Donahaye, E.J., Navarro, S. and Leesch J.G. [Eds.] (2001) Proc. Int. Conf. Controlled Atmosphere and Fumigation in Stored Products, Fresno, CA. 29 Oct. - 3 Nov. 2000, Executive Printing Services, Clovis, CA, U.S.A. pp. 79-89

METABOLISM OF CARBONYL SULFIDE TO HYDROGEN SULFIDE IN INSECTS IS CATALYSED BY CARBONIC ANHYDRASE

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ABSTRACT

Carbonyl sulfide (COS) is a new fumigant under development as a methyl bromide replacement for fumigation of durable commodities. COS has been shown to be relatively fast acting and toxic to a broad range of stored-product pests but little is known of the mechanism of toxicity of COS to insects. In rats, COS is metabolised to hydrogen sulfide by the enzyme carbonic anhydrase, a widely distributed family of enzymes that usually catalyse the reversible hydration of carbon dioxide. The present study investigated whether hydrogen sulfide was the toxic agent of COS in insects. Firstly, adult stored-product insects *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (F.) were fumigated with hydrogen sulfide for 6 h at a range of concentrations from 1-50 mg L^{-1}. The percentage mortality of insects was ≥84% at concentrations of 5 mg L^{-1} and above suggesting hydrogen sulfide is potently toxic to insects. To determine the role of metabolism in COS toxicity, 17-21 day old larvae of *T. castaneum* were raised on culture medium containing carbonic anhydrase inhibitors, acetazolamide or methazolamide, at concentrations up to 20 mg g^{-1} media. Larvae raised on inhibitors and untreated larvae were then fumigated with 60 mg L^{-1} COS for 5 h, a concentration - time product that had been shown to produce approximately 90% mortality in untreated larvae. The mortality of larvae raised on acetazolamide-containing medium (20 mg g^{-1}) was much lower than untreated larvae (40% versus 95%) after fumigation. Similarly, mortality was reduced in insects administered methazolamide (20 mg g^{-1}) resulting in 53% mortality. Administration of carbonic anhydrase inhibitors to insects protected them from the acute toxicity of COS. This work demonstrates that the acute toxicity of COS to insects is dependent on carbonic anhydrases metabolism to hydrogen sulfide, the toxic agent of COS.

INTRODUCTION

Carbonyl sulfide (COS) is a new fumigant under development as a methyl bromide replacement for fumigation of durable commodities (Desmarchelier, 1994). COS is relatively fast acting toward a broad range of stored-product and timber pests (Desmarchelier, 1994; Plarre and Reichmuth, 1997; Zettler *et al.* 1997). These authors also report that the egg stage is the most tolerant stage of the common stored product insects to COS. The lethal concentration - time profile for COS fumigation over a range of time, concentration and temperature

conditions has been characterised for both adult and egg stages of the rice weevil, *Sitophilus oryzae* (L.) (Weller and Morton, 2001).

Although the effective concentration range for fumigation of stored-product insects has been established, little is known of the mechanism of toxicity of COS in insects. In rats, COS administered by injection of the gas into the peritoneum was lethal at doses of 20 mg kg^{-1} and above; COS was converted to hydrogen sulfide which was detected in the blood (Chengelis and Neal, 1980). However, when rats were pretreated with the enzyme inhibitor acetazolamide (at 200 mg kg^{-1}) prior to administration of COS, they were protected from the lethal effects of COS. Acetazolamide is a specific inhibitor of carbonic anhydrases - an enzyme group that catalyses the reversible hydration of carbon dioxide (CO_2) (Maren and Sanyal, 1983). Therefore it appeared that the acute toxicity of COS in the rat was a result of its metabolism to hydrogen sulfide and by inhibiting its metabolism with acetazolamide, COS toxicity was prevented.

Carbonic anhydrases are ubiquitous enzymes. They are found in vertebrates, plants, microorganisms and insects (Tashian, 1989). The usual substrate for carbonic anhydrases is CO_2, which is metabolised with the addition of water to bicarbonate and hydrogen ion; an essential reaction for maintaining pH balance, transport of CO_2 and production of ions for secretory fluids in animals (Tashian, 1989). The presence of the same family of enzymes in rats and insects implies that a similar metabolic pathway for COS to that in rats may occur in insects. Support for this notion includes the finding that whole body homogenates of adult *S. oryzae* and larval *Ephestia kuehniella* (Zeller) catalysed the formation of hydrogen sulfide when exposed to COS (Haritos, unpublished data). However, it is not clear whether the toxicity of COS to insects is due to its metabolism to hydrogen sulfide. This paper describes the role of carbonic anhydrases in the toxicity of COS and the toxicity of the proposed metabolite, hydrogen sulfide to stored-product insects.

EXPERIMENTAL

Chemicals

COS (97.5%) and hydrogen sulfide (99%) were supplied by BOC Gases Australia Ltd. (Chatswood, Australia). The purity of COS with respect to hydrogen sulfide was checked using a GowMac gas density balance combined with a Tracor MT150 gas chromatograph and found to be <1%. Acetazolamide and methazolamide were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Insect culturing

Tribolium castaneum (Herbst) were cultured on wholemeal flour containing brewers yeast (10% w/w) and maintained in controlled temperature rooms at 30°C and 60% r.h. Early 3rd-4th instar larvae (17-21 d) or adult insects were used in experiments. Adult *S. oryzae* were obtained from cultures reared on soft wheat, and *Rhyzopertha dominica* (F.) from cultures raised on soft wheat containing flour at 25°C and 65% r.h.

Hydrogen sulfide toxicity to insects

Fifty adult *T. castaneum*, *R. dominica* and *S. oryzae* were placed in separate glass jars without media in a 2.5 L glass desiccator fitted with a gas sampling port and septum. Hydrogen sulfide was added by gas-tight syringe, after removal of the corresponding volume of air, to achieve a final concentration of 0, 1, 5, 25 or 50 mg L^{-1}. Nominal concentrations of hydrogen sulfide were used in the experiment. There was a single desiccator for each hydrogen sulfide concentration. After a 6 h exposure, the desiccators were aired in a fume cabinet. Culturing medium was added to each jar, then the jars were covered and returned to culture rooms. Mortality was assessed 7 d after fumigation.

Determination of COS toxicity to larval *T. castaneum*

The acute toxicity of COS to *T. castaneum* larvae (approximately 3rd-4th instar) was tested over the concentration range of 50 to 80 mg L^{-1} for a duration of 3 to 5 h. Larvae (50) and culture medium were added to glass jars and placed in glass desiccators (2.5 L). The required volume of COS was added by gas-tight syringe through a septum port in the lid. The concentration of COS in each desiccator was measured by gas chromatography at the beginning and end of the exposure period. Each concentration/time interval for COS was tested in duplicate. After fumigation, the culture medium and insects were aired for at least one hour in a fume cabinet and then returned to culture rooms at 30 C, 60% r.h. Insect mortality was assessed 1, 7 and 21 d after fumigation.

Effect of dietary enzyme inhibitors on COS toxicity

In the initial screening experiment, 100 *T. castaneum* larvae per treatment were placed on culture medium (11 g) containing acetazolamide at 0, 1, 2.5 or 5 mg g^{-1} medium. Acetazolamide had been mixed by hand as a dry powder with flour and yeast. The insects were raised on the medium for 4 d at 30 C and 60% r.h.. Each acetazolamide concentration was tested in duplicate. All cultures were fumigated with COS at 80 mg L^{-1} for 4 h in glass desiccators fitted with gas sampling ports, and each desiccator held control and inhibitor-fed insects. Gas concentrations were measured by gas chromatography at the start and end of the experiment. One treatment group of larvae (2.5 mg acetazolamide g^{-1} medium) was placed on fresh media without inhibitors before fumigation to determine whether the inhibitors themselves had any direct effect on mortality during fumigation. After fumigation the jars were aired, insects placed on fresh media and returned to culture rooms. Mortality was assessed 7 d and 21 d following fumigation.

In the second experiment, 100 *T. castaneum* larvae per treatment were placed in glass jars containing culture medium (11 g) and acetazolamide (0, 0.5, 1, 2 and 4 mg g^{-1} medium) for 3 d at 30 C, 60% r.h., in duplicate. The untreated and inhibitor-fed insects were exposed to 60 mg L^{-1} COS for 5 h. Gas concentrations were measured by gas chromatography at the start and end of the experiment. After fumigation the jars were aired and insects placed on fresh media and returned to controlled temperature and humidity rooms. Mortality was assessed 5 d following fumigation.

In the final acute exposure, 100 *T. castaneum* larvae were raised on medium containing 0, 10 or 20 mg g^{-1} of acetazolamide or methazolamide for 36 h mixed

in half the original amount of medium (5.5 g medium). The cultures were then fumigated with 60 mg L^{-1} COS for 5 h. The fumigated containers were aired and the larvae were placed on fresh medium (11 g) and returned to the culture room (30 C, 60% r.h.) for 5 d after which mortality was assessed.

In a separate experiment designed to investigate the effect of carbonic anhydrase inhibitors during a longer fumigation period (24 h), 100 *T. castaneum* larvae per treatment were cultured on media containing acetazolamide (0 or 2 mg g^{-1} medium) for 2 or 3 d. This was followed by fumigation with 15, 20 or 25 mg L^{-1} COS in replicate experiments. Gas concentrations were monitored at the start and end of the experiment. The mortality of larvae was assessed 4 d after airing and removal of insects to fresh media.

Analysis of COS by gas chromatography

The concentration of COS in the vessels was measured by gas chromatography using a Tracor MT-220 instrument fitted with a flame photometric detector. The samples were injected onto a 6 ft glass column packed with HayeSepQ 80/100 mesh operated at an oven temperature of 100°C, with the injector and detector temperature at 150°C. The concentration of COS in each vessel was calculated from a calibration curve prepared over the range 40-80 mg L^{-1} (typically achieving an r^2 = 0.989) or between 1-30 mg L^{-1} for the lower exposure groups (r^2 = 0.992). According to this analysis, the loss of COS from the fumigation chambers during the exposure periods was negligible.

RESULTS

Hydrogen sulfide toxicity

Hydrogen sulfide was found to be acutely toxic and fast acting towards adult stored-product insects as most were killed at concentrations between 5 and 25 mg L^{-1} for 6 h. The percentage mortalities of the insects exposed to hydrogen sulfide over the concentration range of 0-50 mg L^{-1} are given in Table 1. *S. oryzae* was the most tolerant insect among the three species tested. There were no signs of agitation from the insects treated with hydrogen sulfide and they appeared sedated. By observation of the insects through the glass chamber, the 50 mg L^{-1} concentration appeared to kill the insects very quickly, within 2 min of addition of gas. At 25 mg L^{-1} hydrogen sulfide, the insects had ceased moving within 5 min and at 5 mg L^{-1}, appeared to be dead within 15 min.

TABLE 1
Adult insect mortality (percentage) following exposure to various concentrations of hydrogen sulfide for 6 h. Mortality assessed 7 d after fumigation

Hydrogen sulfide concentration (mg L^{-1})	Mortality (%)		
	R. dominica	*S. oryzae*	*T. castaneum*
0	2	0	0
1	98	52	22
5	100	84	100

25	100	98	100
50	100	100	100

Determination of COS toxicity to larval *T. castaneum*
The mortality of *T. castaneum* larvae exposed to COS was determined at a range of concentrations and short exposure times to find a concentration and time (Ct) product of COS that would result in approximately 90% mortality. These findings were used in subsequent experiments. In Fig. 1, the percentage mortality of larvae exposed to COS at a range of Ct products from 162 to 365 mg h L^{-1} are given by a sigmoidal-shaped curve. The Ct product that gave 86% mortality was 315 mg h L^{-1}. Fumigation of 60 mg L^{-1} COS for 5 h (300 mg·h L^{-1}) was used in successive experiments.

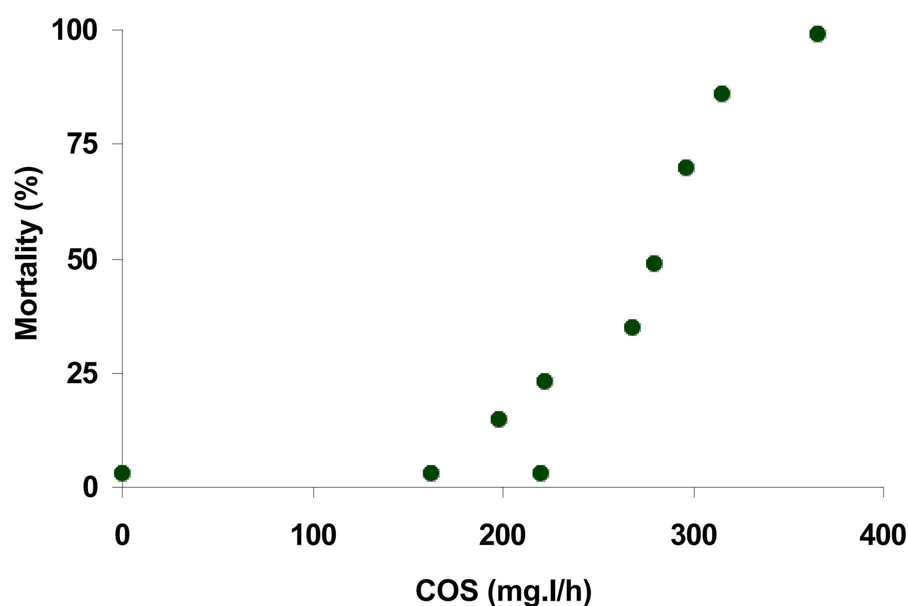


Fig. 1. The relationship between mortality of *T. castaneum* larvae and the concentration-time product of COS; concentrations between 50 and 80 mg L^{-1} and exposure periods of 3 to 5 h.

Effect of dietary enzyme inhibitors on COS toxicity
T. castaneum larvae were raised on a diet containing acetazolamide at concentration levels up to 5 mg g^{-1} for 4 d with no mortality or obvious detrimental effect on the insects. In the initial COS fumigation, untreated larvae exposed to COS at 80 mg L^{-1} for 4 h resulted in 99% mortality. Larvae raised on

a diet containing acetazolamide at 1 to 5 mg g^{-1} media had reduced mortality (73-79%) compared with untreated larvae. In one treatment group, larvae were raised on acetazolamide (2.5 mg g^{-1}) for 4 d then placed on fresh media for the fumigation. The mortality following COS fumigation in this sample was 77%, close to the mortality obtained for other acetazolamide treated groups.

After exposure to 60 mg L^{-1} COS for 5 h the percentage mortality of untreated larvae was 95% but the mortality of the acetazolamide-treated larvae was reduced by the presence of inhibitor in the diet (Fig. 2). The reduction in mortality was proportional to the amount of inhibitor at concentrations of 1 mg g^{-1} and below but appeared to level off at the higher concentrations. Mortality following fumigation with COS was reduced by approximately one third in larvae fed with acetazolamide-containing diet (> or equal to 1 mg g^{-1}) compared with larvae raised on a normal diet. This result was reproduced in replicated experiments (data not shown). A group of *T. castaneum* larvae that were raised on acetazolamide in the diet (2.5 mg g^{-1}, 3 d) but not fumigated with COS, showed no signs of toxicity and developed normally after returning to culture medium.

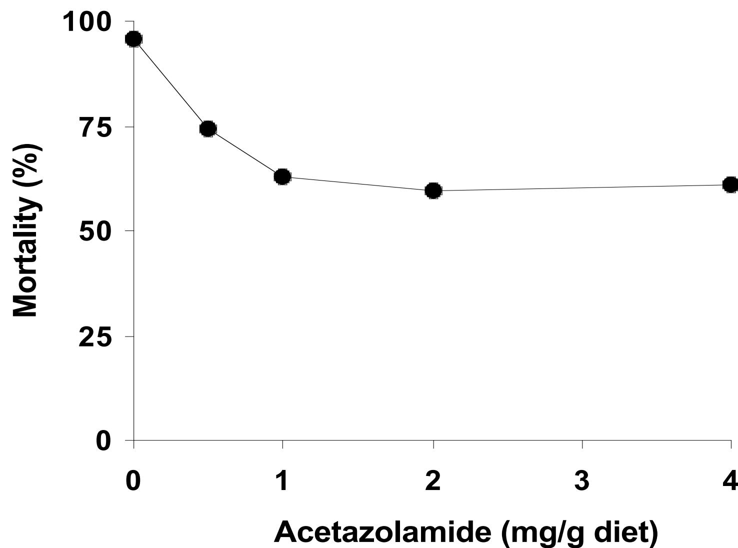
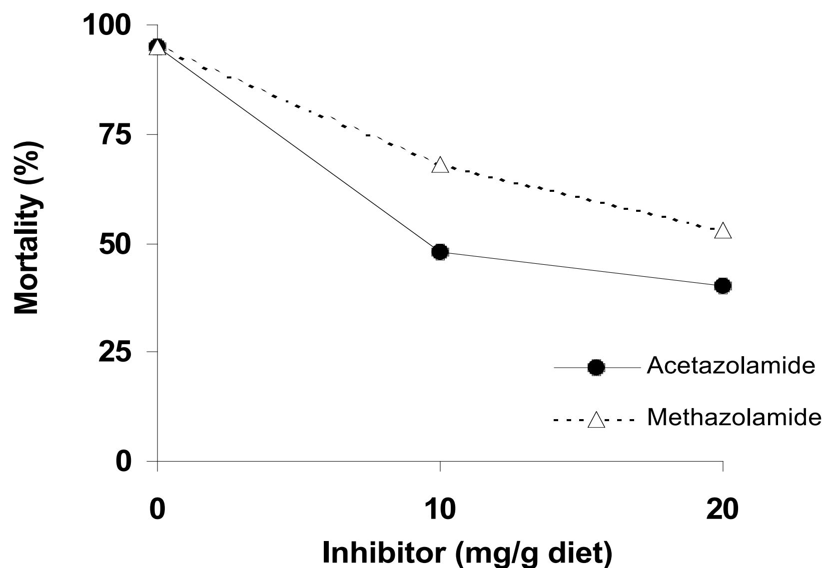


Fig. 2. Effect of dietary administration of acetazolamide (0.5-4 mg g^{-1}) on the percentage mortality of *T. castaneum* larvae exposed to COS (60 mg L^{-1}, 5 h) compared with a control diet.

In a further experiment, *T. castaneum* larvae were raised on a diet containing higher concentrations of carbonic anhydrase inhibitors (10 or 20 mg g^{-1}) and the mortality after exposure to COS was compared with larvae raised on a control diet. Larvae raised on either acetazolamide or methazolamide were protected from the lethal effects of COS fumigation as shown in Fig. 3. Fifty-five percent

of the *T. castaneum* larvae raised on the highest dietary level of acetazolamide were protected from a lethal COS exposure. In a similar result, up to 42% of



methazolamide (20 mg g^{-1}) treated larvae were protected from COS toxicity.

Fig. 3. Protection of *T. castaneum* larvae from the acute toxicity of COS (60 mg L^{-1}, 5 h) by administration of carbonic anhydrase inhibitors, acetazolamide and methazolamide in the diet compared with larvae raised on a control diet.

The effect of dietary acetazolamide on the toxicity of COS during a 24 h fumigation was investigated in *T. castaneum* larvae. The larvae were raised on control medium or medium containing acetazolamide (2 mg g^{-1}) for 2 or 3 d prior to COS fumigation at 15 or 20 mg L^{-1}. The mortality of larvae raised on untreated media and then exposed to COS (15 mg L^{-1}) was 87% but for acetazolamide-treated larvae, mortality was reduced by 17-23% (Table 2). Larvae raised on acetazolamide-containing medium for 3 d experienced slightly higher mortality when fumigated with COS in comparison with those raised on acetazolamide medium for 2 d.

In all experiments where *T. castaneum* larvae were raised on a diet containing carbonic anhydrase inhibitors and then fumigated with COS, the surviving larvae were placed on culture medium and appeared to recover fully and develop normally into adult insects.

TABLE 2
Mean mortality of *T. castaneum* larvae fumigated with COS for 24 h and the effect of dietary exposure to acetazolamide

Dietary acetazolamide (mg g^{-1})	Number of days on medium	Mortality (%) COS 15 mg L^{-1}	Mortality (%) COS 20 mg L^{-1}
0	2	87	99
2	2	63	100
0	3	87	100
2	3	69	99

DISCUSSION

COS is highly toxic to all stages of stored-product insects with the exception of young eggs which appear to be the most tolerant (Desmarchelier, 1994). As a fumigant COS can be effectively used in short-term fumigation using high concentrations or lower concentrations for longer exposure periods (Weller, 1999). The current work examines the basis for the toxicity of COS to insects. Homogenates of stored-product insects have been shown to metabolise COS to hydrogen sulfide *in vitro* but it was not known whether hydrogen sulfide was the toxic agent of COS and to what degree metabolism was involved in COS toxicity. This question was approached by first confirming that hydrogen sulfide was toxic to stored-product insects (Table 1). Hydrogen sulfide is highly toxic to animals as it has a high affinity for metallic ion-containing proteins, particularly cytochrome *c* oxidase, a key component of cellular respiration. It is thought that the inhibition of this enzyme is the main acute toxic action of hydrogen sulfide (Beauchamp Jr *et al*. 1984). It is highly likely that hydrogen sulfide is acting by a similar toxic mechanism in insects.

The next step was to determine the role of metabolism in COS toxicity to insects, particularly the role of carbonic anhydrase which was known from the work of Chenglis and Neal (1980) to catalyse the conversion of COS to hydrogen sulfide in rats. This question was approached by administering inhibitors of carbonic anhydrase to insects, followed by fumigation with COS, and monitoring the effect on mortality or development. Acetazolamide, a crystalline sulfonamide derivative, is one of the most widely used inhibitors of carbonic anhydrases but it is only moderately diffusible through tissues and has low water solubility (Maren and Sanyal, 1983). A structurally related compound, methazolamide, is considered a superior inhibitor for experimental work, as it is more diffusible through tissues, it inhibits two major forms of carbonic anhydrase equally and is slowly excreted from animals compared with acetazolamide (Maren and Sanyal, 1983; Maren, 1991). In homogenised tissues of tobacco hornworm acetazolamide is a potent inhibitor of carbonic anhydrase activity at

concentrations in the nanomolar range (Jungreis *et al.* 1981). *Drosophila melanogaster* and *D. hydei* administered acetazolamide by injection or mixed with the diet showed a significant decrease in potassium, magnesium and chloride in the cytoplasm of Malpighian tubule cells, a function that is supported by carbonic anhydrases (Wessing *et al.* 1997).

There are several methods of introducing inhibitors of carbonic anhydrase into insects such as through incorporation with food or water or by injection of a solution into the body or by inhalation of a gas or aerosol. One benefit of the dietary route of administration is that the cuticle remains intact unlike administration by injection where it is punctured. This is of particular relevance for fumigation where disruption of the insect cuticle may give rise to higher gas penetration and toxicity. In the present study, larval *T. castaneum* were selected for the dietary administration of enzyme inhibitors as the larvae are actively feeding and the inhibitor was easily mixed into the culture medium. No obvious adverse effects were observed in the larvae raised on carbonic anhydrase inhibitors in the diet although prolonged exposure to the inhibitors may detrimental to the insects.

T. castaneum larvae that were given carbonic anhydrase inhibitors in the diet were protected against the acute lethality of COS; the percentage mortality of untreated larvae fumigated with COS was more then double that of larvae given dietary acetazolamide (20 mg g^{-1}) (Fig. 3). This result suggests that the main mechanism of COS toxicity is through the toxic action of the metabolite hydrogen sulfide. Complete protection of insects from toxicity of COS by dietary administration of inhibitors would be difficult to achieve because individual insects probably consume different amounts of food containing inhibitor and the amount required to inhibit the carbonic anhydrases of insects is not known. Dietary administration of methazolamide was not as effective as acetazolamide in protecting larvae from the toxic effects of COS (Fig. 3). The reason for this result is not clear. Methazolamide would be expected to diffuse more easily in the insect and cause more potent, longer lasting inhibition of carbonic anhydrases based on its action in mammals (Maren and Sanyal, 1983).

In longer exposures to COS, larvae fed on a diet containing acetazolamide were moderately protected from the toxicity (Table 2). The level of protection observed was lower than for insects fed a similar amount of inhibitor in the diet but fumigated with 60 mg L^{-1} COS for 5 h (Fig. 2). The difference could be due to the larvae becoming moribund or ill from COS exposure and no longer consuming the food containing the inhibitor, and as acetazolamide is rapidly eliminated from the body of animals (Maren 1991), protection was not continued throughout the exposure period. Alternatively, carbonic anhydrase metabolism of COS to hydrogen sulfide may not be the only mechanism of toxicity to insects in longer-term exposures.

The knowledge of the mechanism of COS toxicity can be used to explain aspects or make some predictions of insect response to the fumigant. Insect species or life stages that are tolerant to COS toxicity, such as the egg stage of *S.!oryzae*, may have a reduced capacity to metabolise COS to hydrogen sulfide. The probability of resistance forming in insects to hydrogen sulfide (from COS) is expected to be low because of the known mechanism of toxicity of hydrogen sulfide. Its action is to potently inhibit metabolic respiration and this mechanism is likely to require a major metabolic shift to result in resistance. Deletion of carbonic anhydrases in mutant insects, as a possible mechanism of resistance,

would most likely be non-viable as these enzymes perform essential endogenous functions in most organisms. The target site of COS toxicity in insects is different to that of protectants and phosphine. In the case of phosphine, the formation of reactive oxygen radicals as a result of its interaction with the electron transport chain is the likely mechanism of toxicity (Chaudhry, 1997; Hsu *et al.* 1998). The mechanism of phosphine resistance in insects is thought to be via active exclusion from the respiratory system combined with a detoxication process (Chaudhry, 1997). These resistance mechanisms appear to be exclusive to phosphine in insects as Chaudhry (1997) found methyl phosphine to be more toxic to resistant than to susceptible insects. Also, from what is known of the resistance mechanisms of protectants, for example increased esterase activity or altered acetylcholinesterase in organophosphate resistance (Guedes *et al.* 1997; Hemingway, 2000) or altered sodium channel structure in pyrethroid resistance (Williamson *et al.* 1996), it is unlikely that cross-resistance would occur between COS and protectants.

In summary, when fed enzyme inhibitors in their diet *T. castaneum* larvae were protected from the acute lethal effects of COS *in vivo* and this finding supports the view that the metabolism of COS to hydrogen sulfide forms the ultimate toxic agent of COS, hydrogen sulfide. Future efforts will be focussed on improving the understanding of COS tolerance in young insect eggs with an understanding of the mechanism of COS toxicity to insects.

ACKNOWLEDGEMENTS

The technical assistance of Greg Dojchinov and Gaye Weller from the Stored Grain Research Laboratory is gratefully acknowledged, as is the financial assistance of the Grains Research and Development Corporation and the Partners to the SGRL Agreement. The author is grateful to Peter Annis and Gaye Weller for their comments on the manuscript.

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