

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. 57-69

## **A METHOD FOR EXPOSURE OF INSECT CELL CULTURES TO PHOSPHINE**

S. PRATT

*Stored Grain Research Laboratory, CSIRO Entomology, ACT 2601, Australia*  
[e-mail: s.pratt@ento.csiro.au]

### **ABSTRACT**

Cell culture promises to facilitate the study of the toxic effects of gases such as phosphine (PH<sub>3</sub>), but to date, application of the gas to the cells has been problematic. This report describes a simple and flexible method for treating insect cell cultures with quantitative doses of the fumigant gas PH<sub>3</sub>. After inoculation with Sf9 cells (*Spodoptera frugiperda* (J.E. Smith) Lepidoptera; Noctuidae), cell culture flasks with loosened caps were placed inside glass desiccating chambers, which were sealed and injected with concentrated PH<sub>3</sub>. The time taken for the gas to enter the culture flasks, and the amount of PH<sub>3</sub> that dissolved in the medium were determined. Cell growth and mortality after PH<sub>3</sub> exposure were monitored for several days by repeatedly carrying out Trypan Blue dye exclusion assays. Phosphine entered cell flasks with an exponential time course. Internal concentrations rose to higher than 90% of the concentration in the exposure chamber in less than 2 h. Phosphine was found to rapidly (equilibrium reached in 10-20 min.) partition into water or cell culture medium under conditions similar to those used during cell exposure, with a partition coefficient ( $C_w / C_a$ ) of 0.15, which is in approximate agreement with the literature value. After exposure of Sf9 cells to PH<sub>3</sub> for 18 h, concentrations of 1,400, 5,200 and 10,400 ppm (1.9, 7.2, 14 mg $\Pi^{-1}$ ) produced mortalities of 20%, 50% and 75% respectively, when measured 8 h after exposure ceased. Establishment of total cell mortality of low doses was made difficult by resumption of growth by surviving cells. Control mortality remained below 5%. The high doses, with respect to whole insects, required to cause high mortality are noted. Comparisons are drawn with earlier cell culture work that used aqueous PH<sub>3</sub>, and implications for the use of the method to expose cells to PH<sub>3</sub> and other gases before conducting other assays and reactive oxygen species studies are discussed.

### **INTRODUCTION**

Since phosphine (PH<sub>3</sub>) was introduced as a grain fumigant more than 50 years ago, many laboratory studies have been conducted on whole insects to ascertain the conditions (concentration, time, temperature, life stages) required for their effective control by fumigation. These studies have been carried out by exposing living insects to PH<sub>3</sub> in static or flow-through experimental apparatuses (Winks and Waterford, 1983). Considerable light has also been shed on the biochemical mode of action, and

mechanisms of resistance in insects using these methods (Price and Chambers, 1990; Chaudhry, 1997). A common practice has been to expose insects to known doses of a fumigant and then homogenise them in order to perform biochemical assays (Nakakita, 1987; Bolter and Cherfuka, 1990a; Chaudhry and Price, 1992).

Several workers have advised caution when dealing with gases, and have gone to great lengths to ensure adequate mixing. Winks (1982) used mechanical stirrers in large exposure chambers and “pumped” smaller flasks by syringe. The choice of materials can also be problematic in fumigant studies. Phosphine was shown to adsorb only slightly to glass, but large amounts of the gas were lost when in contact with teflon, nylon or polyvinyl chloride (PVC). It passes freely through silicone rubber tubing (Waterford and Winks, 1986). In contrast, *in vitro* PH<sub>3</sub> studies have generally relied on the addition of the fumigant dissolved in culture medium or buffer (Potter *et al.*, 1991). The low solubility of PH<sub>3</sub> in aqueous media (Weston, 1954) means that nominal dosages have been applied by adding saturated buffer to the tissues or extracts under investigation (Hobbs and Bond, 1989).

Cell cultures can be thought of as occupying the “middle ground” between sub-cellular biochemical extracts and whole organisms and as such, are becoming widely used as models for toxicity studies. The finding that apoptosis (“programmed cell death”) was induced in insect cell culture by addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or exposure to UV irradiation (Hasnain *et al.*, 1999) is relevant to earlier findings that isolated insect and mammalian mitochondria release H<sub>2</sub>O<sub>2</sub> after treatment by PH<sub>3</sub> (Bolter and Cherfuka, 1990b). In a study of PH<sub>3</sub> on mammalian cells, measured amounts of solutions of aluminium or magnesium phosphide were added to the flasks and the caps sealed, again producing nominal concentrations of PH<sub>3</sub> (Hsu, Quistad and Casida, 1998). In spite of these difficulties, internally consistent determinations of inhibition and mortality rates were obtained. One of the problems with these methods is that exposure periods are short (generally 1-6 h) and difficult to extend, whereas whole insect studies have shown that the duration of exposure can be an important factor, and long exposure times are often required.

Some apparatuses, both complex and rudimentary, have been constructed to expose cell cultures to gases. In order to study the effects of photochemical smog on cell cultures, Knebel *et al.* (1998) built a large flow-through chamber to supply gaseous compounds in sterile air to the cultures. Araki *et al.* (1994), placed bacteria on agar plates on a rack inside a 10 L Tedlar (gas-tight plastic) bag.

Early attempts by this author at determining cell mortality relied on the use of static cultures (in which cells adhered to the floor of the culture flask) in a medium that contained animal blood serum. This had several shortcomings. Repeated mortality assessment was difficult because of the disruption entailed in resuspending the cells, resulting in erratic cell counts and mortality assays. There were also limitations in the maximum cell density that could be attained and hence the length of time that cultures would remain in exponential growth after exposure. Some assays (such as Lactate Dehydrogenase (LDH) leakage, another cell death assay) cross-react due to protein activity in the added serum.

This report describes a simple method for treating insect cell cultures in suspension with quantitative amounts of  $\text{PH}_3$ , which is flexible enough to be used with other gases. Cell cultures in flasks with loosened caps were placed inside glass chambers, which were sealed and injected with  $\text{PH}_3$ . The time taken for the gas to enter the culture flasks and the amount of  $\text{PH}_3$  that dissolved in the medium was determined. Cell growth and mortality after  $\text{PH}_3$  exposure were monitored for several days.

## EXPERIMENTAL METHODS

### Exposure chambers

Glass desiccating chambers of 2.5 L nominal volume were used. The volumes of the chambers had already been accurately determined using the weight of contained water method. Quickfit® screw-top adaptors fitted with rubber septa were placed in the holes in the lids.

### Phosphine generation and analysis

Phosphine was generated by immersing a pellet (1 g) of a commercial aluminium phosphide formulation (Detia, Frankfurt) in 5%  $\text{H}_2\text{SO}_4$  under a water-filled 100 mL gas burette, with a septum at the top for withdrawing samples of gas (Anon. 1975). The  $\text{PH}_3$  content (*ca.* 90%) of this 'source' gas was determined by gas chromatography analysis using a gas density balance detector (GC/GADE). Column: 1.8 m x 6.25 mm OD, packed with Porapak Q. Oven temperature: 100°C. Carrier gas: R134a (tetrafluoroethane).

The  $\text{PH}_3$  concentrations inside the exposure chambers were measured by GC with a flame photometric detector (GC/FPD). Peak areas were compared with those from mixtures obtained by quantitative dilution of source gas into glass flasks of known volume. GC conditions were: Column: GSQ (J&W Scientific, USA) 30 m x 0.53 mm ID, carrier gas:  $\text{N}_2$ , head pressure: 130 kPa. Oven temperature: 100°C.  $\text{PH}_3$  retention time: 25 s.

GADE and FPD analyses were performed in at least in triplicate, where standard deviations less than 0.5% of the reading were the norm.

### Validation of phosphine exposure

*Permeation of gases into cell culture flasks:* Phosphine was injected into sealed empty chambers in order to establish that they were gas-tight and maintained constant  $\text{PH}_3$  concentrations.

According to cell culture flask manufacturers, loosening caps so that a marked band was uppermost allows gas exchange without undue risk of microbial contamination. Caps of 25 cm<sup>2</sup> tissue culture flasks (Falcon, Cat. 3082) were loosened as directed before exposure.

The time required for equilibration of  $\text{PH}_3$  concentrations between headspace and flasks was measured. Holes (4 mm) were carefully drilled in the sides of culture flasks so as not to crack the plastic, and cylindrical septa (Alltech) were fitted and

sealed in place with epoxy resin (Araldite brand, Ciba-Geigy). Gas-tightness of flasks with septa was confirmed by placing the flasks with their caps firmly closed in an exposure chamber, and injecting source  $\text{PH}_3$  (1.0 mL, achieving a concentration of 350 ppm ( $0.5 \mu\text{g L}^{-1}$ )). After overnight exposure, the  $\text{PH}_3$  concentrations in the exposure chambers were measured by GC/FPD, the flasks were removed from the desiccating chambers, and the interiors of the flasks were measured for  $\text{PH}_3$ .

The exposures were then repeated with the caps loosened. The flasks were exposed to  $\text{PH}_3$  for periods of time between 10 min and 3 h. The ratio of the  $\text{PH}_3$  concentrations in the exposure chambers and inside the culture flasks were recorded, and plotted. The rate of entry of  $\text{PH}_3$  when culture flask caps were fitted with air filters ( $0.2 \mu\text{m}$ , Falcon Cat No. 3109) was also determined.

*Partitioning of phosphine into cell culture medium:* In order to determine the amount of  $\text{PH}_3$  present in the aqueous phase, the partition coefficient was determined under conditions similar to that of cell culture exposures. Pairs of Boston jars (250 mL, the smallest volume in which  $\text{PH}_3$  concentrations can be maintained reliably (unpublished data) were charged with 0, 40, or 100 mL of distilled water or 40 mL fresh HiQ SFX serum-free insect cell culture medium (Promega). Gas-tight Mininert (SKC Corp.) caps with septa were fitted, and the same amount of concentrated source gas was injected into all the bottles, to achieve headspace concentrations of approx. 3,000 ppm. After gentle orbital shaking overnight at 27-28°C (cell culture conditions), the  $\text{PH}_3$  concentrations in the headspace and aqueous phase were determined by GC/FPD. It had been found that injections of up to 10  $\mu\text{L}$  water did not disturb the detector flame and it was assumed that the detector gave the same molar response to  $\text{PH}_3$  after aqueous or gaseous injection. Injections of cell culture medium were not performed to avoid damaging the GC column. The partition coefficient  $P_k$  was calculated by dividing the aqueous  $\text{PH}_3$  concentration (w/v) by the headspace concentration (w/v). Mass balances and experimental errors of the derived values were calculated where water was used.

### Cell culture

Cell cultures (Sf9, *Spodoptera frugiperda* (J.E. Smith), ATCC CRL 1711) were grown in suspension in 'shaker culture' (orbital rotation at approx. 100 rpm) and maintained in HiQ SFX serum-free insect cell culture medium (Promega) and incubated at 26-28°C. Cell densities were counted with a hemocytometer. When passaging, (inoculating new cultures), cultures were centrifuged (1,000 rpm for 3 min), the supernatant was discarded and the pellet of cells resuspended in fresh medium. New cultures were seeded at a starting density of about  $0.8 \times 10^6$  cells  $\text{mL}^{-1}$  and allowed to incubate for some hours so that they were exposed to  $\text{PH}_3$  during "log. phase" growth. Exponential growth normally continued with a doubling time of about 17 h until limiting densities of about  $1 \times 10^7$  cells  $\text{mL}^{-1}$  were reached.

*Exposure of cell cultures:* Glass 50 mL Erlenmeyer screw-cap flasks containing 10 mL of medium were inoculated with  $0.8 \times 10^6$  cells mL<sup>-1</sup>. After 4-14 h incubation, the flasks were placed in pairs in chambers, which were then sealed and dosed with the requisite volume of 'source' PH<sub>3</sub>. In a typical experiment, volumes of 0, 4, 15 and 30 mL of ca 90% PH<sub>3</sub> were injected, giving headspace concentrations of 1,400, 5,200 and 10,400 ppm (1.9, 7.2, and 14 mg m<sup>-3</sup>.)

The flasks were placed on a trolley, which was rocked by the motion of an orbital shaker in a circular motion, similar to that used in the incubator. Temperature was monitored with a data logger, which was kept with the flasks throughout the experiment. The chambers were incubated at 26±0.7°C in a constant-temperature room for 18 h. Phosphine concentrations were measured before the chambers were opened. The cell culture flasks were then assayed for cell mortality. It was assumed that PH<sub>3</sub> quickly escaped from the culture flasks, especially since they were opened for assay purposes shortly after removal from PH<sub>3</sub> exposure.

In a separate experiment, the ability of medium to sustain cell growth after exposure to PH<sub>3</sub> was examined. Medium in a sterile flask was placed in an exposure chamber alongside flasks that were inoculated with cells. After airing, the flask was inoculated with cells and their growth recorded.

*Cell mortality assay:* Cell mortality was determined using Trypan Blue exclusion. An aliquot of cells was removed from flasks and an equal volume of Trypan Blue solution was added. Cells were counted using a hemocytometer. Cell mortality was expressed as the proportion of stained (dead) cells to total (stained and unstained) cells. One determination was made for each sample in order to perform all the counts within a suitable time period.

## RESULTS

### Permeation of gases into cell culture flasks

Phosphine levels in empty chambers were recorded over intervals up to 100 h. The PH<sub>3</sub> levels in the chambers did not decrease by more than 1% (which was within the experimental error).

The entry of PH<sub>3</sub> into the flasks followed an exponential time course (Fig. 1), as expected. The data were plotted logarithmically as the ratio of the concentration inside the flasks to that in the exposure chamber, subtracted from 1. Thus, at commencement of exposure, the internal concentration was zero, so the curve begins at 1. Where caps were loosened, it took about half an hour for the internal concentration to rise to half, and almost 2 h to reach 90% of the external concentration. When caps with filters in them were used, equilibration was faster, with  $t_{50} \approx 20$  min and  $t_{90} \approx 1$  h. No case of microbial contamination of flasks was observed. Levels of PH<sub>3</sub> inside culture flasks with closed caps were not measurable after exposure, indicating that the caps were gas tight. Later mortality assessments (see below) used different conical glass culture flasks, but it is assumed that the rate

of gas permeation past their loosened caps would be similar to those observed in the 'flat' type flasks.

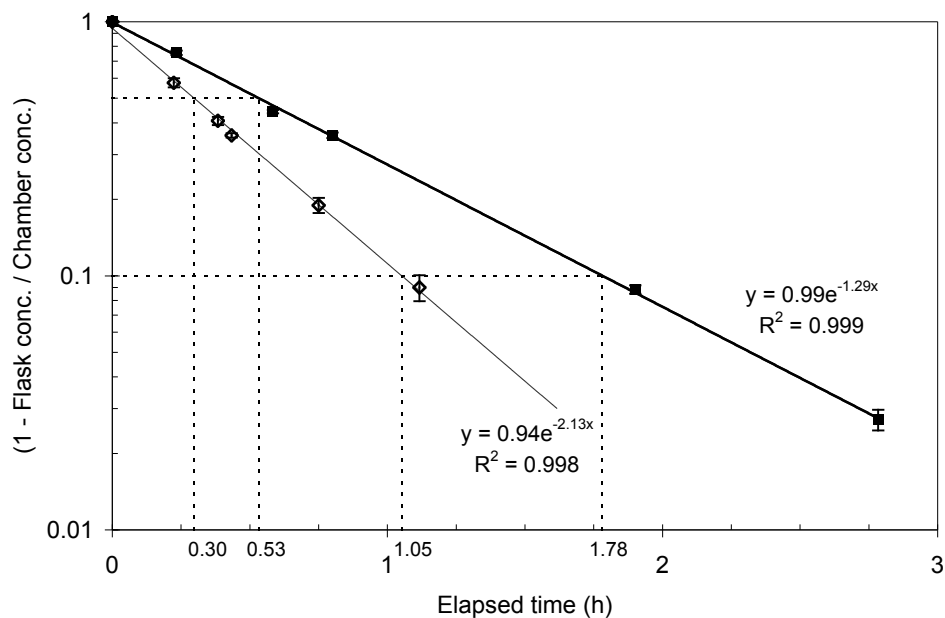


Fig. 1. Time-course of  $\text{PH}_3$  entry into cell culture flasks placed in exposure chambers. Values expressed as the ratio of the  $\text{PH}_3$  concentration inside the flasks to that of the exposure chamber on a logarithmic scale. ■ Flasks with solid caps. □ Flasks with filters in caps. Vertical lines indicate  $t_{50}$  and  $t_{90}$  for each type of cap. Vertical bars represent the ranges of the duplicate measurements.

### Partitioning of $\text{PH}_3$ into cell culture medium

The  $\text{PH}_3$  determinations in gas and aqueous phases are shown in Table 1. The partition coefficient ( $P_k = C_{\text{water}}/C_{\text{air}}$ ) determinations were reproducible, and the average was 0.15.

Some of the aqueous  $\text{PH}_3$  determinations also showed the presence of a small peak with a longer retention time, but attempts to produce the peak consistently or to characterise it failed. In this experiment no effort was made to quantify the amount of oxidation of  $\text{PH}_3$  to hypophosphite, phosphite and phosphate. The stability of the headspace concentrations over periods of time longer than the 18 h used here (unpublished data) indicates that the oxidation rate is low. The coefficient was determined at concentrations in the lower range of those used in the mortality assays and assumed to be constant over the entire range, for the reason that addition of large volumes of  $\text{PH}_3$  without introducing pressure effects becomes difficult, as does FPD analysis at concentrations of 10,000 ppm.

TABLE 1  
Derivation of partition coefficient,  $P_k$ , in water and HiQ cell culture medium

Filling ratio (%)	PH <sub>3</sub> concentration (ppm)		Partition coefficient ( $P_k$ )	Mass balance (%)
	Water	Air		
0		2766		100
16	470	3041	0.15 ± 0.01	95 ± 7 <sup>c</sup>
40	607	3599	0.17 ± 0.01	87 ± 5
16 HiQ	<sup>a</sup>	3080	0.16 <sup>b</sup>	

Notes:

- Liquid concentration not determined with HiQ medium, to avoid column contamination.
- Partition coefficient calculated by estimating dissolved PH<sub>3</sub> concentration.
- Mass balance as proportion of amount of PH<sub>3</sub> in waterless flasks (0% filling ratio).

It was noted that the rate of equilibration was very rapid. When PH<sub>3</sub> was injected into the headspace of jars, which were then clamped upside down, so that water samples could be withdrawn with minimum disturbance of the water, stable aqueous PH<sub>3</sub> levels were attained within 10-20 minutes.

### Cell exposure and mortality assessment

After exposure to PH<sub>3</sub>, Trypan Blue cell mortality assays were performed at intervals over three days. This allowed the time course of cell mortality (Fig. 2) and cell density (Fig. 3) to be simultaneously determined. In both graphs, the commencement of exposure to PH<sub>3</sub> is denoted as time zero. As is shown in Fig. 2, control mortality remained below about 5%, but there was a dose-dependant increase in mortality in the treated cultures immediately after exposure (t=20 h). An assay at a later time point (t=26 h) indicated a higher level of cell mortality, and a third assay 18 h later showed that the proportion of dead cells continued to rise after treatment at 5,200 or 10,400 ppm.

The fraction of dead cells in the lowest treatment (1,400 ppm) fell between the second and third assays, as a result of growth and division of the cells that survived PH<sub>3</sub> treatment. This is borne out in Fig. 3, which plots the cell density (cells mL<sup>-1</sup>) of the cells over the same period. The control cells grew at a steady logarithmic rate, except for a slight decline during their period in the constant temperature room where the exposure was carried out. In this case, the room was slightly colder than the cell culture incubator. (In other experiments, where the temperatures were better matched, the control growth rate was constant.) The growth rate of the control cells slowed after t = 48 h, as they exhausted their nutrient supply. The cells that survived the lowest exposure (1,400 ppm) resumed growth at a rate similar to the control cells. The cells subjected to the highest treatment (10,400 ppm) failed to resume growth. The post-exposure behaviour of the intermediate treatment showed a period of low or no growth, then a resumption of growth by the survivors (Fig. 3), along with continued cell death (Fig. 2).

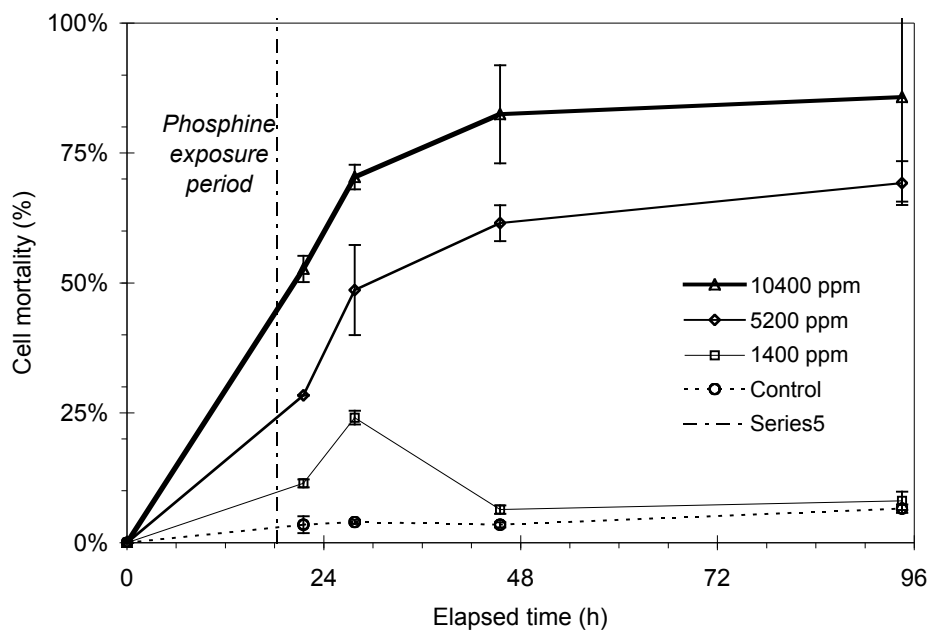


Fig. 2. Cell mortality after 18 h exposure to 3 concentrations of PH<sub>3</sub>. Mortality was expressed as the fraction of cells stained by Trypan Blue with respect to the total number of cells counted. Mortalities rose in the first day after cessation of exposure. The apparent decline in cell mortality in the lowest dose is a result of the continued growth of viable cells. Vertical bars represent the ranges of the duplicate measurements.

In culture medium had been exposed to PH<sub>3</sub>, cell growth rates and mortality levels were consistent with cells that had not been exposed to PH<sub>3</sub> (data not shown).

## DISCUSSION

This paper shows that it is possible to use gaseous PH<sub>3</sub> to achieve high mortality in cell culture toxicity tests, and that the method is very suitable for the purpose. Cultures remained free of infection and untreated (control) cells continued to grow normally after being handled and transferred to and from the exposure room.

The slow penetration of PH<sub>3</sub> into the cell flasks observed in this study (about 2h) would make the estimation of the effective exposure (i.e. that experienced by the cells) difficult for short exposures, but this was not a concern because the high levels of tolerance of the cells to the toxicant required long exposure periods and high concentrations. Shorter exposures would perhaps be feasible if culture flask caps were replaced by a film of semi-permeable membrane to increase gas equilibration rates without compromising sterility.

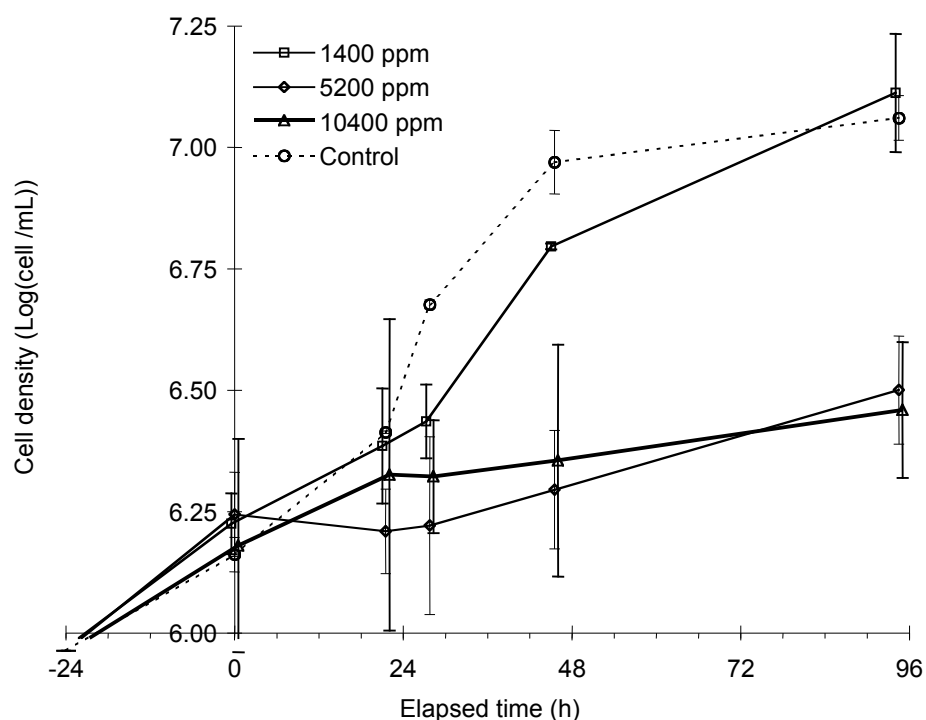


Fig. 3. Cell growth after 18 h exposure to 3 concentrations of  $\text{PH}_3$ . Exposure was from  $t=0$  to  $t=18$  h. Cell density is the total of live and dead cells, as determined by Trypan Blue exclusion. The exposure chamber was slightly colder than the recovery incubator, which caused the increase in control cell growth after  $\text{PH}_3$  exposure. Vertical bars represent the ranges of the duplicate measurements.

The observed partition coefficient,  $P_k = 0.15$ , is acceptably close to the lower of the literature values, which range from 0.22-0.26 (Weston, 1954; Weston and Bigeleisen, 1954). It should be noted that the higher figure of 0.26, which is the source of the widely quoted statement that 26 mL of pure  $\text{PH}_3$  will dissolve in 100 mL of water, was determined under low pressure binary phase conditions, where the only substances present in the chamber were mixtures of  $\text{H}_2\text{O}$  and  $\text{PH}_3$  in gaseous and liquid phases. The aqueous phase only becomes saturated with  $\text{PH}_3$  when the gas occupies 100% of the headspace. The highest concentration used in this study, 10,400 ppm, is equivalent to 1% v/v, so it would be expected that the aqueous medium was far from saturated. The lower coefficients reported in the literature were measured when dissolved salts and weak acids were present in solution. A low rate of proton exchange of  $\text{PH}_3$  with  $\text{H}_2\text{O}$  at near-neutral pH was noted by Weston (1954) and the unreactivity of  $\text{PH}_3$  towards oxidation is evidenced by the maintenance of stable headspace concentrations over water which were stable over several days.

Phosphine's low partition coefficient highlights the limitations of liquid applications of  $\text{PH}_3$ -saturated buffer. Indeed, the rapid equilibration of  $\text{PH}_3$  implies that studies which exposed mammalian cells to  $\text{PH}_3$  by addition of phosphide-treated buffer (e.g. Hsu *et al.*, 1998) may have actually subjected the cells to a short burst of  $\text{PH}_3$ , before most of it partitioned into the headspace of the culture flask, and the effects (cell death, lipid peroxidation) that continued to increase over several hours may have merely been delayed responses. The 'preference' by  $\text{PH}_3$  for the gas phase, coupled with the large volume of headspace with respect to culture medium in a typical flask, makes it doubly inevitable (there is no such thing as doubly inevitable) that most of the toxicant leaves the medium, becoming unavailable to the cells. In fact, the authors acknowledge that the concentrations were, "nominal and maximal". Their highest applied concentration was based on a tenfold dilution of saturated buffer. It is assumed by this author that cells were not treated directly with saturated medium because the counter ion ( $\text{Mg}^{2+}$  &  $\text{Al}^{3+}$ ) concentrations would have been directly cytotoxic. A calculation based on relative volumes of medium and headspace in culture flasks indicates that at the maximum concentration applied by Hsu *et al.* (1998), which was 1 mM, resulting in 31% mortality after 6 h, the effective aqueous concentration (neglecting any losses) after partitioning would have been 20  $\mu\text{M}$ . This is comparable to the mid-range test concentration used in this report (5,200 ppm, equivalent to 32  $\mu\text{M}$ ), which caused 28% mortality when assayed immediately after 18 h exposure, but which rose to greater than 50% several days post-exposure. Cell mortality of 18% was reported by Hsu *et al.*, (1998) after addition of stock solution even after degassing it with  $\text{N}_2$ . This mortality would seem not to be due entirely to residual  $\text{PH}_3$ , because one would expect that almost all the dissolved  $\text{PH}_3$  would have escaped, based on the observations reported here of equilibration times in an undisturbed air/water system. In fact, Hsu *et al.*, (1998) noted an unexplained difference in toxicity between solutions derived from AIP and  $\text{Mg}_3\text{P}_2$ . Gaseous  $\text{PH}_3$  treatment would make it possible to apply  $\text{PH}_3$  alone and in conjunction with  $\text{Mg}^{2+}$  and  $\text{Al}^{3+}$  to cells to confirm that the ROS production observed by Hsu *et al.* (1998) were due to  $\text{PH}_3$  rather than a combination of factors. Mammalian cell lines, with their requirements for elevated  $\text{CO}_2$  levels with respect to air, could be treated in exposure chambers with small adjustments.

The finding in this study, that there was a continued rise in cell death for at least 24 h after exposure is reminiscent of the importance placed on mortality response times of whole insects to  $\text{PH}_3$  (Howe, 1974; Winks 1982; Winks and Waterford, 1986), where it was found that it took up to 14 days for insects to die after exposure. This is an encouraging indication that the mechanism of cell death may be relevant to the way in which insects succumb to the poison.

Cell death (necrosis) may not be the best measure of  $\text{PH}_3$  toxicity, particularly as Trypan Blue and LDH are known to under-estimate cell death, because loss of membrane integrity is one of the last stages of death. The problem of delayed mortality also makes assessment of the effect of low doses difficult, since unaffected cells resume growth after the exposure. Further work is envisaged using other

assays, which give a measure of cell *viability* as opposed to cell death (Mosmann, 1983), which may give more definite information as to  $\text{PH}_3$ 's effects when performed soon after exposure.

The  $\text{PH}_3$  concentrations required to elicit cell death were very much higher than those that kill whole insects over the same period of time. This serves as a reminder that cells are not directly comparable with whole organisms. An organism relies on the action of complex grouping of cells to control nutrient supplies, waste removal and prevention of infection (among other things), and death of an organism may be effected by selective damage to certain organs or nerves, whereas cells are deemed to be alive if they continue to metabolise and divide, in an environment (the culture medium) that is kept free of infection and periodically replenished by an outside party. Nevertheless, given the obvious differences between organisms and their cells in culture, and the different criteria (or endpoints) for mortality, comparisons can be made between the effects of  $\text{PH}_3$  (and other poisons) on cells and on whole insects.

A possible explanation of the cell's high tolerance to  $\text{PH}_3$ , on a molecular level, may rest on the knowledge that reactive oxygen species (ROS), especially  $\text{H}_2\text{O}_2$ , leave the cell and enter the cell culture medium (Flanagan, Moseley and Buettner, 1998). Cells suspended in medium are in a highly diffuse state, compared to those in an organism, which have about a thousand-fold more cells per unit volume. Thus, ROS emitted by a cell in an organism are much more likely to enter and cause oxidative stress to a neighbouring cell. The high tolerance of cultured cells bears a similarity to that of young moth eggs (Bell, 1976). This may indicate that egg cultured cells and egg cells are living under similar circumstances, with large volumes of essentially 'non-biological' liquid (the culture medium or egg yolk) that are in contact with the sensitive biological components inside the cells. It may also indicate that undifferentiated cells are inherently less sensitive to  $\text{PH}_3$  toxicity. This comparison warrants further investigation.

The method described here is shown to be an effective means of exposing insect cell cultures to  $\text{PH}_3$ . Phosphine concentrations in the cell medium can be determined quantifiably, and kept constant over lengthy periods of time. The highest concentration that can be applied is higher than is possible by application of saturated buffer, and without the confounding problem of adding counterions to the cell medium. Cell culture also lends itself to rapid screening of compounds that may enhance (e.g. other ROS producers) or reduce (e.g. antioxidants, (Hsu *et al.*, 2000)) the toxicity of  $\text{PH}_3$ .

Work is now progressing to use this method to measure  $\text{PH}_3$ -induced ROS production by a fluorescence assay (indirect) or by EPR (direct). Other effects, such as the influence of cell density on  $\text{PH}_3$  toxicity and the effects of sublethal doses, are being investigated. It seems reasonable to expect that other gases could be investigated under similar conditions, so  $\text{PH}_3$  analogues will also be tested for toxicity and ROS production using this method.

### ACKNOWLEDGEMENTS

Travel to the conference was funded by the Grains Research and Development Corporation. The author was also in receipt of a GRDC Grains Industry Research Scholarship. S. Dorrian is thanked for provision of cell stocks and advice in cell culture technique. J.M. Desmarchelier, V.S. Haritos, E.J. Wright and P.C. Annis are thanked for guidance, fruitful discussions and critical reading of the manuscript.

### REFERENCES

- Anon. (1975) Recommended methods for the detection and measurement of agricultural pests to pesticides. 16: Tentative method for adults of some stored cereals, with methyl bromide and phosphine. Food and Agriculture Organization of the United Nations **23**, 12-25.
- Araki, A., Noguchi, T., Kato, F. and Matsushima, T. (1994) Improved method for mutagenicity testing of gaseous compounds by using a gas sampling bag. *Mutation Research*, **307**, 335-344.
- Bell, C.H. (1976) The tolerance of developmental stages of four stored product moths to phosphine. *J. stored Prod. Res.*, **12**, 77-86.
- Bolter, C.J. and Chefurka, W. (1990a) The effect of phosphine treatment on superoxide dismutase, catalase and peroxidase in the granary weevil, *Sitophilus granarius*. *Pesticide Biochem. and Physiol.*, **36**, 52-60.
- Bolter, C.J. and Chefurka, W. (1990b) Extramitochondrial release of hydrogen peroxide from insect and mouse liver mitochondria using the respiratory inhibitors phosphine, myxothiazol and antimycin and spectral analysis of inhibited cytochromes. *Archives of Biochemistry and Biophysics*, **278**, 65-72.
- Chaudhry, M.Q. (1997) A review of the mechanisms involved in the action of phosphine as an insecticide and phosphine resistance in stored-product insects. *Pesticide Science*, **49**, 213-228.
- Chaudhry, M.Q. and Price, N.R. (1992) Comparison of the oxidant damage induced by phosphine and the uptake and tracheal exchange of <sup>32</sup>P-radiolabelled phosphine in the susceptible and resistant strains of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). *Pesticide Biochem. and Physiol.*, **42**, 167-179.
- Flanagan, S.W., Moseley, P.L. and Buettner, G.R. (1998) Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonance spin trapping. *FEBS Letters*, **431**, 285-286.
- Hasnain, S.E., Taneja, T.K., Sah, N.K., Mohan, M., Pathak, N., Sahdev, S., Athar, M., Totey, S.M. and Begum, R. (1999) *In vitro* cultured *Spodoptera frugiperda* insect cells: Model for oxidative stress-induced apoptosis. *Journal of Biosciences*, **24**, 13-19.
- Hobbs, S.K. and Bond, E.J. (1989) Response of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) to sublethal treatments with phosphine. *J. stored Prod. Res.*, **25**, 137-146.
- Howe, R.W. (1974) Problems in the laboratory investigation of the toxicity of phosphine to stored product insects. *J. stored Prod. Res.*, **10**, 167-181.
- Hsu, C.H., Han, B.C., Liu, M.Y., Yeh, C.Y. and Casida, J.E. (2000) Phosphine-induced oxidative damage in rats: attenuation by melatonin. *Free Radical Biology and Medicine*, **28**, 636-642.

- Hsu, C.H., Quistad, G.B. and Casida, J.E. (1998) Phosphine-induced oxidative stress in Hepa 1c1c7 cells. *Toxicological Sciences*, **46**, 204-210.
- Knebel, J.W., Ritter, D. and Aufderheide, M. (1998) Development of an in vitro system for studying effects of native and photochemically transformed gaseous compounds using an air/liquid culture technique. *Toxicology Letters*, **96-7**, 1-11.
- Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application for proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**, 55-63.
- Nakakita, H. (1987) The mode of action of phosphine. *Journal of Pesticide Science*, **12**, 299-309.
- Price, N.R. and Chambers, J. (1990) Biochemistry of phosphines. In: The chemistry of organophosphorus compounds, primary, secondary and tertiary phosphines, polyphosphines and heterocyclic organophosphorus (III) compounds. (Edited by Hartley, F.R), Wiley, Chichester, 643-661.
- Potter, W.T., Rong, S., Griffith, J., White, J. and Garry V.F. (1991) Phosphine-induced Heinz body formation and hemoglobin oxidation in human erythrocytes. *Toxicology Letters*, **57**, 37-45.
- Waterford, C.J. and Winks, R.G. (1986) Loss rates of phosphine associated with various materials used in laboratory fumigation apparatus. *J. stored Prod. Res.*, **22**, 25-27.
- Weston, R.E. and Bigeleisen, J. (1954) Kinetics of the exchange of hydrogen between phosphine and water: a kinetic estimate of the acid and base strengths of phosphine. *Journal of the American Chemical Society*, **76**, 3074-3078.
- Weston, R.E. (1954) The solubility of phosphine in aqueous solutions. *Journal of the American Chemical Society*, **76**, 1027-1028.
- Winks, R.G. (1982) The toxicity of phosphine to adults of *Tribolium castaneum* (Herbst): Time as a response factor. *J. stored Prod. Res.*, **18**, 159-169.
- Winks, R.G., and Waterford, C.J. (1983) Multi-chamber apparatus for laboratory studies with gases. *Laboratory Practice*, **31**, 62-65.
- Winks, R.G., and Waterford, C.J. (1986) The relationship between concentration and time in the toxicity of phosphine to adults of a resistant strain of *Tribolium castaneum*. *J. stored Prod. Res.*, **22**, 85-92.