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DISTRIBUTION OF RADIO-LABELLED PHOSPHORUS IN SUSCEPTIBLE AND RESISTANT *TRIBOLIUM CASTANEUM* AFTER FUMIGATION

C. WATERFORD

Stored Grain Research Laboratory, CSIRO Entomology, Canberra, ACT. 2601 Australia [e-mail: colin.Waterford@ento.csiro.au]

ABSTRACT

The total amount of phosphine taken up by susceptible insects for equi-toxic exposure, is generally much higher in susceptible than resistant insects. On a strict uptake basis this would appear to make susceptible insects more tolerant of phosphine. However Ct product data show higher Ct is required to kill resistant insects. One hypothesis for the uptake being so low in resistant strains is active exclusion. This study looked at the distribution of uptake, after fumigation, to gain some insight into the toxicology. Phosphine-resistant and -susceptible *Tribolium castaneum* (Herbst) were exposed to 0.1 mg L^{-1} phosphine labeled with isotope ^{32}P at their respective LD_{50} and LD_{99} response levels. Uptake and distribution of ^{32}P, after separation of treated samples into sugars, amino acids, organic acids, protein and an insoluble fraction, were assessed using a scintillation counter. Uptake or final distribution in the susceptible strain at the LD_{50} and LD_{99} was greater in every fraction than the equivalent resistant fraction except for the LD_{99} level of the insoluble fraction. This was so despite the increased exposure for the resistant over the susceptible strain at the LD_{99}, 36.5 h *vs*. 4.25 h. This result is consistent with the mechanism of active exclusion. It also suggests that the lethal part of the uptake may be associated with an insoluble residue of phosphorus.

INTRODUCTION

Phosphine (PH_3) is one of the few remaining fumigants used widely on stored products to control insect pests. Though used for many years, the site of toxic action remains uncertain and the mechanisms for resistance are not fully understood. Banks (1975), Price (1981), Nakakita and Kuroda (1986) and Reichmuth (1994) reported that gross uptake in susceptible strains is much higher than in resistant strains for a number of stored-product pests. Moreover, this uptake is initially at a much higher rate in the susceptible strains.

Price (1984) proposed active exclusion as a resistance mechanism. However, PH_3 has a range of possible reactive pathways within the insect (Banks 1975; Lam *et al.*, 1991) and given that the uptake is so high in susceptible insects not all uptake is toxic. Hsu et *al.*, (1998) has proposed PH_3 induced oxidative stress as the main toxic mechanism leading to cell death in liver cells. Quistad *et al.*, (2000) proposed a chemical model for PH_3 induced lipid peroxidation as a

pathway for PH_3 toxicity. Here, cellular hydrogen peroxide reacts with PH_3 to produce elevated levels of radical oxidative species. These radicals then go on to degrade lipids.

This study aimed to see if the uptake associated with non-toxic reactive pathways could be identified in susceptible and resistant strains of *Tribolium castaneum* (Herbst) exposed to radio labelled PH_3 to investigate the possible site of the uptake associated with toxicity.

MATERIALS AND METHODS

Test insects used were a susceptible strain of *T. castaneum* (CTC_4) held in laboratory culture since collection in 1965 from a produce merchant's store in Brisbane Australia, and a PH_3 resistant selection from this strain. Culturing and general handling techniques follow those described in Winks (1982).

Radioactively labelled PH_3 was produced according to the method described in Reichmuth (1994). This was then diluted by non-labelled PH_3, produced according to a published method (Anon. 1975), to provide a source of suitable activity for the experiment. This source was handled using gas-tight syringes. The concentration of PH_3 was determined by gas chromatography using the response of a Gowmac gas density detector. All dosing and handling was carried out at 25°C. The insects were starved and conditioned in an incubator at 25°C, 57% r.h. overnight prior to exposure to PH_3.

The susceptible and resistant strains used had previously been assessed for mortality response when exposed to a fixed concentration of 0.1 mg L^{-1} of PH_3 for a range of exposure times in a multi-chamber apparatus described in Winks and Waterford (1983). Groups of 200 adults were used at each exposure time. End-point mortality response was determined from successive observations using the method recommended by Winks (1982) and results were analysed using the method of Finney (1971). The response lines for these strains are shown in Table .

TABLE 1

Dosage estimates and parameters of regression of probit mortality on log dosage for adults of *T. castaneum* exposed to 0.1 mg L^{-1} phosphine for various exposure times at 25°C, 57% r.h.

Strain	LD_{50} mg·hr L^{-1}	LD_{99} mg·hr L^{-1}	Slope ±SE	Mean probit response (Y)	Heterogeneity	
					χ^2	*d.f.*
Susceptible	0.249	0.425	10.0± 0.48	5.26	2.83	7
Resistant	1.84	3.653	7.8±0.41	5.01	1.18	4

The phosphine exposures on the insects were carried out in Lux tissue culture flasks, approximately 40 mL and 266 mL in volume. The smaller flasks were used for the short exposure periods and the larger flasks for the longer exposure periods to prevent excessive reduction in oxygen levels, and increased levels of CO_2 and to reduce the amount of ^{32}P label used. The flasks were placed in an incubator conditioned to 57% r.h. The flasks were then sealed and dosed through the lid, fitted with a septum, with 0.1 mg L^{-1} of ^{32}P-labelled phosphine.

The volume injected into the flasks was calculated from the source concentration and the volume of the flasks. The two strains of *T. castaneum* were dosed with equi-toxic exposure at the LD_{50} and LD_{99} according to the Ct exposures in Table 1. The resistant strain was also exposed for 2.5 and 4.25 h, to the LD_{50} and LD_{99} of the susceptible strain. At the end of exposure, PH_3 was rapidly removed and the insects washed in water to collect any external, soluble label. The ^{32}P activity in these washings and whole insects was then assessed prior to a series of further extractions of the whole insects by a technique that broadly followed the techniques described in Annis *et al*. (2000). These extractions were done in parallel and as only five extraction columns were available the resistant strain dosed at the LD_{50} of the susceptible was not included in this set of extractions. First, lipids were removed from the washed insects by Soxhlet extraction with the solvent chloroform. The remaining sample was water extracted to remove all the water-soluble components, which were further divided into amino acids, organic acids and sugars by ion exchange on Dowex-50w and Dowex-1 with suitable solvents. Proteins were released from the insoluble fraction by protease digestion leaving a final insoluble remainder. All fractions were then brought to a common volume in 25 mL volumetric flasks and assessed using Cherenkov counting.

RESULTS

As expected from the results of Banks (1975), Price (1981), Nakakita and Kuroda (1986) and Reichmuth (1994), gross uptake was greatest in the susceptible strain. The gross rate of uptake on the surface of the insects, as reflected by the content of the washings, and the whole insect is shown in Fig. 1. The rate of uptake for each of the extracted components is shown in Fig. 2. Again the rates were generally lower in the resistant strain. Considering the extensive possibilities for phosphorus chemistry via phosphatides in lipids there was, surprisingly, no uptake in any lipid fraction. Figure 3 shows, that despite the increased exposure time of the resistant strain, at equi-toxic exposure (LD_{50} and LD_{99}), the ratio of uptake between resistant and susceptible (R/S), as reflected by counts per minute, is least in every fraction except the insoluble fraction. Assuming zero uptake at the commencement of exposure there are sufficient points to indicate the relative progress of uptake in the extracted fractions of the resistant strain as compared with total uptake at the end of exposure for each extracted fraction. This is shown in Fig. 4.

DISCUSSION

Despite the more than eightfold increase in exposure time at the LD_{99} the distribution and relative uptake of ^{32}P in resistant over susceptible *T. castaneum* was less in the resistant strain. Even though the resistant strain is a phosphine resistant selection of the susceptible strain, it is likely that most of the biochemistry and physiology within the cells of the two strains is similar. Given the overall reduced uptake in a wide range of possible reactive pathways, one way this could come about is by a lower overall PH_3 concentration within the cells of the resistant strain during the exposure. An impervious barrier, to prevent entry, or an active respiratory driven transport of phosphine out of the cell could both achieve the lowered PH_3 concentration within the cell. However, Price (1984) showed a negative gas exchange between alive and dead resistant *Rhyzopertha*

dominica (F.) suggesting an active metabolically-dependant transport mechanism rather than a simple diffusive barrier. The data that is reported in this paper seems to support active exclusion as well.

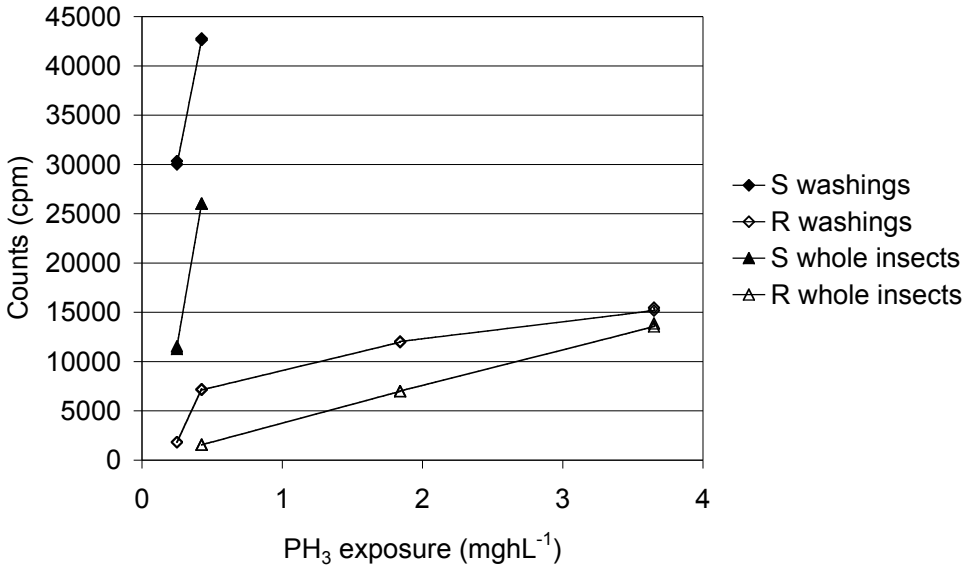

Fig. 1. Soluble uptake onto the surface of *T. castaneum* as reflected by the counts in the water washings at the end of exposure and the remainder of the gross uptake in susceptible and resistant whole insectsÆ

The uptake on the surface of the insects, though much slower overall in the resistant strain, had an initial rapid rate on the surface of the resistant strain which then declined (Fig. 1). Banks (1975) included a strain of melanotic stink gland mutants in his studies on phosphine uptake since this strain does not produce defensive quinones. Quinone production by *T. castaneum* often follows the stress of PH_3 exposure. It is thought that the presence of the defensive quinones is one reason why *T. castaneum* has such a high uptake. This is supported by the results of Banks (1975). It may be that the resistant strain was initially stressed by the presence of PH_3 leading to an initial flush of quinone production. Then the active exclusion mechanism was switched on lowering the internal PH_3 concentration of the insect. With the PH_3 stress reduced further quinone production was slowed or stopped. This is also consistent with the switch being inside the cell.

The absence of label in the lipid fraction is consistent with the destructive toxicity via lipid peroxidation put forward by Quistad *et al*. (2000). Here the proposed toxic pathway is the production of excessive radical oxidative species (ROS) when PH_3 reacts with cellular hydrogen peroxide. The resulting abnormal level of ROS destroys organic systems via lipid peroxidation and other adverse reactions and the cell dies. The label, in this case, presumably ends up in the soluble fraction as phosphoric acid. This complicates the use of uptake as a measure of toxicity, since it seems much of the soluble label in susceptible insect uptake is not associated with toxicity.

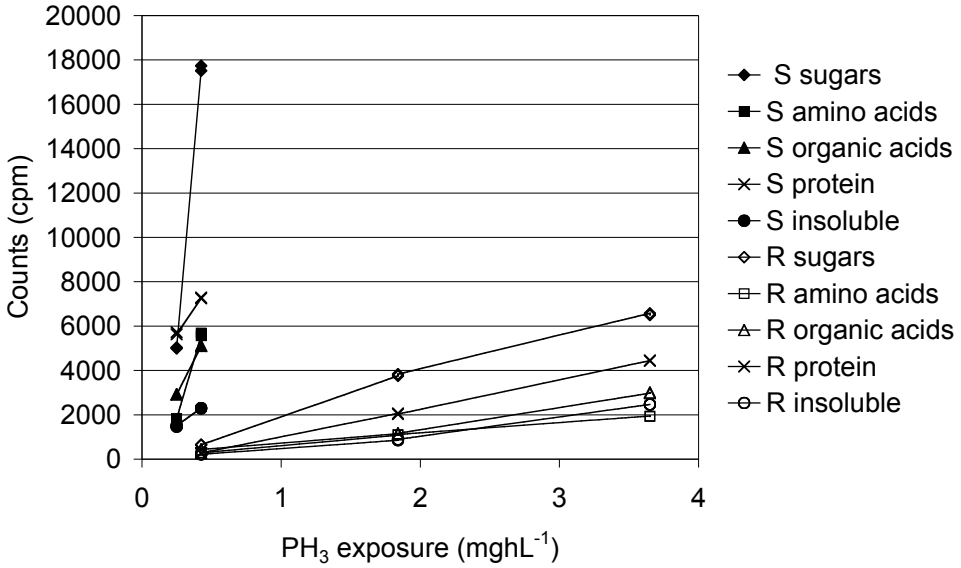

Fig. 2. Relative uptake in extracts from resistant and susceptible strains of *T. castaneum* at the LD_{50} and LD_{99} of these strains.

The relative progress of uptake in the various fractions of the resistant strain, as shown in Fig. 4, suggests early accumulation in some fractions and a late accumulation in others, particularly the protein and insoluble fraction. It might be that this is because the accumulation in these two fractions particularly in the insoluble fraction reflects the onset of cell death. This was the only fraction that was the same in both susceptible and resistant strains at the LD_{99}. Since calcium phosphate is insoluble this is suggestive of the failure of the calcium pump and subsequent flooding of the cytosol with excessive calcium ions from outside the cell.

In terms of resistance, if insects can acquire or increase the ability to keep the PH_3 concentration within the cell low, and further reduce this concentration of PH_3 with non-toxic reactive pathways, then the scope for production of ROS may be reduced to a level where the natural capacity of the insect to deal with them is sufficient to protect the insect from exposure to phosphine. This would be a formidable resistance.

The data suggest that a comprehensive picture of the dynamics of PH_3 uptake could be built up using techniques that separate the label into identifiable reaction sinks. To finally confirm what resistance mechanism is in place in resistant insects an experimental technique that can directly measure the concentration of phosphine within the cell is needed.

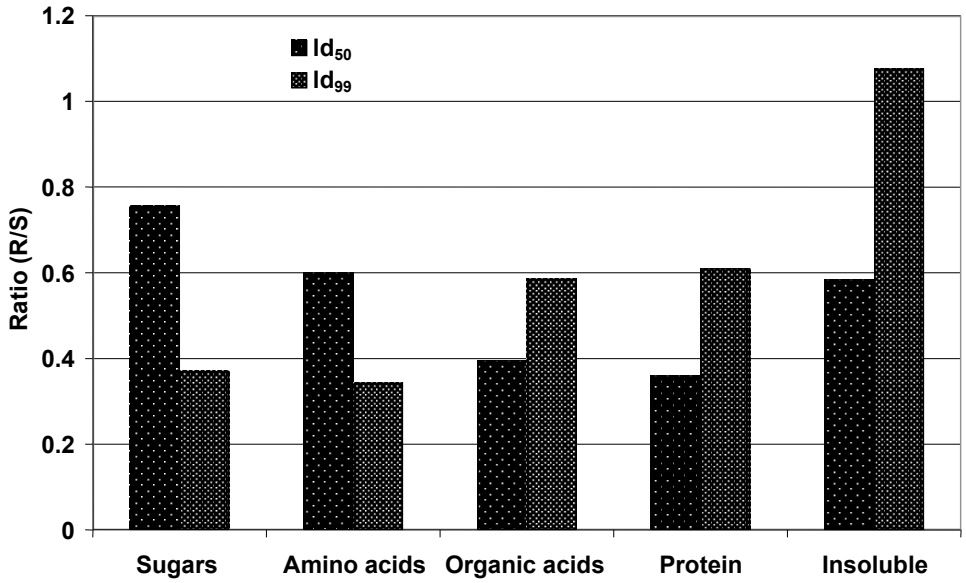

Fig. 3. Ratio of resistant to susceptible uptake (R/S) for equi-toxic exposure to phosphine for a resistant and susceptible strain of *T. castaneum*.

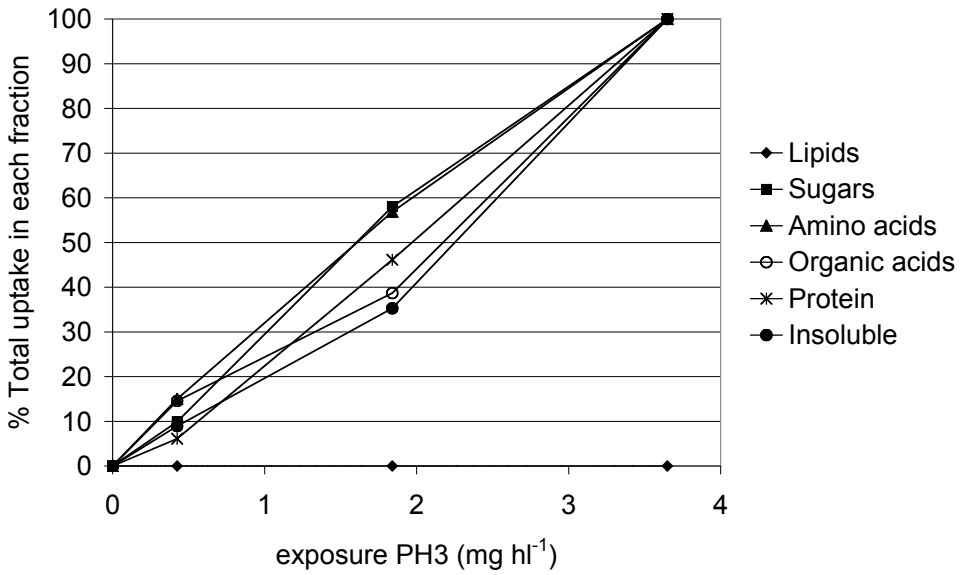

Fig. 4. Progress of phosphine uptake against dosage for each extract from a resistant strain of *T. castaneum* compared with final uptake at the LD_{99}.

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