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ANTIPEROXIDE ACTIVITY OF SODIUM METABISULFITE

A DOUBLE-EDGED SWORD

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Abstract—Sulfites are chemical substances that are used widely in the pharmaceutical industry to reduce or prevent oxidation. Sodium metabisulfite ($Na_2S_2O_5$) is still present in several parenteral amino acid solutions. Since intravenous lipid emulsions are contaminated by hydroperoxides, we evaluated whether metabisulfite had an antioxidant activity against hydroperoxides. *In vitro*, $Na_2S_2O_5$ inhibited the oxidant activity of H_2O_2, *tert*-butyl-, and cumene hydroperoxides. The antioxidant capacity of metabisulfite was supported *in vivo* by the lower ($P < 0.01$) excretion of malondialdehyde, a stable end product of lipid peroxidation, in babies receiving metabisulfite in their parenteral nutrition. However, for concentrations outside the range found in solutions for parenteral nutrition, the reduction of hydroperoxides by $Na_2S_2O_5$ could transform this compound into an oxidant, like a sulfite radical. It is suggested that metabisulfite has antiperoxide properties that, under specific conditions, contribute to the generation of toxic oxidants.

Key words: parenteral nutrition; hydroperoxides; metabisulfite; reactive oxygen species; malondialdehyde; antioxidant

Oxidation can cause instability and loss of potency in certain drugs [1], especially those intended for intravenous administration. This reaction can be inhibited by the inclusion of antioxidants in drug products. Sulfites are chemical substances that are used widely in the pharmaceutical industry to reduce or prevent oxidation [1, 2]. Because adverse reactions to sulfites have occurred in certain sensitive subjects [3–5], the Food and Drug Administration has proposed requiring a warning on the label of all prescription drugs containing sulfiting agents [1].

Sulfites commonly used as antioxidants are sodium or potassium metabisulfite, sodium or potassium bisulfite, and sodium or potassium sulfite. Sodium metabisulfite is still present in several parenteral amino acid solutions [2] at concentrations varying from 1.5 to 3×10^{-3} M. In view of recent reports that intravenous lipid emulsions are contaminated by hydroperoxides [6, 7], it would be of interest to evaluate whether bisulfite can quench this source of oxidants known to cause tissue injury [8]. This question is of clinical relevance since amino acid solutions containing metabisulfite (S_2O_5) are often infused concomitantly with lipid emulsions. Despite the wide use of sulfites, their antioxidant properties are poorly documented. Others have even suggested that bisulfite could become an oxidant [9–11].

We previously studied the local vascular effects of metabisulfite on a human umbilical vein model infused with clinically relevant amino acid solutions [12]. We documented that metabisulfite interfered with vascular tone as well as prostaglandin synthesis, a marker of endothelial oxidative injury [13–15]. It remains unclear whether these findings are related to a direct effect of metabisulfite on vascular mediators or to its antioxidant properties. The direct effect of metabisulfite could be related paradoxically to oxidant properties.

Therefore, the aim of this study was to determine *in vitro* and *in vivo* whether metabisulfite, used at concentrations found in parenteral alimentation, has an antiperoxide activity.

MATERIALS AND METHODS

The antioxidant activity of metabisulfite was tested by measuring, first, *in vitro*, its inhibitory effect on oxidant activities of H_2O_2, *tert*-butyl- and cumene hydroperoxide; and second by researching *in vivo*, a reduction in the urinary excretion of malondialdehyde (MDA)† in neonates receiving metabisulfite in their parenteral alimentation. MDA is a widely used and well documented index of lipid peroxidation [16]. Urinary excretion of MDA has been shown in animals and in humans to be affected by dietary antioxidants [17] and to increase under the influence of oxidants [18]. Furthermore, urinary MDA has been shown to correlate with the so-called free radical mediated complications of prematurity [19].

Materials. *Tert*-butyl hydroperoxide (TBH), hydrogen peroxide (H_2O_2) and cumene hydroperoxide (Cum-OOH) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Xylenol orange [*o*-cresolsulfonphthalein-3′,3″-bis-(methyliminodiacetic acid sodium salt)], scopoletin (7-OH-6-methoxycoumarin) and ferrous chloride

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† Abbreviations: MDA, malondialdehyde; TBH, *tert*-butyl hydroperoxide; HPO, horseradish peroxidase; TPN, total parenteral nutrition; and TBA, thiobarbituric acid.

were also purchased from Aldrich. 1,1,3,3-Tetraethoxypropane was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase (HPO, EC 1.11.1.7), xanthine oxidase (EC 1.1.3.22) and catalase (EC 1.11.1.6) were obtained from Boehringer Mannheim (Laval, Quebec, Canada). Sodium metabisulfite (*m*-bisulfite) and thiobarbituric acid were purchased from Fisher Scientific Ltd. (Ottawa, Ontario, Canada). The Intralipid 10%™ emulsion, the amino acid solution Travasol 10%™ in Viaflex™ containing no *m*-bisulfite (TIV), and the amino acid solution Travasol 10%™ in glass containing 300 mg/L of *m*-bisulfite (Blend C) were obtained from Clintec Canada Inc. (Mississauga, Ontario, Canada).

Since *m*-bisulfite ($S_2O_5^=$) is linked to bisulfite ($SO_3^=$) by the following equation $2HSO_3^- \Leftrightarrow S_2O_5^= + H_2O$ [20], *m*-bisulfite was used instead of bisulfite [10, 11] to generate sulfite radicals. Sulfite radicals were generated either by adding 0.1 mM hydrogen peroxide to 10 mM *m*-bisulfite and 0.5 U/mL xanthine oxidase [10] or by adding a trace metal [11] such as iron (1.0 μM ferrous chloride) to *m*-bisulfite (10^{-6} to 10^{-3} M).

In vitro *study*. Hydrogen peroxide was detected by the HPO-mediated oxidation of scopoletin [21]. Briefly, reduced scopoletin is a compound that fluoresces at 460 nm with an intensity directly proportional to its concentration if activated by light at 350 nm (Perkin–Elmer luminescence spectrometer LS50); a drop in fluorescence is related to the oxidation of scopoletin catalyzed by HPO in the case of H_2O_2 [21]. Preparations containing H_2O_2 were assayed by adding 3 μM scopoletin and 5 U HPO in a phosphate buffer (pH 7.4) for a total volume of 2 mL. Between 0 and 2 μM H_2O_2, the readings were linear and were abolished by the addition of 20 μg catalase. Concentration–response curves were sought between a fixed concentration of H_2O_2 (2 μM) and increasing *m*-bisulfite (10^{-6}–10^{-3} M). The intra- and interassay coefficients of variation were 3.4 and 3.2%, respectively.

A further technique that detects a wide range of hydroperoxides [22] was used. Briefly, under acidic conditions Fe^{2+} is oxidized in the presence of hydroperoxides; the Fe^{3+} that is formed reacts with xylenol orange, which absorbs at 560 nm (Beckman spectrophotometer DU-6) proportionally to the concentration of hydroperoxides [22]. For the assays, 22.5 mM H_2SO_4, 90 μM xylenol orange and 225 μM Fe^{2+} were added to 100 μL of sample, for a total volume of 1 mL. The absorbance was read after a 30-min incubation at room temperature. The linearity was tested for H_2O_2 (0–250 μM), cumene hydroperoxide (0–100 μM) and TBH (0–100 μM). Concentration–response curves were sought between fixed concentrations of the three peroxides (H_2O_2: 200 μM; Cum-OOH: 100 μM; TBH: 50 μM) and increasing *m*-bisulfite (0–500 μM). The intra- and interassay coefficients of variation were 4.3 and 5.3%, respectively.

To test the hypothesis that the reaction between *m*-bisulfite and hydroperoxides generates an oxidant, we verified first, whether scopoletin would be oxidized spontaneously (in the absence of HPO) by sulfite radical generating systems [10, 11]; and second, whether *m*-bisulfite plus a hydroperoxide would generate a similar response. Therefore, a response was sought between reduced scopoletin and a preparation in which the ratio of *m*-bisulfite to TBH varied from 1:1000 to 1000:1. For this purpose, three concentrations of TBH (10^{-5}, 10^{-4} and 10^{-3} M) were used in the presence of increasing concentrations of *m*-bisulfite (10^{-7} to 10^{-2} M).

In vivo *study*. To evaluate the potential antioxidant effect of *m*-bisulfite, urinary MDA excretion was measured in infants receiving two total parenteral nutrition (TPN) regimens differing only by the *m*-bisulfite content (0 vs 300 mg/L) of the infused amino acid solutions.

Sixteen infants with gastrointestinal disorders (11 necrotizing enterocolitis, 5 gastroschisis) precluding oral feeding were included in this study. They had a mean ± SD birth weight of 1.80 ± 0.72 kg, a gestational age of 32 ± 2 weeks, and post-natal age of 19 ± 6 days. These infants had no congenital anomalies other than abdominal wall defects, and they were studied while clinically stable as they required no oxygen supplementation and had no documented or suspected infection and no hyperbilirubinemia. Thirteen of these infants were part of a clinical study previously reported [23].

To minimize the impact of catabolism on study results, the protocol was initiated at least 5 days after the acute surgical or medical events that led to the indication of TPN. The experimental design consisted of a Latin-square cross-over [24] in which each infant served as his/her own control. This enabled us to evaluate separately for statistical significance ($P < 0.05$), by ANOVA, the effects of treatment (TIV vs Blend C), time, and sequence of administration. Each patient received during two 4-day periods, isoenergetic, isolipidic (Intralipid 10%) and isonitrogenous regimens differing only by the *m*-bisulfite content of the infused amino acid solutions: TIV (0 mg/L) and Blend C (300 mg/L). All solutions were supplemented with electrolytes and trace elements, as previously described [25]. The total volume of multiple vitamins (MVI-Ped, from USV Inc., Mississauga, Ontario, Canada) added to the amino acid solutions was based on the weight at study entry and was kept identical for each patient throughout the study (1.5 mL < 1.5 kg; 3.25 mL if 1.5 to 3.0 kg; 5 mL > 3.0 kg) since lipid-soluble vitamins are well known to affect lipid peroxidation. The lipid emulsion was "piggy-backed" on the amino acid dextrose solution. The measurements were made on the last day of each period. To evaluate the effect of time and of the sequence of administration on MDA, patients were randomly assigned to start with either regimen (half started the protocol with Blend C and the other half with TIV). Approval by the institutional ethics committee and informed parental consent were obtained before the investigation.

On the last day of each period, a 24-hr timed urinary collection was performed by a previously described technique [25], using a leak-proof adhesive collector. Samples were stored at −70° until analysis. The infants had no gastric aspirates nor losses in stools since they were on TPN for over 8 days at the time of sample collection.

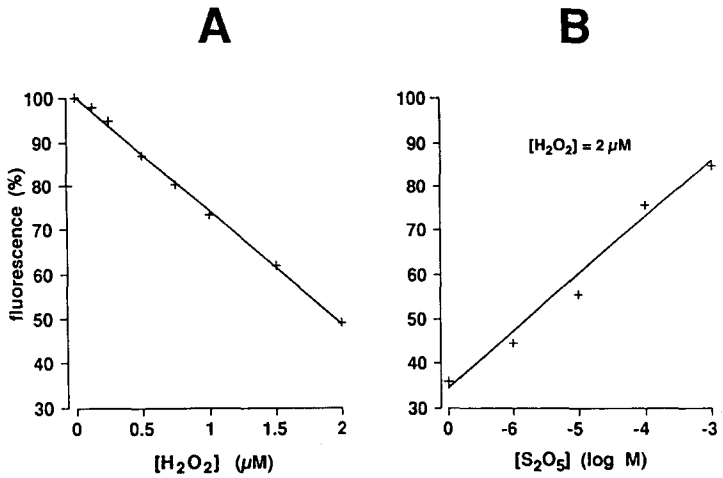

Fig. 1. (A) Relationship between the intensity of fluorescence (% of the blank) of 3 μM scopoletin in the presence of 5 U of HPO and increasing concentrations of hydrogen peroxide. (B) Relationship between the intensity of fluorescence (% of the blank) of 3 μM scopoletin in the presence of fixed amounts of HPO (5 U) as well as H_2O_2 (2 μM) and increasing concentrations of *m*-bisulfite.

MDA was measured after isolation of the MDA–thiobarbituric acid complex (MDA–TBA) by HPLC (Waters 680 Millipore Corp., Milford, MA, U.S.A.) as described by Lepage *et al.* [26]. The separation occurred on a C_{18} column (Waters Microbondapak C_{18}, Millipore Corp.) using a linear gradient of CH_3OH:H_2O as the mobile phase; the ratio changed from 1:9 to 4:6 after 10 min. The MDA–TBA had a maximal absorbance at 532 nm (Waters 441 detector, Millipore Corp.). 1,1,3,3-Tetraethoxypropane was used as the standard [26].

RESULTS

In vitro. In the presence of the peroxidase, HPO, the fluorescence of scopoletin was inversely proportional to the concentration of H_2O_2 between 0 and 2 μM (Fig. 1A). This oxidation of scopoletin with 2 μM H_2O_2 was inhibited by increasing concentrations of *m*-bisulfite (Fig. 1B). The oxidation of scopoletin catalyzed by HPO is specific to H_2O_2, as this reaction did not detect Cum-OOH or TBH at concentrations up to 1 mM.

To confirm this antiperoxide activity of *m*-bisulfite, the xylenol orange technique was used. Hydrogen peroxide, Cum-OOH and TBH produced an absorbance of the complex "xylenol orange–Fe^{3+}" proportional to their concentrations (Fig. 2A). The oxidative properties of all three peroxides were inhibited by increasing concentrations of *m*-bisulfite (Fig. 2B). One mole of *m*-bisulfite was needed to quench the effect of 2 moles of H_2O_2, whereas the reaction with the other two hydroperoxides was equimolar.

The sulfite radical generating systems exhibited an oxidant activity as documented by a drop in fluorescence of scopoletin (Figs. 3 and 4), which increased with time (Fig. 4). Separately, *m*-bisulfite, H_2O_2, hydroperoxides, Fe^{2+}, and xanthine oxidase had no effect on scopoletin, in the absence of HPO.

The reaction of *m*-bisulfite with TBH produced spontaneously (in the absence of HPO) an oxidation of scopoletin. The highest oxidant activity was reached with 10^{-3} M TBH (Fig. 5). This oxidant activity reached a maximum when the concentration of hydroperoxide was higher than that of *m*-bisulfite. When the concentration of *m*-bisulfite exceeded that of hydroperoxide, fluorescence returned towards baseline (100% of the blank).

In vivo. The Blend C (with *m*-bisulfite) and TIV regimens differed only by their *m*-bisulfite content, as energy (313 ± 8 vs 322 ± 4 kJ/kg/day), amino acid (2.6 ± 0.1 vs 2.7 ± 0.1 g/kg/day) and lipid (2.2 ± 0.2 vs 2.2 ± 0.1 g/kg/day) intakes were similar. There was no difference in the volume of urine collections between both treatments (102 ± 4 vs 99 ± 3 mL/kg/day). MDA excretion showed important inter-individual variation, underlining the relevance of performing the measurements in a paired fashion. No clinical correlation was found with individual MDA excretions. The statistical analysis revealed no carry-over effect of time nor of the sequence of administration on urine MDA excretion, allowing for the presentation of the effect of treatment in a paired fashion.

The 24-hr urinary excretion of MDA differed significantly ($F_{(1,14)} = 12.8$, $P < 0.005$) between regimens. The presence of *m*-bisulfite (Blend C) was associated with a lower MDA output (68 ± 6 vs 81 ± 7 nmol/kg/day). These values are comparable to those reported by others in a similar population [19].

DISCUSSION

This study documents that *m*-bisulfite has antioxidant activities against hydroperoxides (Figs. 1 and 2), but under specific conditions it may have oxidant properties (Figs. 3–5). Previous work has shown that SO_3^{2-} generates sulfite radicals [9–11].

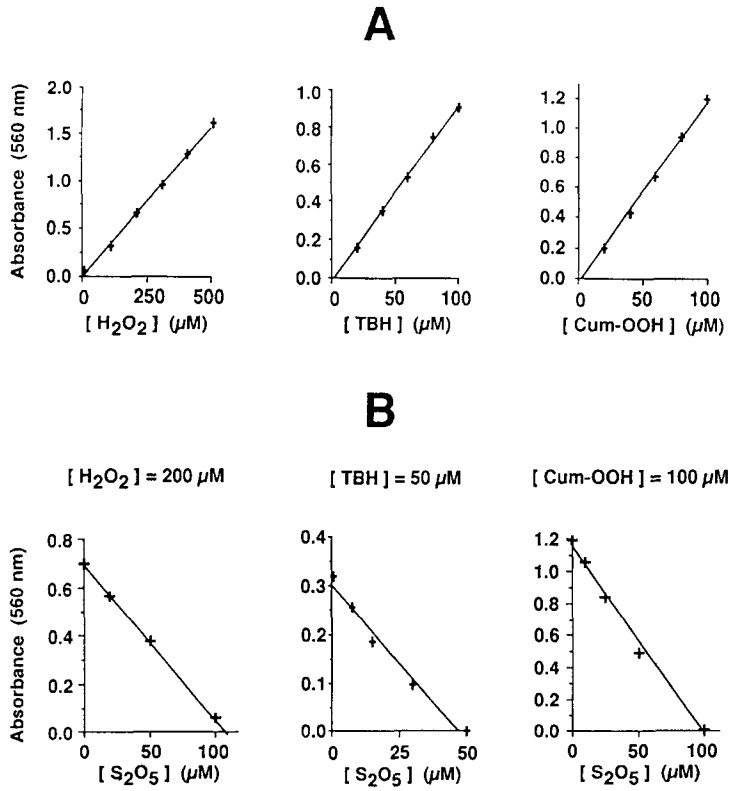

Fig. 2. (A) Concentration–response curves between the absorbance of the xylenol orange–Fe^{3+} complex and increasing concentrations of hydrogen peroxide, cumen hydroperoxide (Cum-OOH), and *tert*-butyl hydroperoxide (TBH). (B) Concentration–response curves between the absorbance of the xylenol orange–Fe^{3+} complex in the presence of fixed concentrations of H_2O_2 (200 μM) or Cum-OOH (100 μM) or TBH (50 μM) and increasing concentrations of *m*-bisulfite.

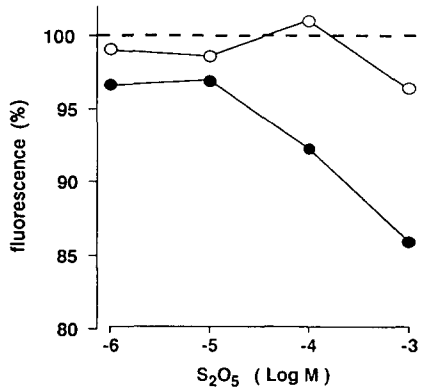

Fig. 3. Concentration–response curves between the intensity of fluorescence (% of the blank) of 3 μM scopoletin and increasing concentrations of metabisulfite in the presence (●) or the absence (○) of Fe^{2+} (1.0 μM).

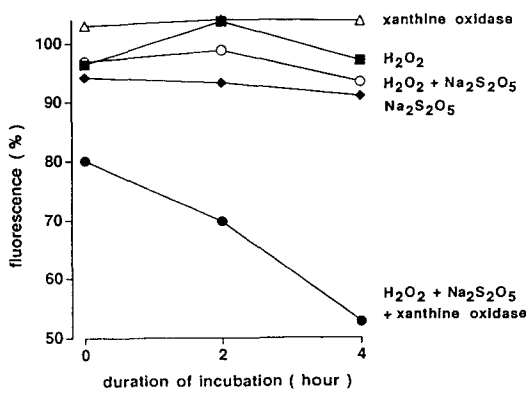

Fig. 4. Relationship between the intensity of fluorescence (% of the blank) of 3 μM scopoletin and the components of the sulfur radical generating system (xanthine oxidase: 0.5 U/mL; H_2O_2: 10^{-4} M; $Na_2S_2O_5$: 10^{-2} M).

Since $Na_2S_2O_5$ is linked to SO_3^{2-}, metabisulfite could also be transformed in a sulfite radical, as suggested by its prooxidant activity.

The antiperoxide activity could be of pharmacological importance, because of the recent reports that there is a spontaneous generation of 0.3 mM hydroperoxides in 20% lipid emulsions used during intravenous alimentation [6]. The results from

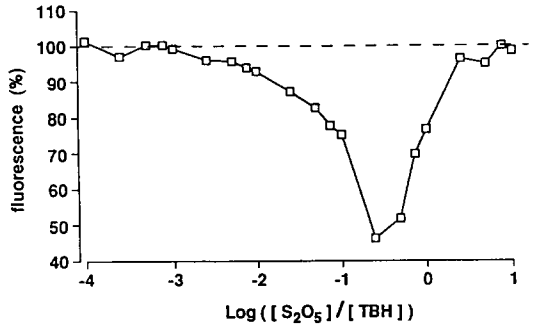

Fig. 5. Relationship between the intensity of fluorescence (% of the blank) of 3 μM scopoletin in the presence of TBH (10^{-3} M) and increasing concentrations of metabisulfite expressed as a ratio (*m*-bisulfite/TBH).

Fig. 2B suggest that the reaction between *m*-bisulfite and TBH or Cum-OOH was equimolar. Based on these data, one can extrapolate that these exogenous lipid hydroperoxides would be quenched by the infusion of clinically relevant volumes of amino acid solutions containing 1.5 to 3 mM *m*-bisulfite. Indeed, since the ratio in volumes of a 10% amino acid solution to a 20% lipid emulsion is usually close to 1:1, *m*-bisulfite would be in excess to hydroperoxide concentrations by a factor of 5 to 10. Figure 5 shows that under those conditions *m*-bisulfite would not have an oxidant activity. This is supported by the results of the clinical study, suggesting that *m*-bisulfite protected the infants against lipid peroxidation, as evidenced by the drop in MDA excretion. However, the solutions of parenteral alimentation contain other possible sources of peroxides such as vitamins and amino acids [27, 28], which could also interfere with *m*-bisulfite.

This study supports previous reports that *in vitro* sulfites can become oxidants [9–11]. Concomitantly to its antiperoxide effect (Figs. 1 and 2), the reaction of *m*-bisulfite with a hydroperoxide such as TBH can produce an oxidation of scopoletin (Fig. 5). This suggests that the reduction of hydroperoxides by $Na_2S_2O_5$ could transform this compound into an oxidant similar to a sulfite radical. Further research is required to explain the mechanisms by which *m*-bisulfite-derived oxidants are formed and also why this oxidant activity disappears when *m*-bisulfite is in excess of TBH (Fig. 5).

We have documented that *m*-bisulfite has systemic effects on MDA excretion as well as biological effects in human neonatal tissue [12]. But the concentration of *m*-bisulfite can be critical, because, in the presence of peroxides, it is a double-edged sword with antioxidant as well as prooxidant properties. This is especially true in neonates who often require parenteral nutrition and who are known to have immature antioxidant capacities [29].

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