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Activation of Chemical Promutagens by *Selenastrum capricornutum* in the Plant Cell/Microbe Coincubation Assay

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The critical balance of organisms living in aquatic environments is influenced by the presence and relationship of plants to those environments. However, even though plants occupy a fundamental trophic level within aquatic ecosystems, few studies have focused upon the effect of xenobiotics on aquatic plants, and even fewer studies have dealt with xenobiotic metabolism by aquatic plants.

It is well established that plants can metabolize chemicals into mutagens (for a review see Gentile and Plewa 1988). In some instances the genotoxicity of the chemical in question can be demonstrated only under the conditions of plant metabolism (Plewa and Gentile 1976, 1982; Gentile and Plewa 1982, 1983). The impact of these unique plant-activated chemical mutagens on ecosystems, food chains and, ultimately, human health is an important question that will require intensive and integrative investigation.

Several techniques have been developed to study plant activation of chemicals. In vivo protocols rely upon extracts from pre-exposed intact plants and subsequent application of these extracts to a microbial genetic indicator organism (Plewa and Gentile 1976; Gentile et al 1982). Two different in vitro protocols have proven effective with several test agents. One protocol employs enzyme extracts from non-exposed plants in conjunction with a genetic indicator system (for a review see Gentile and Plewa 1988) while the other relys upon the incubation of plant cells in culture with a genetic indicator organism (for a review see Plewa and Gentile 1988). This latter assay is known as the plant cell/microbe co-incubation assay.

All of the approaches to studying plant activation have both advantages and disadvantages. However, the plant cell/microbe coincubation assay is particularly advantageous for use with unicellular algae. The conditions of this assay are such that chemical metabolism and subsequent mutagen detection can be followed in intact algal cells under simulated field conditions. The purpose of our research was to demonstrate that a unicellular algal species could be used effectively in the plant cell/microbe coincubation assay to activate model chemical mutagens.

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MATERIALS AND METHODS

We used Selenastrum capricornutum in our studies because it is common to fresh water ecosystems, it readily absorbs organic agents (Casserly et al 1983), it has been used successfully as a indicator organism for toxicity studies in aquatic systems (Ska et al 1984; Blaise et al 1986), and it has been demonstrated previously to oxidize polyaromatic hydrocarbons (Schoeny et al 1988). S. capricornutum was generously provided by Dr. C. Blaise (Quebec). Axenic stocks were maintained in a synthetic nutrient medium as defined by Miller et al (1978) at room temperature on a gyratory shaker under constant, cool-white light (300 uE $m^{-2}sec^{-1}$).

The chemicals chosen for investigation were 4-nitro-o- phenylenediamine (NOP) and benzo(a)pyrene (BaP). NOP is a direct-acting mutagen that is enhanced in activity in the presence of plant peroxidase enzymes (Gentile et al 1985). BaP is a mammalian and plant promutagen that is activated by cytochrome P-450-dependent monooxygenases (Higashi 1988). BaP was purchased from Sigma Chemical Co., Missouri, and NOP was purchased from Aldrich Chemical Co., Wisconsin. All other chemicals were purchased from Sigma Chemical Co., while bacteriological media was purchased from Hazelton Laboratories, Kansas.

Salmonella typhimurium strain TA98 was obtained from Dr. B. N. Ames (Berkley) and maintained in our laboratory according to the protocols outlined by Maron and Ames (1983).

For a typical experiment, algal cells were grown to either mid-log or stationary-phase and harvested by centrifugation (10 min at 1500 xg). The supernatant fluid was removed and the cells were resuspended and washed 3x in 100 mM potassium phosphate buffer (pH 7.0). Following the final wash, cells were resuspended in buffer to a concentration of 3.0×10^8 cells/mL.

Bacterial cells were grown from a single colony isolate. Cells were grown aerobically in 100 mL nutrient broth at 37 C. The bacterial suspension was centrifuged; the bacterial pellet was resuspended and washed 3x in 100 mM potassium phosphate buffer and the pellet harvested following the final wash. The bacterial pellet was resuspended in buffer to a titer of 2×10^9 cells/mL and iced for no greater than 1 h prior to use.

For a coincubation experiment algal cells (3.0 mL), bacterial cells (0.5 mL) and test agent (50 uL solvent) were combined in a reaction vessel and incubated in a gyratory shaker (200 rpm) at 28 C for desired periods of time. Negative controls consisted of algal and bacterial cells without the test agent, solvent alone, and the test agent added to heat-killed algal cells coincubated with viable bacteria. Positive controls consisted of NOP alone or BaP incubated with Arochlor 1254-induced rat hepatic homogenates (Hazelton Laboratories, Maryland). After treatment time, the reaction tubes were placed on ice to quench the reaction (Plewa et al 1988). Triplicate 0.5 mL aliquots of the reaction mixture were removed and added to 2 mL of molten top agar supplemented with histidine and biotin. The

top agar was poured onto minimal medium plates and incubated for 48 h at 37 C. Salmonella his^+ revertants were scored using a New Brunswick Biotran III Automated Colony Counter (New Jersey). All experiments were conducted a minimum of three times.

RESULTS AND DISCUSSION

The activation of NOP by Selenastrum is demonstrated in Figure 1. NOP activation was enhanced with increasing concentrations of algal cells (Figure 2). While the maximum concentration of cells used in our study was 3×10^8/mL, one could anticipate more activation using even greater cell concentrations. Coincubation reactions were conducted for varying periods of time in an effort to establish an optimal time-frame for the reaction (Figure 3). Near optimal activation was obtained using reaction times of either 120 or 180 min. A 120-min reaction time was established as standard since a longer reaction time could allow for incipient bacterial growth in the reaction tube under some assay conditions.

The growth stage of the algae used in coincubation studies was evaluated. Selenastrum cells, like other cells in culture, exhibit a typical growth response with a lag-, log-, and stationary-phase of growth. We had previously established that plant cells in culture differentially metabolize selected promutagens relative to the stage of the growth cycle at which the plant cells are harvested (Gentile et al 1986, 1987). Selenastrum cells were harvested at different stages of their growth curve and used in the coincubation assay. Results from these experiments are presented in Figure 4. Nine-day-old Selenastrum cultures were most efficient at activating NOP into its mutagenic form. Nine-day-old cultures are, under conditions within our laboratory, in a stationary-phase of growth. These results with Selenastrum and NOP are similar to those reported previously with other plant cell lines (Gentile et al 1987). NOP is activated by plant-mediated perodactic oxidation (Gentile et al 1985). Plant peroxidases are stable and accumulative within cells and within cell cultures (Dohn and Krieger 1981). Thus, late-log-phase and stationary-phase cell cultures contain larger amounts of peroxidases than early or mid-log-phase cell cultures, a fact that may account for the increased competency of stationary-phase Selenastrum cultures to activate NOP.

BaP was not activated by Selenastrum using the assay conditions identified for NOP. In an effort to enhance the ability of Selenastrum cells to activate BaP, we used the maximum number of algal cells amenable to our assay (3.0×10^8 cells/mL), and we modified reaction conditions to allow for increased incubation time between algae and BaP. In these experiments we incubated BaP and algae together in the absence of bacterial cells for 48 h. At 12-h intervals throughout the incubation period, we harvested aliquots of the reaction mixture and added these materials to S. typhimurium cells in a fresh reaction tube. The new reaction mixture (algal cells, chemical and bacteria) was allowed to coincubate for 1 h and then bacterial cells were plated and scored as previously described. A pre-incubation period of 48 h proved best for BaP activation (Figure 5) with mid-log-phase cells. Using this protocol we

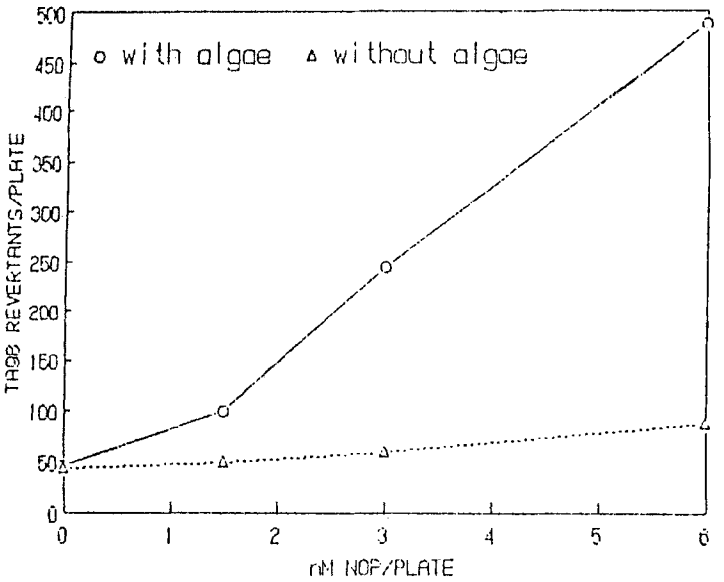

Figure 1. The activation of NOP by Selenastrum.

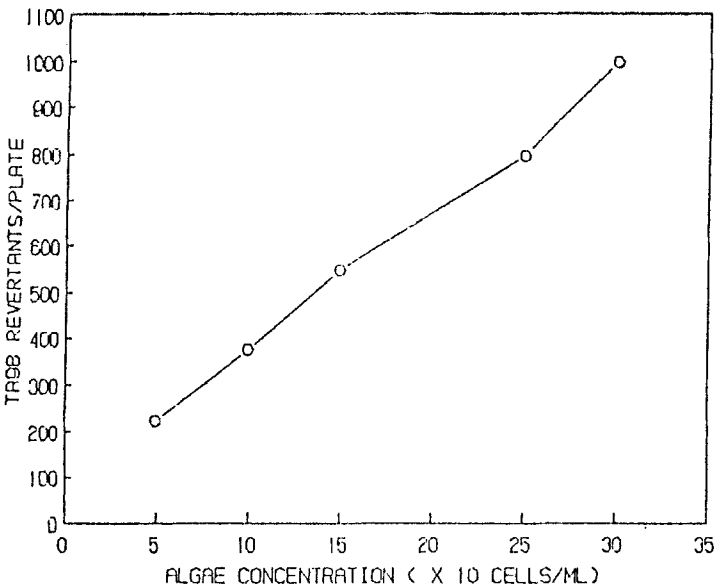

Figure 2. Effect of Selenastrum cell concentration on NOP (6 nm/plate) activation.

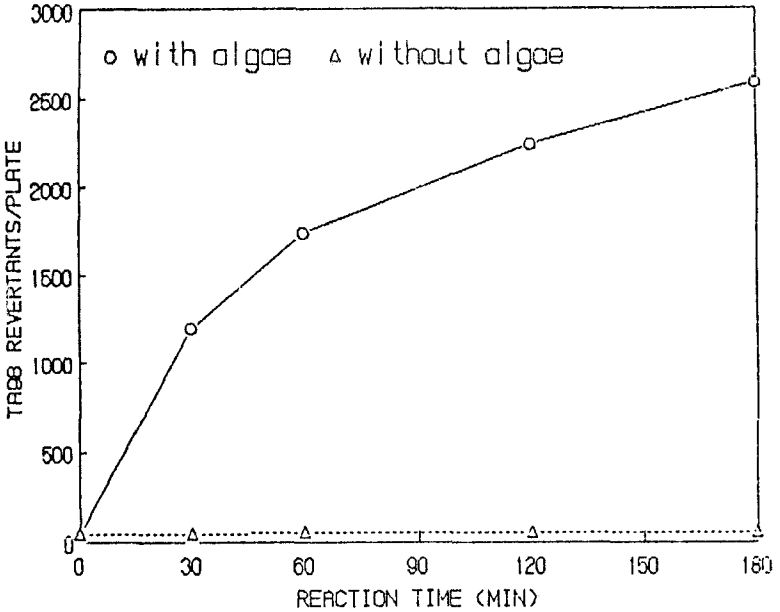

Figure 3. Effect of coincubation time on NOP (6 nm/plate) activation by Selenastrum.

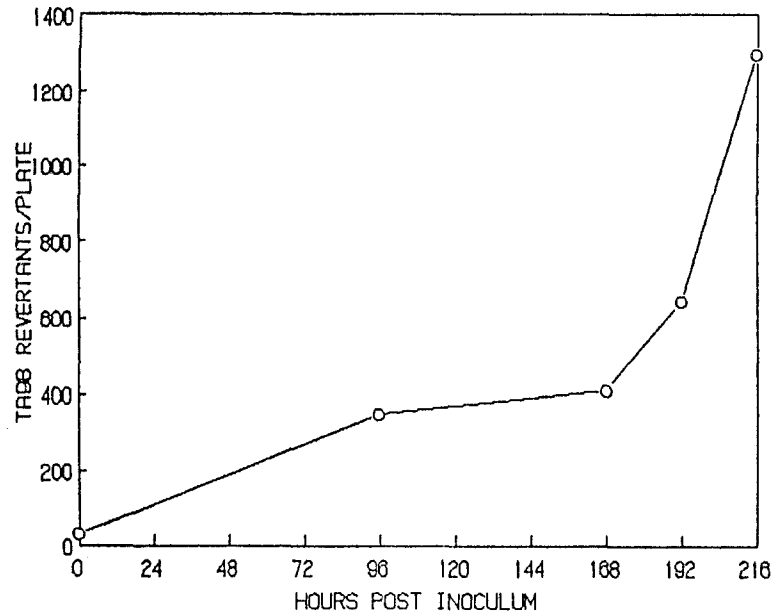

Figure 4. Activation of NOP (6 nm/plate) by Selenastrum cells harvested from different stages of their growth curve.

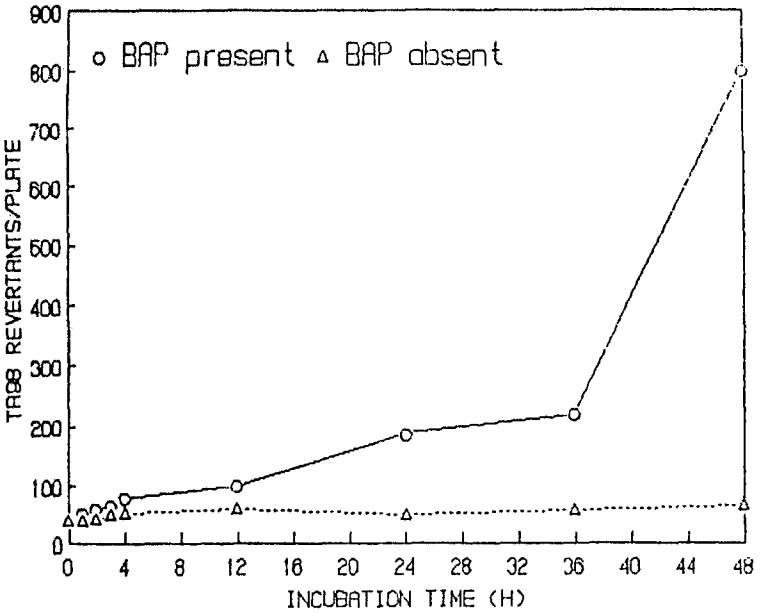

Figure 5. Effect of extended preincubation time between BaP (20 ug/plate) and Selenastrum cells on the ability of Selenastrum to activate BaP into a mutagen.

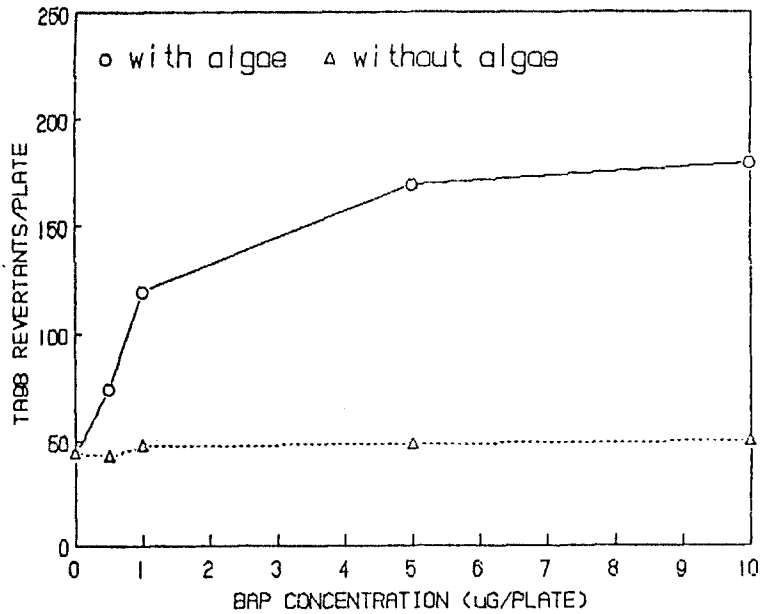

Figure 6. Dose-dependent response for BaP activation by Selenastrum using a 48-h preincubation period followed by a 1-h coincubation period with Salmonella.

generated a reproducible dose-dependent response curve for BaP (Figure 6). These data indicating the need for prolonged algal cell exposure to BaP for BaP activation are consistent with the observation of Schoeny et al (1988) who found that a minimum of 24 h was necessary before algal cells metabolized BaP into an oxidized form. In addition, the fact that mid-log-phase algal cultures were most efficient at BaP activation correlates well with the observation that cytochrome-P-450-dependent monooxygenases, the enzyme complex associated with BaP metabolism, express their highest activity in log-phase cell cultures (Higashi 1988).

Public concern about the degradation of surface and groundwater quality has stimulated efforts to develop reliable methods for quantifying aquatic toxicity. The presence of genotoxic agents in many aquatic environments has been established by chemical analysis of water and sediment extracts and by genetic bioassays. Although most freshwater systems may contain low levels of genotoxic agents, contamination from industrial, municipal, and agricultural wastes can dramatically increase these levels (Sloof and Van Kreij 1983).

Most aquatic genotoxicity assays are still in the developmental stage and have been tested with a relatively few compounds. Nevertheless, there is good evidence that exposure of aquatic animals to chemical mutagens has a deleterious effect (Harshbarger et al 1984). In some instances, the observed effects have been linked to exposures to specific classes of compounds including polyaromatic hydrocarbons and aromatic amines. Many fewer studies have been conducted on the effect of mutagenic agents on aquatic plants. The metabolism of chemical mutagens by aquatic plants and the subsequent effect of these plant-derived genotoxic metabolites on other organisms is a virtually unexplored area of study.

The coincubation assay with Selenastrum offers a powerful tool with which to integrate plant activation studies into an overall assessment of the impact of mutagens in aquatic systems. Now that we have demonstrated the effectiveness of this assay with Selenastrum, we will direct out attention towards using a spectrum of physiologically-different Selenastrum cultures to activate other model promutagens. This will allow us to define a comparative data base for assessing the activation potential of Selenastrum with undefined environmental pollutants.

Conceivably, waters contaminated with genotoxins and concomitantly having a dense algal bloom could produce significant quantities of previously undefined mutagens. The Selenastrum/microbe coincubation assay offers the opportunity to investigate this concern. It can be used for the rapid screening of chemicals or complex mixtures as well as for more in-depth analysis of specific metabolic reactions.

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