A METHOD FOR EXPOSURE OF INSECT CELL CULTURES TO PHOSPHINE

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ABSTRACT

Cell culture promises to facilitate the study of the toxic effects of gases such as phosphine (PH_3), 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_3. 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_3. The time taken for the gas to enter the culture flasks, and the amount of PH_3 that dissolved in the medium were determined. Cell growth and mortality after PH_3 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_3 for 18 h, concentrations of 1,400, 5,200 and 10,400 ppm (1.9, 7.2, 14 mg!L^{-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_3, and implications for the use of the method to expose cells to PH_3 and other gases before conducting other assays and reactive oxygen species studies are discussed.

INTRODUCTION

Since phosphine (PH_3) 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_3 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_3 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_3 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_2O_2), or exposure to UV irradiation (Hasnain *et al*., 1999) is relevant to earlier findings that isolated insect and mammalian mitochondria release H_2O_2 after treatment by PH_3 (Bolter and Cherfuka, 1990b). In a study of PH_3 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_3 (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 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 PH_3. The time taken for the gas to enter the culture flasks and the amount of PH_3 that dissolved in the medium was determined. Cell growth and mortality after 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% H_2SO_4 under a water-filled 100 mL gas burette, with a septum at the top for withdrawing samples of gas (Anon. 1975). The 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 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: N_2, head pressure: 130 kPa. Oven temperature: 100°C. 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 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^2 tissue culture flasks (Falcon, Cat. 3082) were loosened as directed before exposure.
The time required for equilibration of 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 PH_3 (1.0 mL, achieving a concentration of 350 ppm (0.5 μg L^{-1})). After overnight exposure, the 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 PH_3.

The exposures were then repeated with the caps loosened. The flasks were exposed to PH_3 for periods of time between 10 min and 3 h. The ratio of the PH_3 concentrations in the exposure chambers and inside the culture flasks were recorded, and plotted. The rate of entry of PH_3 when culture flask caps were fitted with air filters (0.2 μm, Falcon Cat No. 3109) was also determined.

Partitioning of phosphine into cell culture medium: In order to determine the amount of 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 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 PH_3 concentrations in the headspace and aqueous phase were determined by GC/FPD. It had been found that injections of up to 10 μL water did not disturb the detector flame and it was assumed that the detector gave the same molar response to 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 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 x 10^6 cells mL^{-1} and allowed to incubate for some hours so that they were exposed to PH_3 during "log. phase" growth. Exponential growth normally continued with a doubling time of about 17 h until limiting densities of about 1×10^7 cells mL^{-1} were reached.

Exposure of cell cultures: Glass 50 mL Erlenmeyer screw-cap flasks containing 10 mL of medium were inoculated with 0.8×10^6 cells mL^{-1}. 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_3. In a typical experiment, volumes of 0, 4, 15 and 30 mL of ca 90% PH_3 were injected, giving headspace concentrations of 1,400, 5,200 and 10,400 ppm (1.9, 7.2, and 14 mg L^{-1}.)

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 $26 \pm 0.7°C$ 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_3 quickly escaped from the culture flasks, especially since they were opened for assay purposes shortly after removal from PH_3 exposure.

In a separate experiment, the ability of medium to sustain cell growth after exposure to PH_3 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_3 levels in the chambers did not decrease by more than 1% (which was within the experimental error).

The entry of PH_3 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_3 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.
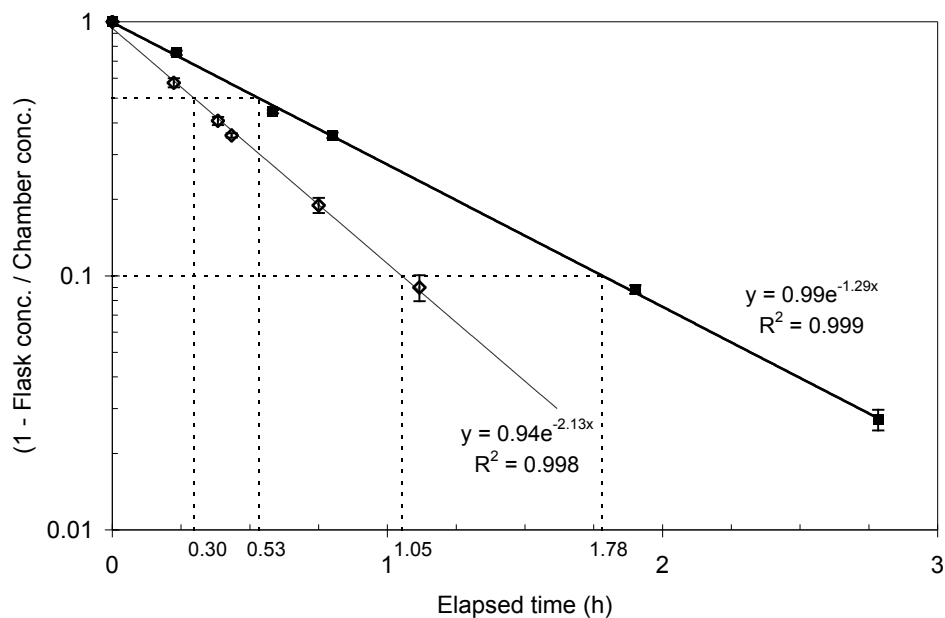

Fig. 1. Time-course of PH_3 entry into cell culture flasks placed in exposure chambers. Values expressed as the ratio of the PH_3 concentration inside the flasks to that of the exposure chamber on a logarithmic scale. ■ Flasks with solid caps. ◊ Flasks with filters in caps. Vertical lines indicate t_{50} and t_{90} for each type of cap. Vertical bars represent the ranges of the duplicate measurements.

Partitioning of PH_3 into cell culture medium

The PH_3 determinations in gas and aqueous phases are shown in Table 1. The partition coefficient ($P_k = C_{water}/C_{air}$) determinations were reproducible, and the average was 0.15.

Some of the aqueous 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 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 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$_3$ concentration (ppm)		Partition coefficient (P_k)	Mass balance (%)
	Water	Air		
0		2766		100
16	470	3041	0.15 ± 0.01	95 ± 7 [c]
40	607	3599	0.17 ± 0.01	87 ± 5
16 HiQ	[a]	3080	0.16 [b]	

Notes:
 a. Liquid concentration not determined with HiQ medium, to avoid column contamination.
b. Partition coefficient calculated by estimating dissolved PH$_3$ concentration.
c. Mass balance as proportion of amount of PH3 in waterless flasks (0% filling ratio).

It was noted that the rate of equilibration was very rapid. When PH$_3$ 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$_3$ levels were attained within 10-20 minutes.

Cell exposure and mortality assessment
After exposure to PH$_3$, 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$_3$ 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$_3$ treatment. This is borne out in Fig. 3, which plots the cell density (cells mL^{-1}) 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).
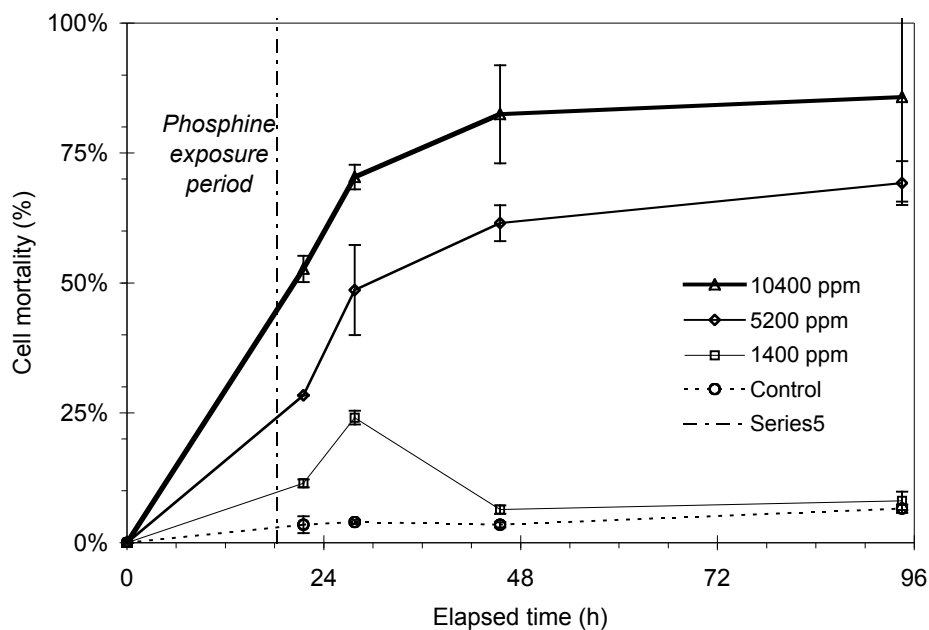
Fig. 2. Cell mortality after 18 h exposure to 3 concentrations of PH_3. Mortality was expressed as the fraction of cells stained by Trypan Blue with respect to the total number of cells counted. Mortalities rose in the first day after cessation of exposure. The apparent decline in cell mortality in the 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_3, cell growth rates and mortality levels were consistent with cells that had not been exposed to PH_3 (data not shown).

DISCUSSION

This paper shows that it is possible to use gaseous PH_3 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_3 into the cell flasks observed in this study (about 2!h) 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.
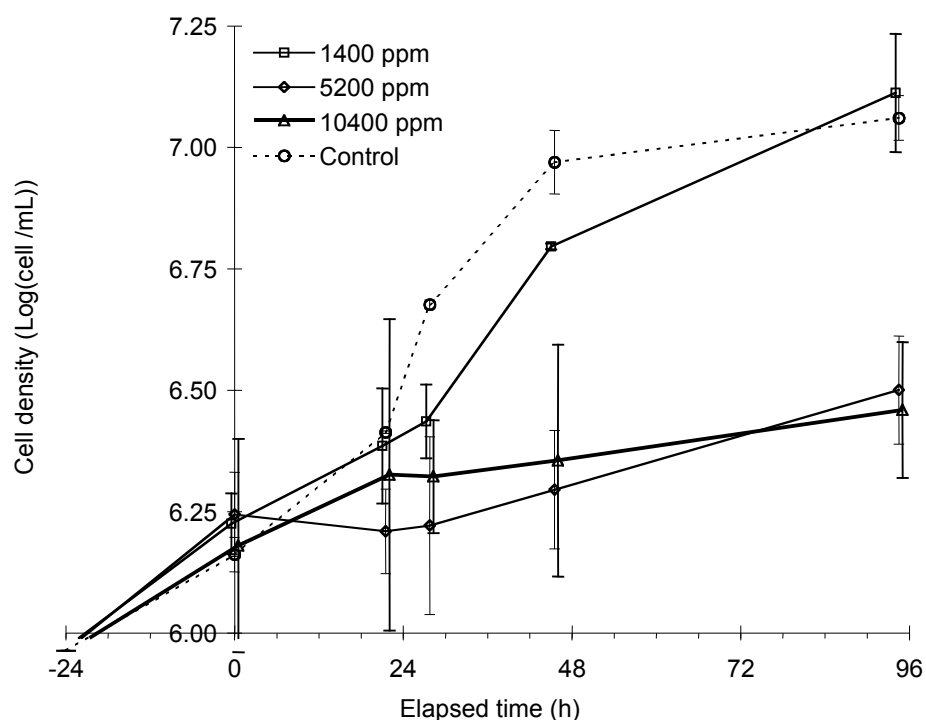
Fig. 3. Cell growth after 18 h exposure to 3 concentrations of PH_3. Exposure was from t=0 to t=18 h. Cell density is the total of live and dead cells, as determined by Trypan Blue exclusion. The exposure chamber was slightly colder than the recovery incubator, which caused the increase in control cell growth after 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 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 H_2O and PH_3 in gaseous and liquid phases. The aqueous phase only becomes saturated with 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 PH_3 with H_2O at near-neutral pH was noted by Weston (1954) and the un!reactivity of 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 PH_3-saturated buffer. Indeed, the rapid equilibration of PH_3 implies that studies which exposed mammalian cells to 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 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 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 (Mg^{2+} & 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 μM. This is comparable to the mid-range test concentration used in this report (5,200 ppm, equivalent to 32 μM), 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 N_2. This mortality would seem not to be due entirely to residual PH_3, because one would expect that almost all the dissolved 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 AlP and Mg_3P_2. Gaseous PH_3 treatment would make it possible to apply PH_3 alone and in conjunction with Mg^{2+} and Al^{3+} to cells to confirm that the ROS production observed by Hsu *et al*. (1998) were due to PH_3 rather than a combination of factors. Mammalian cell lines, with their requirements for elevated 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 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 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 PH_3's effects when performed soon after exposure.

The 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 PH_3 (and other poisons) on cells and on whole insects.

A possible explanation of the cell's high tolerance to PH_3, on a molecular level, may rest on the knowledge that reactive oxygen species (ROS), especially H_2O_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 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 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 PH_3.

Work is now progressing to use this method to measure PH_3-induced ROS production by a fluorescence assay (indirect) or by EPR (direct). Other effects, such as the influence of cell density on 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 PH_3 analogues will also be tested for toxicity and ROS production using this method.

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