regard to the coagulation time determined according to the method of Lee–White, rabbit blood was not affected by 10^{-2} *M* bisulfite-sulfite.

Bisulfite-sulfite has been used as an antioxidant in injections (up to 0.5%) and as an antiseptic, but little has been known about its actions on blood cells. It is, therefore, noteworthy that bisulfite–sulfite alters the functions of platelets. Furthermore, a simple chemical such as bisulfite-sulfite might be an excellent tool for the investigation of the mechanisms of platelet aggregation and its inhibition.

SUMMARY

1. Bisulfite-sulfite (10^{-3}–10^{-2} *M*) inhibited adenosine diphosphate- and collagen-induced aggregation of rabbit platelets in platelet-rich plasma.

2. Inhibitory activity of the agent was characterized by a strong deaggregation induced by unclear mechanisms.

3. The extent of inhibition was dependent on the interval of treatment of the platelet-rich plasma with the agent, and the inhibition was reversible.

4. The inhibition was due to the direct action of the agent on platelets, not to the degradation of the inducer nor to the production of other inhibitors in plasma.

5. The effects of the agent on platelets were observed in the presence of other blood cells.

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▲ To whom inquiries should be directed.