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Metabolism of Sulfur Dioxide in 'Thompson Seedless' Grape Berries

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Abstract. **'Thompson Seedless' grapes (*Vitis vinifera* L.) were fumigated with 0.5% SO_2 at 0°C, and the amount of residual sulfite in the berries was followed over time. To prevent autooxidation during analysis, sulfite was stabilized using a solution of tetrachloromercurate-glycine, pH 10. After 72 hr, over 90% of the sulfite was oxidized to sulfate. Kinetics of sulfite loss indicate 2 different rates of loss, giving half-lives for sulfite of about 4 hr (70% of the total sulfite) and 20 hr (30% of the total sulfite). Presumably this reflects 2 forms, free and bound sulfite.**

Exposure of table grapes to SO_2 to control decay by *Botrytis cinerea* Pers. has been practiced for more than 50 years. One of the earliest reports of its use was by Winkler and Jacob (17). Since the SO_2 treatment does not kill the fungus inside the berry but only sterilizes the surface (5), repeated exposure at 7 to 10 day intervals to SO_2 is necessary to prevent the spread of the fungus from infected berries during storage (10). Sulfur dioxide also can inflict damage to the grapes, and a compromise must be reached between the beneficial and harmful properties of the gas.

Gaseous SO_2 is soluble in water and ionized depending on the pH of the solution. At pH 7 it exists largely in the form of bisulfite and sulfite ions (13):

$$SO_2(g) \longleftrightarrow SO_2(aq) \underset{pK = 1.76}{\overset{H_2O \quad H^+}{\rightleftharpoons}} HSO_3^- \underset{pK = 7.20}{\overset{H^+}{\rightleftharpoons}} SO_3^{2-}.$$

Although SO_2 fumigation of table grapes is an important commercial practice and is used extensively, little information is available on the fate of SO_2 on the grapes after fumigation. In one study (9), "free" and "total" SO_2 were determined on 'Flame Tokay' grapes 1–2 hr and 24–30 hr after fumigation. During the crushing of the berries, however, no precautions were taken to stabilize the sulfite, which is known to be very labile. Our study was conducted to determine the lifetime and fate of SO_2 on table grapes, taking necessary precautions to stabilize sulfite during analysis. Unless otherwise noted, sulfite will be used to denote the total of aqueous SO_2, bisulfite, and sulfite.

Materials and Methods

Table grapes were harvested from the Univ. of California, Davis Farm, and were held at 0°C up to 3 weeks with no exposure to SO_2 until experimental treatment.

Grapes were exposed to nonradioactive SO_2 or radioactive $^{35}SO_2$. For both treatments, berries were cut individually from clusters with about 2–4 mm of pedicle remaining. Only berries with a fully intact pedicle and skin were used. Individual berries instead of clusters were used to allow for uniform SO_2 exposure to all berries. All exposures to SO_2 and subsequent storage was at 0°C. In the experiment with nonradioactive SO_2, berries were distributed evenly at the bottom of a 6 liter glass jar, and pure SO_2 was added with mixing to give 0.5% (by volumes) SO_2. Although the concentration of SO_2 used commercially in the initial fumigation depends on many factors, it is about 0.5% for 20–30 min (10). In our experiment, SO_2 exposure was for 30 min, then the jar was flushed with air. Triplicate samples of 3 berries each were taken immediately (designated "0" time) and at subsequent intervals to determine sulfite content. Berries were homogenized in 15 ml of solution containing equal volumes of 0.2 M glycine pH 10 and 0.04 M tetrachloromercurate. After homogenization, the pH was about 8–8.5. Following centrifugation, SO_2 was distilled from the supernatant in Conway diffusion vessels to eliminate interfering substances. An aliquot of the supernatant placed in the outer well was acidified with sulfuric acid, and the liberated SO_2 was reabsorbed in 0.04 M tetrachloromercurate placed in the inner well. Sulfite was determined using the pararosaniline assay (12).

Berries were exposed to $^{35}SO_2$ in a 125 ml Erlenmeyer flask. [^{35}S]sulfur dioxide was generated by acidification of Na[^{35}S]sulfite (79 μCi with specific activity of 50 μCi/μmol) plus 29 μmol of unlabeled sulfite in a 5 ml shell vial and transferred via tubing to the partially evacuated Erlenmeyer flask. The resulting SO_2 concentration was about 0.5% with a specific activity of 2.56 μCi/μmol. After exposure for 35 min, the flask was vented in a hood, and duplicate samples of 2 berries were taken immediately (designated "0" time) and subsequently at 3.5 and 12 hr. Berries were homogenized using a mortar and pestle in 6 ml of solution containing equal volumes of 0.2 M glycine pH 10 and 0.04 M tetrachloromercurate. After centrifugation, an aliquot of the supernatant was taken and [^{35}S]sulfite and [^{35}S]sulfate were separated with high voltage paper electrophoresis at pH 7, and the ratio of these ions were determined using a radioscanner.

Results

Tetrachloromercurate often has been used to stabilize sulfite and prevent its oxidation to sulfate (16). In preliminary experiments, we tried various concentrations of tetrachloromercurate to prevent the oxidation of sulfite during the homogenization of grape berries, but found them ineffective to inhibit sulfite oxidation completely. We found, however, a solution of equal volumes of 0.04 M tetrachloromercurate and 0.2 M glycine (pH 10) almost completely prevented sulfite oxidation during the homogenization of grape berries and subsequent analysis by paper electrophoresis (Table 1). The stabilization of sulfite during homogenization of the berries seemed to be the most critical step. When a freshly distilled sample of [^{35}S]sulfite, which was free

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Table 1. Stability of [^{35}S]sulfite during homogenization of grape berries with various media.

Medium	$^{35}SO_3^{2-}$ remaining[z] (%)
H_2O	12
0.04 M Tetrachloromercurate (TCM)	40
0.02 M TCM, 47% ethanol	73
0.02 M TCM, 0.1 M gly pH 10	95

[z]A small (about 0.15 g) segment of a grape berry was homogenized in 0.5 ml of the indicated solutions (concentrations are final concentrations) containing 4 nmol, 0.26 μCi [^{35}S]sulfite. The amount of [^{35}S]sulfite remaining in the homogenates was determined with a radioscanner after separation with high voltage paper electrophoresis.

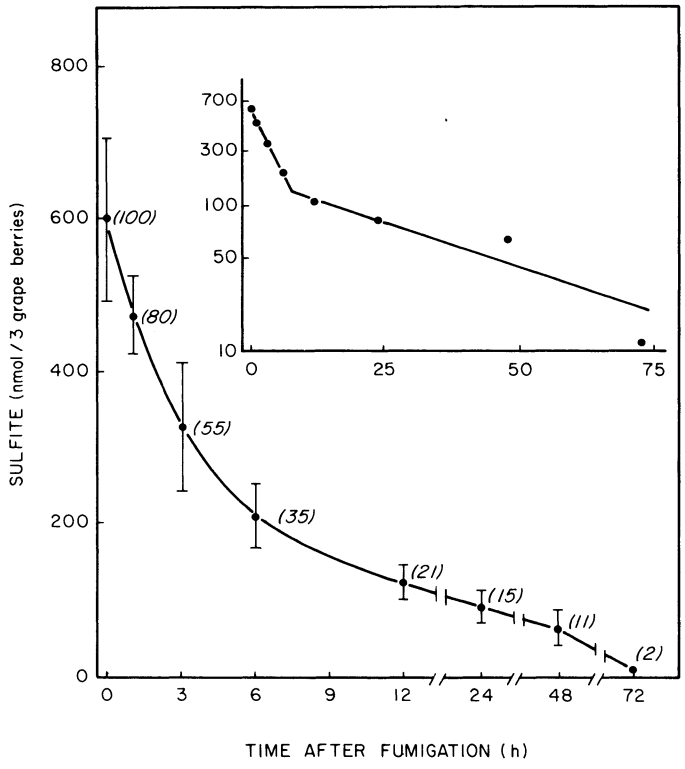

Fig. 1. Time course for loss of sulfite in grape berries fumigated with 0.5% SO_2 for 30 min. Each point is the mean of 3 samples, each sample composed of 3 berries, with indicated SD. The values in parenthesis are the percentages of sulfite remaining at the indicated times. The insert is of the data presented in semi-log form.

from [^{35}S]sulfate, was run on electrophoresis, no [^{35}S]sulfate was found, indicating that no oxidation occurred during electrophoresis. The high pH of the solution seemed to be important since the combination of tetrachloromercurate and glycine at pH 2.4 showed no improvement over tetrachloromercurate alone.

In the experiment using nonradioactive SO_2, sulfite in the grape berries decreased rapidly, and only about 2% of the sulfite remained after 72 hr (Fig. 1). About 1.25 mmol (80 mg) of SO_2 was applied in this experiment, and about 17 μmol (1 mg) of sulfite was found in the berries measured immediately after the fumigation (0 hr). This is an underestimate, since, during the fumigation, some sulfite would have been oxidized or converted into other forms which are not accounted for in this calculation.

When berries were fumigated with $^{35}SO_2$ (Table 2), the pattern for sulfite loss was very similar to the results of the previous experiment. About 3 to 4 hr were required to lose half of the sulfite, and only about one-fourth of the sulfite remained after 12 hr. The radioactive $^{35}SO_2$ experiment provides 2 pieces of information not easily obtainable with nonradioactive SO_2. First, it is apparent that about 16% of the sulfite was lost during the 35 min fumigation. Secondly, sulfate was the only soluble product recovered. Even after 7 days, [^{35}S]sulfate was the only soluble compound found; no [^{35}S]sulfite remained, and no organic forms of ^{35}S were found in the soluble fraction. Based on the specific radioactivity, it was estimated that 31 μmol (2 mg) of SO_2 was applied and about 3 μmol (0.19 mg) was in the berries immediately after the fumigation (0 hr). The amount of SO_2 absorbed by the berry in both radioactive and nonradioactive SO_2 experiment was similar (16 μg and 14 μg per berry, respectively).

Table 2. Time course for the oxidation of [^{35}S]sulfite to [^{35}S]sulfate on grape berries.[z]

Time after fumigation (hr)	$^{35}SO_3^{2-}$ (%)	$^{35}SO_4^{2-}$ (%)
0	84	16
3.5	49	51
12	26	74

[z]Fumigation was for 35 min at 0°C with 0.5% $^{35}SO_2$ with specific activity of 2.56 μCi/μmol.

Discussion

Sulfite in grape berries is very unstable during homogenation (Table 1), and it is very important to have a protective agent to prevent its oxidation. Sulfite can be oxidized through either an ionic or a free radical mechanism. Oxidation via the ionic mechanism can be catalyzed by sulfite oxidase which has been reported in animals (2, 14) and plants (1, 7, 15), and is associated with mitochondria (1, 2, 14, 15). Free radical oxidation of sulfite can be initiated by various means, including enzymes (4, 8), metal ions (18), and photosensitized chlorophyll (3, 11). It is difficult from our data to determine the mechanism of sulfite oxidation during homogenization of grape berries in the absence of tetrachloromercurate-glycine mixture. Addition of ethanol, a free radical scavenger, to the tetrachloromercurate inhibited the oxidation of sulfite when compared to tetrachloromercurate alone. Also, ethanol could inhibit oxidation of sulfite by inhibiting enzymes that either initiate the free-radical oxidation of sulfite or cause the ionic oxidation of sulfite, via sulfite oxidase.

Sulfite reacts with many organic compounds to form addition products. With aldehydes, methyl ketones and cyclic ketones, sulfite forms hydroxy sulfonic acids, and this reaction generally is readily reversible (13). Thus, when sulfite is removed, (e.g., by oxidation), the equilibrium between the sulfite and hydroxysulfonic acid will be shifted toward sulfite formation. In foods and beverages treated with SO_2, the "free" and "bound" SO_2 (sulfite) often are measured with the free SO_2 being liberated from an acidified solution very quickly and the bound SO_2 being liberated over a prolonged time period. Presumably, the bound SO_2 is composed of hydroxy sulfonic acids and other readily reversible complexes. Under our analysis conditions, only [^{35}S]sulfite and [^{35}S]sulfate were observed, and there were no bound forms of sulfite. Thus, there may have been little bound sulfite existing in the berries, or the bound sulfite may have released sulfite in the presence of tetrachloromercurate during the extraction procedures. The latter view is supported by the data from the nonradioactive sulfur dioxide experiment (Fig. 1).

If only one form of sulfite was present, it should disappear following 1st-order kinetics.

When the amount of sulfite remaining in the berries is plotted in log-scale vs. time (Fig. 1 insert), there are 2 distinct linear curves. A likely interpretation is that there are 2 forms of sulfite, free and bound: the free sulfite (70% of the total sulfite) is lost rapidly, with a half-life of about 4 hr, whereas the bound sulfite (30% of the total sulfite) is relatively stable and has a half-life of about 20 hr. Presumably, readily reversible bound sulfite can be regarded as a ''storage form'', which will release sulfite when the concentration of free sulfite becomes low, according to the mass action law.

The commercial application of SO_2 to control the spread of *Botrytis* employs weekly fumigations with 0.5% SO_2. This treatment kills *Botrytis* on the surface but not in internal tissue of the berry (5). Our results show that the level of sulfite falls quickly, with only about 15% remaining after 1 day, yet low amounts of sulfite remain, presumably in the bound form, up to about 5 days. Possibly low levels of sulfite released from the bound form afford some control to the spread of *Botrytis*, and the high level of SO_2 used in fumigations may be needed to provide a minimum level of SO_2 over a 5–7 day period following the fumigation. It is to be noted that the level of atmospheric SO_2 needed to inhibit spore germination of *Cladosporium* and *Botrytis* by 50% is only 5 μl/liter (L. Strand and S. F. Yang, unpublished data).

Which species of the sulfur compounds, SO_2, bisulfite or sulfite, is most important in preventing the spread of *Botrytis* on grapes? Our research on the effect of sulfite upon spore germination of *Cladosporium* and *Botrytis* at various pHs revealed that inhibition of spore germination correlated with the calculated concentration of SO_2, but not with that of bisulfite or sulfite (L. Strand and S. F. Yang, unpublished data). This indicates that the rate limiting step in the fungicide action may be the passage of SO_2/sulfite across the cell membrane of fungi, and that the passage of the nonionic form, SO_2, would be favored over that of the ionic species (6). However, the possibility that the ionic species are the agents causing biochemical injury cannot be excluded.

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