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Molecular Basis of the Biological Function of Molybdenum. The Relationship between Sulfite Oxidase and the Acute Toxicity of Bisulfite and SO_2

(rat/tungsten/molybdenum deficiency/systemic and respiratory toxicity/enzyme induction)

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ABSTRACT **The administration of tungsten to rats maintained on a low molybdenum diet resulted in a dose- and time-dependent loss of sulfite oxidase (EC 1.8.3.1) and xanthine oxidase (EC 1.2.3.2) activities and hepatic molybdenum. These tungsten-treated animals appeared healthy, but were more susceptible to bisulfite toxicity. The median lethal dose for intraperitoneal bisulfite was found to be 181 mg of $NaHSO_3$ per kg for the animals deficient in sulfite oxidase, compared to 473 mg/kg for normal rats.**

The survival time of rats exposed to SO_2 at concentrations of 590 ppm and higher was seen to be inversely related to the level of SO_2. At 590 ppm and 925 ppm, control animals displayed symptoms of severe respiratory toxicity before death. At 2350 ppm of SO_2, death was preceded by seizures and prostration, symptoms observed with the systemic toxicity of injected bisulfite. At 590 ppm, animals deficient in sulfite oxidase were indistinguishable from control animals. However, at 925 ppm and 2350 ppm, the deficient animals displayed symptoms of systemic toxicity and had much shorter survival times. It is concluded that sulfite oxidase is instrumental in counteracting the toxic systemic effects of bisulfite, either injected or derived from respired SO_2. Respiratory death probably results from the toxicity of gaseous SO_2 before absorption as bisulfite and cannot be alleviated by sulfite oxidase. Sulfite oxidase does not appear to be inducible by either bisulfite or SO_2.

Animals exposed to SO_2 (1) or given parenteral bisulfite (2) excrete 80–90% of the sulfur as sulfate in the urine. It has also been shown with rats that 70–95% of the radioactivity of ingested [^{35}S]sulfite is absorbed through the intestines and voided within 24 hr with no detectable sulfite in the urine (3). Sulfite oxidase (EC 1.8.3.1) an enzyme capable of oxidizing sulfite to sulfate, has been purified from animal livers and characterized as a molybdoprotein (4–7). Presumably the normal function of this enzyme is the oxidation of endogenous sulfite arising from the degradation of sulfur amino acids. The only evidence for the essentiality of sulfite oxidase is the report of the complete deficiency of the enzyme in a child who excreted no sulfate in his urine and who suffered from severe neurologic defects (8, 9).

Recently we have been able to produce deficiencies of sulfite oxidase and xanthine oxidase (EC 1.2.3.2) the two major molybdoproteins of rat liver, by maintaining animals on a low molybdenum diet and treating them with tungsten, which has been shown to be a competitive antagonist of molybdenum utilization in animal systems (10–12). Using these rats deficient in sulfite oxidase, we have been able to demonstrate that this enzyme is involved in the oxidative metabolism of SO_2 and bisulfite which results in the detoxification of these compounds.

MATERIALS AND METHODS

Male CD outbred rats weighing about 150 g were obtained from Charles River, Wilmington, Mass. Animals were housed in plastic cages, and food and water were freely available. The temperature of the animal colony was maintained at 22°, and a 12-hr light-dark cycle was used. Except as indicated, animals were maintained on a normal protein diet obtained from Nutritional Biochemicals. The diet contained sucrose in place of starch so that it could be supplied in pelleted form. The molybdenum content of the diet was about 30 μg/kg. Animals were given deionized drinking water rendered palatable by the addition of 10 g of sucrose per liter. For producing sulfite oxidase deficiency, the water was supplemented with 100 ppm of tungsten as sodium tungstate. Water consumption by the rats was unaffected by the presence of tungsten. Animals were maintained on this regimen for 3–5 weeks before toxicity studies were performed. Tissues were processed for enzyme activity assays as described (12). Sulfite oxidase activity in liver and lung was measured by following the reduction of cytochrome *c* (6). Hepatic xanthine oxidase activity was measured spectrophotometrically by following the conversion of xanthine to urate. Succinate-cytochrome *c* reductase activity (13), which is not dependent on molybdenum, was monitored as a control. Molybdenum analyses were performed on ashed liver samples, either by atomic absorption spectroscopy with a Perkin Elmer model 107 atomic absorption spectrometer or by a colorimetric assay (14).

Bisulfite Toxicity Studies. Fresh solutions of $NaHSO_3$ at a concentration of 150 mg/ml were used. Animals were given intraperitoneal injections at dose levels ranging from about 0.1 to 1.0 g/kg and observed for 2 days. All fatalities, however, occurred within 1 hr after injection. Median lethal dose (LD_{50}) was calculated according to the method of Weil (15).

SO_2 Toxicity Studies. Rats were exposed to controlled atmospheres in a portable stainless steel chamber of the type described by Hinners *et al.* (16). Atmospheres of SO_2 were maintained by metering SO_2 directly into the incoming air and were monitored at frequent intervals by an iodometric procedure. The characteristics of the SO_2 metering system

Abbreviation: LD_{50}, median lethal dose.

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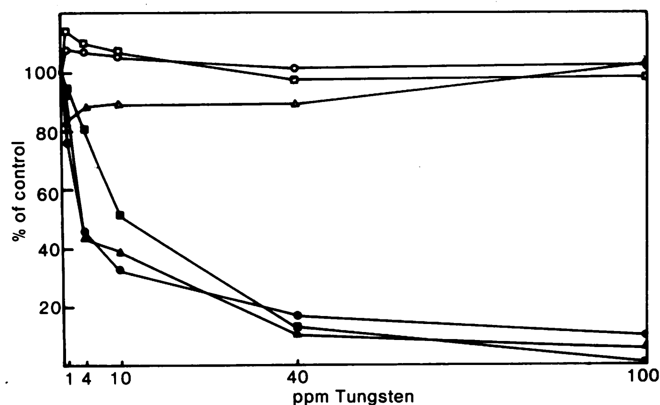

FIG. 1. The effect of tungsten dose on hepatic sulfite oxidase (●), xanthine oxidase (▲), and molybdenum content (■). Also shown are body weight (O), liver weight (□), and hepatic succinate-cytochrome *c* reductase (Δ). Rats were treated as described in the *text*. Each point represents the average of three experimental values.

were such that once the desired concentration was achieved, it varied less than 5% throughout the exposure.

Inducibility of Sulfite Oxidase. 6-Week-old rats maintained on Purina rat chow and tap water were used. SO_2 exposures were performed as described above. For induction studies with phenobarbital, rats were given a dose of 80 mg/kg every day for 3 days and then every other day during the course of SO_2 exposures. For bisulfite administration, animals were weighed daily and given intraperitoneal injections of 150 mg of $NaHSO_3$ per kg. Animals were killed by decapitation the day after the final exposure or injection.

RESULTS

Effect of Tungsten on Hepatic Sulfite Oxidase, Xanthine Oxidase, and Molybdenum Content. Rats were maintained for 3 weeks on 1–100 ppm of tungsten. Fig. 1 shows the effect of such treatment on various parameters. There was a dose-dependent decrease in both sulfite oxidase and xanthine oxidase activities and a concomitant loss of hepatic molybdenum. The animals remained healthy, and as can be seen in Fig. 1, tungsten treatment had no effect on body weight, liver weight, or succinate-cytochrome *c* reductase activity. Using 100 ppm of tungsten, the time-course for the loss of enzyme activity

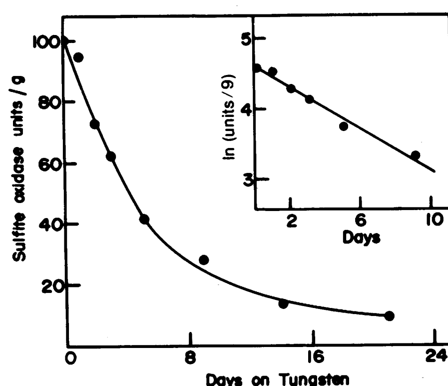

FIG. 2. Time-course of loss of sulfite oxidase activity from livers of rats treated with 100 ppm of tungsten. *Inset* shows the log of activity against exposure time.

was obtained. Fig. 2 shows that the loss of hepatic sulfite oxidase activity followed first-order kinetics. The $t_{1/2}$ calculated for the decay of enzyme activity under these conditions is 4.7 ± 0.4 days. After 5 weeks of tungsten treatment, the residual sulfite oxidase activity was less than 3% of control, in keeping with the fact that about seven half-lives had elapsed.

Sulfite oxidase activity, although highest in the liver, is also present in other tissues. The kidney contains 60–80 units/g, which is about half the activity found in liver. The terminal intestinal ileum contains 15–30 units/g, the heart 10–15 units/g, and the lung 4–6 units/g. Tungsten treatment decreased the activity of sulfite oxidase in all of these tissues to the same extent.

Effect of Sulfite Oxidase Deficiency on the Toxicity of Bisulfite. Animals were maintained on 100 ppm of tungsten for 3 and 5 weeks, and the LD_{50} for intraperitoneal bisulfite was determined by injecting various amounts of a 150-mg/ml solution of $NaHSO_3$. The results are summarized in Table 1. As can be seen, animals deficient in sulfite oxidase are much more sensitive to bisulfite toxicity than controls. There is no overlap in the ranges of LD_{50} within 95% confidence limits between the control group and the two tungsten-treated groups. There is also an indication of a difference in sensitivity between the group treated with tungsten for 3 weeks and the group treated for 5 weeks, reflecting the difference in residual sulfite oxidase activity in the two groups. There was no significant difference in LD_{50} between the control group and rats given Purina rat chow and tap water.

Effect of Sulfite Oxidase Deficiency on the Survival of Animals Exposed to Various Levels of SO_2. Groups of eight control rats and eight rats treated with tungsten for 5 weeks were exposed to various levels of SO_2 for 4 hr and then observed for 2 weeks. The results are summarized in Table 2. Although there was no significant difference in mortality between the two groups, there was some indication of a difference in survival time. It was therefore decided to determine if the survival time on exposure to SO_2 was significantly shortened for tungsten-treated animals. Experiments designed to see if decreased sulfite oxidase activity alters the response to 50% and 5% SO_2 in oxygen were performed under static conditions in a

TABLE 1. *LD_{50} for intraperitoneal bisulfite in rats*

	Mortality			
	Control		Tungsten-treated	
Dose (mg of $NaHSO_3$/kg)	Normal protein diet	Rat chow	3 weeks	5 weeks
89	—	—	—	0/4
133	—	—	0/4	0/4
200	—	—	1/4	1/4
300	0/8	0/4	2/4	4/4
450	4/8	2/4	4/4	—
675	7/8	2/4	—	—
1012	8/8	4/4	—	—
LD_{50}(mg/kg)	475	551	271	181
95% Confidence range of LD_{50}	394–569	396–768	199–369	148–221

Dashes, not tested.

very simple exposure chamber. No difference in survival time was observed between the two groups. At 50% SO_2, all the animals in both groups were dead within 2 min, and at 5% SO_2 all the animals died within 10 min.

Exposures were conducted at lower levels of SO_2, as described in *Methods*, and survival times were recorded. Fig. 3 shows the results obtained on exposure to 2350 ppm of SO_2. The mean survival time for the tungsten-treated group was 63 ± 3 (SE) min compared with 176 ± 9 min for the control group. It thus appears that sulfite oxidase deficiency renders animals much more susceptible to this level of SO_2. Similar results were obtained upon exposure of animals to 925 ppm of SO_2. In this experiment (Fig. 4), an additional group of animals (stock group) was included to determine if there was any effect of the special diet or the sucrose-containing drinking water on SO_2 sensitivity. The stock group was maintained on Purina rat chow and tap water and was indistinguishable from the other two groups in body weight, liver weight, and hepatic protein content. Hepatic sulfite oxidase activity was 141 ± 9 (SE) units/g in this group compared with 120 ± 10 units/g in the control group. The average survival times on exposure to 925 ppm of SO_2 for the tungsten-treated group, the control group, and the stock group were 6.1 ± 0.6, 12.5 ± 0.9, and 11.0 ± 0.7 hr, respectively. It can be seen that animals deficient in sulfite oxidase were much more sensitive to this dose of SO_2 than animals in either the control or the stock group. The difference in survival times between control and stock groups was not significant.

Survival times of control and tungsten-treated animals exposed to 590 ppm of SO_2 were determined (Fig. 5); at this level of SO_2 no marked difference between the two groups was apparent. The experiment was discontinued after 65 hr, with one survivor in each group. Thus, it appears that sulfite oxidase provides a measure of protection against the toxicity of SO_2 over a range of SO_2 concentrations but not at either very high or very low levels.

Attempted Induction of Sulfite Oxidase by Bisulfite and SO_2. It has recently been reported that oral administration of bisulfite for 30 days failed to increase the level of rat liver sulfite oxidase (3). Since the small intestine is known to contain sulfite oxidase, the possibility exists that the lack of hepatic induction could have been due to the metabolism of the bisulfite at the intestinal level. We have examined the ability of subtoxic doses of injected bisulfite and respired SO_2 to induce hepatic and pulmonary sulfite oxidase. Exposure of either control or phenobarbital-treated rats to 50 ppm of SO_2 for 6 hr a day for 10 days or to 100 ppm of SO_2 continuously for 72 hr produced no change in either hepatic or pulmonary

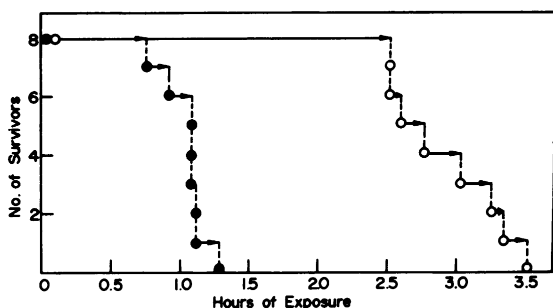

FIG. 3. Survival times of rats exposed to 2350 ppm of SO_2. Groups of eight tungsten-treated animals (●) and eight control animals (○) were used. *Vertical lines* indicate times of death.

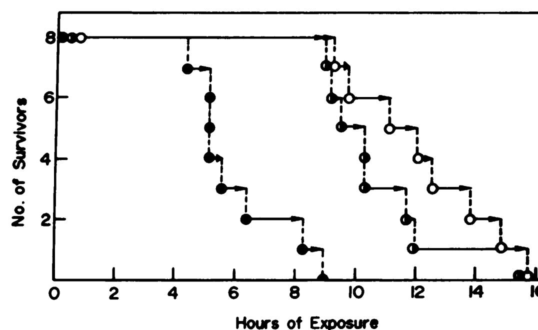

FIG. 4. Survival times of rats exposed to 925 ppm of SO_2. Groups of eight tungsten-treated animals (●), controls on normal protein diet (○), and stock animals on Purina rat chow (◑) were used. *Vertical lines* indicate times of death.

sulfite oxidase activity or the intracellular locus of the enzyme (17). Daily intraperitoneal injections of bisulfite at a dose of 150 mg of $NaHSO_3$ per kg for 6 days similarly failed to induce higher levels of enzyme.

DISCUSSION

Much circumstantial evidence exists for the role of sulfite oxidase in the metabolism and detoxification of bisulfite and SO_2. The observations that inhaled SO_2 (1) and injected bisulfite (2) appear as sulfate in the urine indicate the existence of an oxidative metabolic pathway. The lack of urinary sulfate noted in the instance of sulfite oxidase deficiency (8) gives credence to the role of this enzyme in the oxidative process. Direct demonstration of the involvement of sulfite oxidase in protecting against SO_2 and bisulfite toxicity, however, has become possible with the discovery of molybdenum as an essential prosthetic group. With the use of tungsten, a competitive antagonist of molybdenum, we have been able to produce experimental sulfite oxidase deficiency in rats. In the absence of a stress from administration of large amounts of SO_2 or bisulfite, tungsten-treated animals remain healthy; however, the LD_{50} for intraperitoneal bisulfite for such animals is markedly lower than for controls. Death from injected bisulfite occurs within a few minutes in susceptible animals, and survivors do not appear to be permanently harmed by the

TABLE 2. *Effect of various concentrations of inhaled SO_2 on the mortality of rats*

Concentration of SO_2 (ppm)	2-Week mortality	
	Control	Tungsten-treated
224	0/8	0/8
593	0/8	0/8
965	3/8	2/8
1168	5/8*	8/8*
1319	8/8†	8/8†

* All tungsten-treated animals died in the chamber during the 4-hr exposure (generally in the last half hour). The five control animals died from 1 to 48 hr after exposure. Those surviving 48 hr survived the entire 2-week period after exposure.

† Seven of the eight tungsten-treated animals died during the exposure. The last one died about 4 hr after the exposure. All of the control animals died from 1 to 24 hr after the exposure was completed.

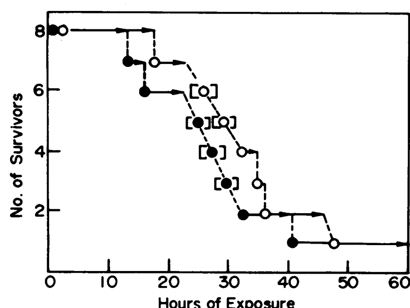

FIG. 5. Survival times of rats exposed to 590 ppm of SO_2. Experimental conditions were as in Fig. 3. *Sloping segments of dashed lines* represent periods when animals were not observed and exact times of death were not recorded.

treatment. It appears that fatality results from the accumulation of bisulfite at an unidentified target tissue. In all cases, death was preceded by prostration and seizures, indicating that the immediate effect of bisulfite may be directed at the central nervous system, as previously suggested (18, 19). Survival depends on the ability of the individual animal to counteract the build-up of bisulfite by rapidly metabolizing it. The properties of the detoxification system must be such that it can handle heavy loads of bisulfite in a short period of time. The fact that in rats deficient in sulfite oxidase the LD_{50} concentration of bisulfite is attained at lower intraperitoneal doses demonstrates that the counteracting, protective reaction is the oxidation of bisulfite to sulfate catalyzed by sulfite oxidase. The LD_{50} for intraperitoneal bisulfite for the control group was no different from that for the stock group (Table I) and is similar to the value reported previously (19). Thus the decreased LD_{50} seen in tungsten-treated animals is independent of the special diet and drinking water required to produce sulfite oxidase deficiency.

The relationship of sulfite oxidase deficiency to inhaled SO_2 toxicity is more complicated. Although there was no statistically significant difference in mortality between control animals and animals deficient in sulfite oxidase exposed to SO_2, at certain levels of inhaled SO_2 there was an observable difference in the survival times. Table 3 summarizes the data on survival times of both groups of animals at various levels of SO_2. At 5% and 50% SO_2, death occurred rapidly, after seizures and prostration, with no differences between the two groups. At 2350 ppm, animals in both groups exhibited these same symptoms before death. At this level, however, the animals deficient in sulfite oxidase survived for a much shorter time than the controls. The difference in survival times was also observed at 925 ppm of SO_2. In addition, a difference in the mode of death was discernible. The animals deficient in sulfite oxidase exhibited symptoms of seizures and prostration similar to those described earlier; animals in the control group developed respiratory difficulties followed by exhaustion and death. The symptoms of seizure and prostration are similar to those exhibited after bisulfite treatment and represent a systemic toxicity, while the respiratory symptoms seen in control animals at 925 ppm of SO_2 are probably due to a direct effect of the gas on the respiratory system. At 590 ppm of SO_2, this latter mechanism of death (respiratory) was observed in both groups with no difference in survival time. It therefore appears that sulfite oxidase alleviates the acute systemic toxicity due to bisulfite but has a minor role, if any, in protection against the subacute and chronic respiratory effects of SO_2.

The apparent inability of sulfite oxidase to protect against levels of inhaled SO_2 in the range of 500 ppm may be because the respiratory type of death observed at these levels is caused by a direct effect of gaseous SO_2 on the respiratory tract before it is absorbed and made available to the enzyme. At higher levels of SO_2, it appears that the bisulfite concentration at the critical site reaches high enough levels that systemic death precedes the otherwise observable respiratory death. Sulfite oxidase activity in the lung and liver is therefore capable of providing a measure of protection against inhaled SO_2 as shown by the much shorter survival time of animals deficient in sulfite oxidase exposed to 2350 ppm of SO_2. At the highest doses of SO_2 used, the toxic bisulfite levels are attained so rapidly as to override the protective effect of the enzyme.

It has been shown that exposure to 1 M bisulfite can alter nucleic-acid bases *in vitro* and produce mutations in phage (20–23). It has also been observed that treatment with this high concentration of bisulfite can sharply diminish the ability of poly(uridylic acid) to form a double helical complex with poly(adenylic acid) and to code for phenylalanine incorporation into cell-free protein-synthesizing system of *Escherichia coli* (24). On the basis of these *in vitro* studies, it has been suggested that SO_2 may pose a long-range genetic hazard to people living in urban areas. It can be calculated that the sulfite oxidase activity of rat lung is capable of detoxifying at least 600 μmol of bisulfite derived from inspired SO_2 per day. This is equivalent to almost 20 ppm of SO_2 in the atmosphere and is based on complete extraction of the SO_2, by rat lung. When total tissue sulfite oxidase activity is considered, a 200-g rat should be capable of oxidizing 150,000 μmol of bisulfite per day, equivalent to continous exposure to 5000 ppm of SO_2. The observation that, at 925 ppm of SO_2, control animals were protected against the systemic toxicity of bisulfite is in good agreement with these calculations. Systemic death seen on exposure to 2350 ppm of SO_2 suggests that at these high levels of SO_2, the limiting factor is likely to be the rate at which the bisulfite is made available to the intracellular sulfite oxidase. However, it is reasonable to conclude that at levels of SO_2 below 925 ppm, there would be no significant accumulation of bisulfite in the animal. The recent report of Gibson and Strong (3) is also relevant in this regard. They have shown that oral administration of sulfite at levels greatly ex-

TABLE 3. *Effect of various concentrations of inhaled SO_2 on survival time of rats*

Concentration of SO_2 (ppm)	Survival time (min ±SE)	
	Control	Tungsten-treated
590	1866 ± 210*	1542 ± 210*
925	750 ± 54	366 ± 36
2350	176 ± 9	63 ± 3
50,000 (5%)	<10	<10
500,000 (50%)	<2	<2

* Mean survival time calculated from individual survival times of seven of the eight animals. Deaths occurring during periods when animals were not observed were assumed to have taken place at the midpoint of the unobserved time.

ceeding the estimated daily oral intake of humans in this country does not saturate the capacity of the rat for sulfite metabolism. Animals are apparently under no stress even when administered these levels of sulfite for several weeks (3). The sulfite oxidase contents of human lung and liver are comparable to those of the corresponding tissues of the rat (Kessler, D. L., & Rajagopalan, K. V., unpublished observations). Thus, at atmospheric concentrations rarely exceeding 5 ppm, present SO_2 levels in urban areas are insufficient to pose any genetic hazard. Any noxious effects of SO_2 at this level may be assumed to result from the immediate surface exposure of epithelial tissues to the gas.

It is well known that the administration of certain drugs and toxic agents causes the induction of enzymes responsible for their metabolism (25, 26). These enzyme systems are microsomal and have low turnover numbers and short half-lives. Sulfite oxidase is a mitochondrial enzyme (17) with a high turnover number (4) and with what appears to be a long half-life. The activity of this enzyme in rat liver and lung could not be increased by either intraperitoneal bisulfite or inhaled SO_2, even in phenobarbital-treated animals. Since sulfite oxidase is the final enzyme involved in oxidative sulfur metabolism, studies involving high sulfur diets are now being undertaken to investigate the capacity of the tissue sulfite oxidase to metabolize endogenously derived bisulfite and to determine whether tissue levels of sulfite oxidase are altered. It would also be of interest to monitor the amount of serum bisulfite and of urinary sulfite and sulfate in tungsten-treated rats before and after exposure to SO_2, bisulfite, and high sulfur diets. Such studies might bring to light the possibility of an endogenous stress, evident only in the animals deficient in sulfite oxidase, which would relate to the reported genetic deficiency of the enzyme (8, 9).

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