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Effect of the Solvent Acetone on Membrane Integrity in the Green Alga *Chlorella pyrenoidosa*

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Organic solvents are widely employed in microbial bioassays involving water-insoluble chemicals, such as pesticides and other pollutants. Although their presence in these bioassays is unavoidable, it creates the potential for interactions between the solvent and the test chemical, since both compounds are xenobiotics. Even though the background solvent concentration is kept low, it has been clearly demonstrated that solvents, at levels well below their toxicity thresholds, can still interact with the test compound being investigated to elicit both synergistic and/or antagonistic responses (Stratton 1985). These can interfere with bioassay results by masking the inherent toxicity of the test compound (Stratton et al. 1982).

Solvent interactions have been widely documented in pesticide bioassays using microorganisms such as algae, fungi, and cyanobacteria (Stratton and Corke 1981; Stratton et al. 1980, 1982), although some data are available for other organisms as well (Dalela et al. 1979; Bowman et al. 1981). To ensure that bioassay data are accurate and not the result of solvent interference, a screening method is available to identify and minimize solvent interactions in pesticide bioassays (Stratton et al. 1982). This method has been expanded into a general procedure for studying the interactive effects of toxicant combinations on microorganisms (Stratton 1988). Such techniques have been used to investigate solvent-pesticide interactions in more detail. For example, bioassay parameters such as pH, temperature and medium composition can have a significant effect on both the magnitude of interaction and the toxicity pattern of the test pesticide (Stratton 1986a, 1986b, 1987a).

In order to understand interaction data it is necessary to determine the mechanisms responsible for solvent-pesticide interactions. One possibility is that the solvent causes some form of membrane damage which leads to either an increase or decrease in the uptake of the pesticide. The purpose of the present study was to develop a method for monitoring the effects of acetone on membrane integrity and cell leakage in the green alga Chlorella pyrenoidosa. Acetone is the most widely used solvent in microbial bioassays (Majewski et al. 1978) and is known to interact with pesticides towards C. pyrenoidosa (Stratton and Corke 1981; Stratton and Smith 1988).

MATERIALS AND METHODS

The green alga Chlorella pyrenoidosa was used as the test organism and was obtained from the National Research Council, Atlantic Research Laboratory, Halifax, Nova Scotia, Canada. Axenic stock cultures were grown in 250-mL flasks sealed with cotton bungs and containing 150 mL of an inorganic, nitrogen-free medium (Stratton 1987b) supplemented with 1.5 g of $NaNO_3$/L. Flasks were incubated at 25±1°C and a light intensity of 7000 lux on a 12 h light-dark cycle. The test solvent used was acetone: glass distilled, pesticide research grade (Caledon Laboratories, Georgetown, Ontario, Canada). All acetone concentrations are given as percent (%) volume/volume.

Acetone-induced leakage from C. pyrenoidosa was monitored by following the loss of carbon compounds from cells using radioisotope techniques. C. pyrenoidosa cells were radiolabelled photosynthetically using ^{14}C-sodium bicarbonate, as outlined below. A standard inoculum was prepared by first adding 1.0 mL of C. pyrenoidosa cells, taken from a 7-day stock culture, into 125 mL of growth medium contained in a 250-mL flask. This was incubated on an orbital shaker (Lab Line model 3520; 125 rpm) for 24 h at a temperature of 25°C and a continuous light intensity of 7000 lux. The cell concentration was then calculated by direct microscopy (Sorokin 1973) and the culture diluted to $1.0x10^6$cells/mL using sterile growth medium. The cell labelling system was prepared by aseptically mixing 125 mL of this standard cell inoculum, 1100 mL of growth medium, and 100 µL of ^{14}C-sodium bicarbonate (Amersham Corp., Oakville, Ontario, Canada; containing 3.72 MBq of radioactivity per mL; 1 MBq=27 µCi) in a sterile 4000-mL flask sealed with Parafilm. This system was incubated for 14 days on an orbital shaker (100 rpm) at a temperature of 25°C and a continuous light intensity of 7000 lux. During incubation 75 to 80% of the ^{14}C was taken up into the cells. Following incubation the cells were harvested by centrifugation. The culture was placed into sterile 250-mL centrifuge bottles in aliquots of 200 mL and centrifuged at 3600 rpm (2000 RCF), using an MSE Centaur 7 centrifuge (John's Scientific, Toronto, Ontario, Canada), for 15 min to pellet the cells. The supernatant was discarded and the pellet resuspended into 200 mL of sterile growth medium and recentrifuged. This process was repeated once more. The pellets were aseptically transferred to a sterile 2000-mL flask and brought up to a volume of 250 mL with sterile medium. The cell concentration was determined as outlined above, and the culture was diluted to $1.0x10^6$ cells/mL. The labelled cells were used immediately.

For cell leakage experiments, 10-mL aliquots of the radiolabelled cell suspension were distributed to sterile 16 x 100 mm test tubes. The cells were then treated with various acetone concentrations ranging from zero to 3.5% (final concentrations adjusted for volume), and incubated for 24, 48, or 96 h under the same growth conditions used for labelling. The test tubes were inclined on a 45° angle during incubation. Each individual experiment tested nine acetone concentrations in replicates of 10 and was repeated three times. Cell leakage was monitored by quantitating the decrease in radioactivity in the cells and the corresponding increase in radioactivity in the growth medium during the incubation period. This was done using a filtration method. The test tube contents were individually filtered through pre-wetted 0.45µm membrane filters (Millipore type HA, 25

mm diameter; Millipore Corp., Bedford, Massachusetts, U.S.A.) housed in a filter manifold (Amicon model VFM-I; Amicon Canada Ltd., Oakville, Ontario, Canada). The filtrate from each test tube was collected and a 0.5- mL aliquot transferred to a 20-mL glass liquid scintillation vial. Each filter pad was then washed with 10 mL of sterile growth medium and placed into a separate glass liquid scintillation vial. One mL of BTS-450 tissue solubilizer (Beckman Instruments Inc., Mississauga, Ontario, Canada) was added and the vials incubated at 40°C for 3 h. Following this, 0.5 mL of 30% hydrogen peroxide was added to each filter pad vial to reduce color quench. Ten mL of Ready-Solv MP liquid scintillation cocktail (Beckman Instruments Inc.), containing 0.7% glacial acetic acid to control chemiluminescence, was added to all filtrate and filter pad vials. Following a two h incubation in the dark at room temperature all vials were counted in a Beckman model 3801 liquid scintillation counter equipped with a random coincidence monitor. Counts were corrected for percentage of counting efficiency using the H-number method and reduced by the background value obtained from appropriate background controls.

From the counts obtained the relationship between acetone concentration and the leakage of radiolabelled carbon compounds from the cell could be determined. These relationships were presented graphically and equations for the lines obtained using a microcomputer-based software package (Cricket Graph, Cricket Software, Malvern, Pennsylvania, U.S.A.). EC_{50} values (concentration causing a 50% increase in leakage) were calculated from these equations. Any significant differences between treatments were determined using an analysis of variance procedure followed by a Tukey's test at P=0.05 (SAS statistics software, SAS Institute Inc., Cary, North Carolina, U.S.A.).

RESULTS AND DISCUSSION

The radiolabelling procedure used was simple and straightforward, resulting in an up to 80% uptake of the isotope during incubation. To label the cells with other isotopes would merely require appropriate additions to the growth medium and possible adjustments to the incubation period. The method employed for counting the algal cells on filter pads was based upon preliminary studies (data not shown). In these experiments maximum counts were obtained by the method used and additional steps, such as drying the filter pads or dissolving them, were unnecessary. This is probably because ^{14}C was the isotope used. For less energetic isotopes the pads may have to be dried and should be dissolved with 1.0 mL of tetrahydrofuran before using the tissue solubilizer. Under the present experimental conditions the filtration procedure was also found to be superior to a centrifugation technique.

The effect of acetone on the retention of ^{14}C-labelled compounds by C. pyrenoidosa is outlined in Figure 1. There was little difference between the counts obtained after the three incubation periods for any given concentration of acetone, and one equation fit the pooled data with a high degree of correlation (Figure 1). There were significant losses of radiolabelled compounds from the cells only at solvent levels greater than 2.0 to 2.5%. It is apparent that following the loss of label from the cells is not a sensitive enough technique, primarily because the cell counts are so high that small decreases go unnoticed. Using the filter pad data an

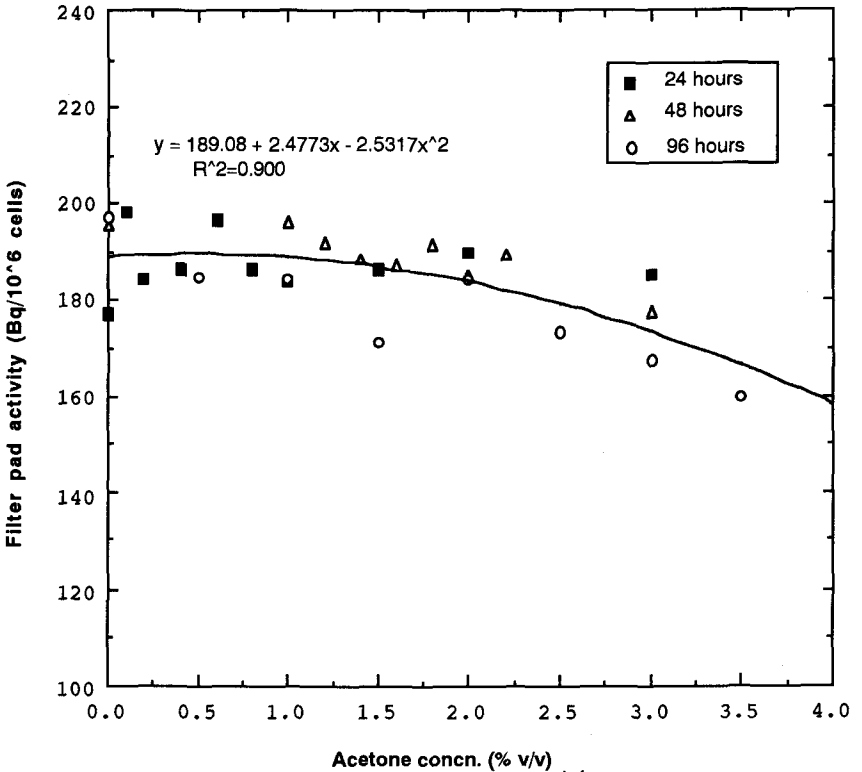

Figure 1. Effect of acetone on the retention of ^{14}C-labelled compounds by C. pyrenoidosa. The figure legend refers to the exposure time to acetone.

extrapolated EC_{50} value (that concentration causing a 50% loss of label) of around 6.5% can be calculated for acetone, which is much higher than estimates obtained using other toxicity criteria. For example, an acetone concentration of 3.33% causes severe cytological damage in C. pyrenoidosa (Parasher et al. 1978) and levels of 2.7 to 3.3% cause a 50% reduction in the growth of this alga (Stratton and Smith 1988). With blue-green algae (cyanobacteria), the EC_{50} of acetone towards growth ranges from 0.4 to 4.4% (Stratton 1987b). Therefore, monitoring membrane damage using the loss of radioactivity from cells is unsuitable.

The effect of acetone on the leakage of ^{14}C-labelled compounds from C. pyrenoidosa into the growth medium is outlined in Figure 2. This technique proved to be far more sensitive, indicating significant differences in the data obtained after all three incubation periods. As the exposure time was increased, the effect of acetone became more pronounced. After 24 h significant cell leakage occurred above 1.5% acetone, but after 48 h significant leakage was evident above 1.0% acetone. After 96 h cell leakage was significant at and above 0.5% acetone. Using the equations listed in Figure 2, EC_{50} values (that concentration causing a 50% increase in filtrate radioactivity) of approximately 3.0, 3.3, and 3.5% can be calculated for acetone after 48, 96, and 24 h exposure, respectively. The consistency of these values indicate that the actual exposure time to acetone may be relatively unimportant. The EC_{50} data are also comparable to those obtained for other toxicity criteria from

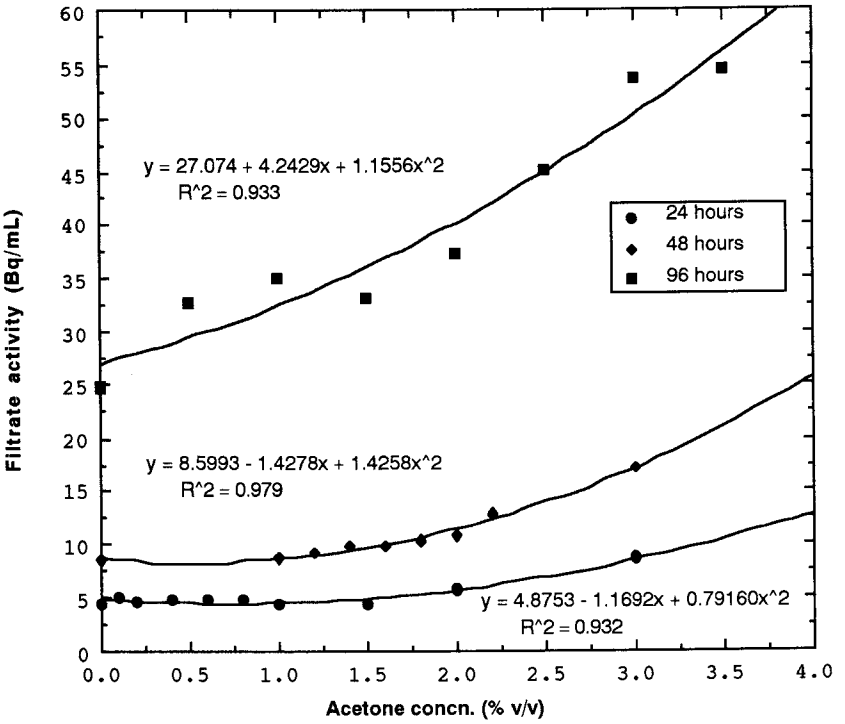

Figure 2. Effect of acetone on the leakage of ^{14}C-labelled compounds from C. pyrenoidosa. The figure legend refers to the exposure time to acetone.

previous studies on C. pyrenoidosa, as summarized above (Parasher et al. 1978; Stratton and Smith 1988). It is important that significant leakage occurred at acetone levels of 1.5% and lower (depending upon the exposure time). Acetone concentrations of 0.1 to 0.4% have notable effects on photosynthesis and nitrogen fixation in blue-green algae (Stratton et al. 1980; Stratton and Corke 1981; Yee et al. 1985), and levels must be lowered below 0.2% to have no effect on green algae such as Chlamydomonas segnis (Yee et al. 1985) and Chlamydomonas eugametos (Hess 1980). These results imply a direct relationship between acetone-induced membrane damage and effects attributed to acetone exposure. These membrane effects occurred at solvent levels normally employed in microbial bioassays. The U.S. Environmental Protection Agency recommends maximum allowable limits of 0.05% solvent for acute toxicity tests and 0.01% for chronic tests (U.S.E.P.A. 1975), but these recommendations apply to aquatic toxicity tests employing fish and macroinvertebrates. In microbial test systems solvent concentrations up to 1.0% are routinely used, due to problems associated with the use of small volumes of test compounds, toxicant solubility, and other technical limitations (Stratton 1987b). At these levels acetone could cause significant membrane damage during the usual incubation periods employed in algal bioassays.

The data presented here indicate that the leakage of radiolabelled compounds from cells of C. pyrenoidosa into the growth medium can be

used as a sensitive measure of solvent toxicity. Results from leakage studies are consistent and comparable with those obtained from other solvent toxicity experiments using toxicity criteria such as growth and photosynthesis. The method outlined here can be applied to any solvent bioassay system employing algae and, with appropriate modifications, can be used to follow the leakage of any radiolabelled compound from the cells. With C. pyrenoidosa, acetone-induced damage to membrane integrity appears to be a primary toxic effect of acetone. This could lead to alterations in the uptake of pesticides present in the same test system, thereby eliciting pronounced interaction responses. To determine whether the presence of acetone causes either a decrease or increase in pesticide uptake would require further studies. This could be accomplished by following pesticide uptake, using radiolabelled pesticides, in both the presence and absence of solvent. Such information is needed before solvent-pesticide interactions can be fully understood.

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