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Plant activation is the process by which promutagenic agents are activated into mutagens by plant systems. With the widespread use of agricultural chemicals on crop plants and with the global exposure of plants to pollutants, the possibility exists that plant-activated agents may be introduced into the human food chain. The plant cell/microbe coincubation assay uses cultured plant cell suspensions as the activating system and bacteria or yeast cells as the genetic indicator organism. After a treatment time, the microbes are plated on selective medium. In this way the activation system and the genetic system can be independently studied. In addition the viability of the plant cells and the microbial cells can be independently determined so that the toxicity of a test agent can be evaluated. Using cytochrome P-450 monooxygenase, and peroxidase inhibitors we are studying the biochemical mechanisms of plant activation of environmental contaminants especially aromatic amines. We developed a model of the TX1-cell activation of aromatic amines. The model integrates our data into a mechanistic framework and serves as a foundation for new experimental designs. The model has seven components. They are, (1) the aromatic amine ($R-NH_2$) is transported into the plant (TX1) cell, (2) TX1 intracellular peroxidase oxidizes the molecule (R-NHOH), (3) the metabolite is conjugated to a macromolecule (R-NHOH-conjugate), (4) the amine-conjugate is secreted into the extracellular medium, (5) the conjugate is absorbed by the bacterial tester strain (TA98), (6) the molecule may be deconjugated and is acetylated ($R-NHO-COCH_3$) and deacetylated by the bacterial acetyl-Co A: *N*-hydroxyarylamine *O*-acetyltransferase, and (7) the deacetylation results in a highly reactive nitrenium ion ($R-NH^+$).

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THE MECHANISMS AND EFFECTS OF THE PLANT ACTIVATION OF CHEMICALS IN THE ENVIRONMENT

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1 OBJECTIVES FOR AFOSR RESEARCH PROJECT Nº AFOSR-88-0336

The original objectives of this project were:

- To compare the plant activation of specific mono- and polycyclic aromatic amines by cultured plant cells and fresh water algae.
- To investigate the biochemical mechanisms of plant activation by the use of specific enzyme inhibitors.
- To analyze the rates of mutagenic product formation.
- To determine if specific inhibitors that constrain the activation of the substrates function by competitive or noncompetitive inhibition.
- To analyze any synergistic effects on plant activation induced by increasing peroxidases by the widely-used herbicide, atrazine.

2 STATUS OF RESEARCH EFFORTS

The results of the research for each objective is presented under the heading that identifies the specific objective.

2.1 Comparison of the Plant Activation of Monocyclic and Polycyclic Aromatic Amines Using Tobacco and Algae Cell Cultures

2.1.1 Plant Cell/Microbe Coincubation Assay

The assay is based on employing living plant cells in suspension culture as the activating system and specific microbial strains as the genetic indicator organism (Plewa et al., 1983). The plant and microbial cells are coincubated together in a suitable medium with a promutagen. The activation of the promutagen is detected by plating the microbe on selective media; the viability of the plant and microbial cells may be monitored as well as other components of the assay (Plewa et al., 1988) (Figure 1). Long-term plant cell suspension cultures of tobacco (*Nicotiana tabacum*), cell line TX1 were maintained in MX medium, a modified liquid culture medium of Murashige and Skoog (1962). *Salmonella typhimurium* strain TA98 was the genetic indicator organism used (Maron and Ames, 1983). A TX1 cell culture was grown at 28°C to early stationary phase, and the cells were washed and suspended in MX^- medium. MX^- medium lacks plant growth hormone. The fresh weight of the plant cells was adjusted to 100 mg/ml, and the

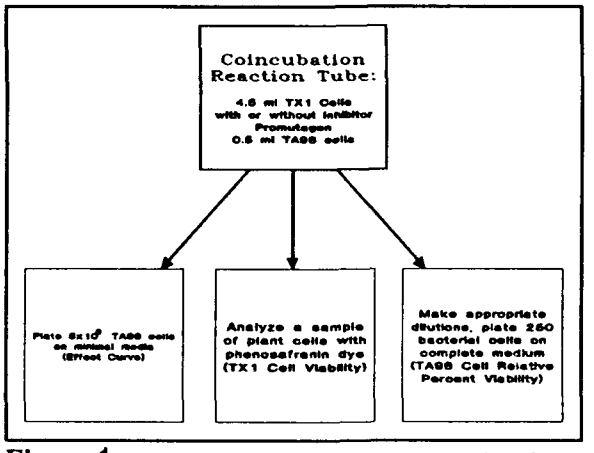

Figure 1 Plating schedule and the analysis of the outputs from a reaction tube.

culture was stored on ice (≤30 min) until used. An overnight culture of *S. typhimurium* was grown from a single colony isolate in 100 ml of Luria broth (LB) at 37°C with shaking. The bacterial suspension was centrifuged and washed in 100 mM potassium phosphate buffer, pH 7.4. The titer of the suspension was determined spectrophotometrically at 660 nm and adjusted to 1×10^{10} cells/ml, and the culture was placed on ice. In the coincubation assay, each reaction mixture consisted of 4.5 ml of the plant cell suspension in MX^- medium, 0.5 ml of the bacterial suspension (5×10^9 cells), and a known amount of the promutagen in ≤25 μl dimethylsulfoxide. Concurrent negative controls consisted of plant and bacterial cells alone, heat-killed plant cells plus bacteria and the promutagen, and both buffer and solvent controls. These components were incubated at 28°C for 1 h with shaking at 150 rpm. After the treatment time, the reaction tubes were placed on ice. Triplicate 0.5 ml aliquots ($\sim 5 \times 10^8$ bacteria) were removed and added to molten top agar supplemented with 550 μM histidine and biotin. The top agar was poured onto Vogel Bonner (VB) minimal medium plates, incubated for 48 h at 37°C, and revertant *his*$^+$ colonies were scored. The remainder of the reaction mixture was used to determine the viability of the plant and bacterial cells. One volume of cold 250 mM sodium citrate buffer, pH 7, was added to each reaction tube which was then placed on ice. 0.5 ml of this suspension was removed and mixed with 2 ml of MX^- medium. The viability of the TX1 cells was immediately determined using the phenosafranin dye exclusion method (Widholm, 1972). The viability of the bacterial cells was determined by adding 1 ml of the cold reaction mixture to 1 ml of cold 100 mM phosphate buffer, pH 7.4. A dilution series using phosphate buffer was conducted so that approximately 300 to 500 cells were added to each of three molten LB top agar tubes and poured upon LB plates (Figure 1). After incubation at 37°C for 24 to 36 h, the bacterial colonies were counted.

2.1.2 The Effect of Toxicity on the Plant Cell/Microbe Coincubation Assay

In the plant cell/microbe coincubation assay, it is essential to monitor the viability of the plant and microbial cells. Our hypothesis was that any agent that was toxic to the cells would cause a reduction of TA98 revertants by killing the activating system or the genetic indicator organism. We tested this hypothesis by preparing a series of reaction tubes in which the TX1-cell populations were composed of a series of different ratios of live to heat-killed TX1 cells. These cells were exposed to 500 μM *m*-PDA and coincubated with 5×10^9 TA98 cells in a total volume of 5 ml. After 1 h at 28°C while shaking, 500 μl of the mixture was added to supplemented top agar and poured onto VB minimal plates. The data indicated a direct linear relationship ($r = 0.99$) of the amount of TX1-cell activation of *m*-PDA and the percent viable TX1 cells in the reaction tube. Thus the coincubation assay is highly sensitive to toxicity in the cultured plant cells. Any test agent, enzyme inhibitor, or presumptive antimutagen that reduced the viability of the plant cells will cause a reduced yield in the number of microbial mutants per plate (Plewa, 1991).

A similar experiment was conducted to evaluate the effect of varying the number of TA98 cells in reaction tubes that contained the normal titer of TX1 cells and 500 μM *m*-PDA. The number of TA98 cells varied from 5×10^9 to 1×10^6 per reaction tube. This reduction of TA98 cells was an attempt to mimic the effect of bacterial cell killing on the number of revertants per plate after 1 h of coincubation. The data demonstrated that only after a reduction from 5×10^8 to 5×10^7 cells plated did a reduction in the resulting number of revertants per plate occur. This phenomenon is due to the number of induced and spontaneous plate mutants per round of cell division that arise after plating in supplemented top agar. Thus the assay is much less sensitive to agents which exert toxic effects only on the bacteria (Plewa, 1991).

2.1.3 Activation of Aromatic Amine Promutagens by *N. tabacum* Cells

In our research we used the polycyclic and monocyclic aromatic amines, 2-AF (CAS № 153-78-6) and *m*-PDA (CAS № 108-45-2), respectively, as model promutagens. 2-AF is a well characterized mammalian promutagen. The first stage in the mammalian activation of 2-AF is a *N*-hydroxylation reaction that is principally dependent upon the cytochrome P-450 enzyme system that functions as the terminal monooxygenase (Lotlikar and Zaleski, 1975; Nagata et al., 1985). 2-AF is also activated by several plant species (Plewa et al., 1988). *m*-PDA is an aniline derivative and is a promutagen in TA98 which is activated by rodent S9 (Ames et al., 1972; Shahin et al., 1983). *m*-PDA is mutagenic in mouse L5178Y lymphoma cells (Palmer et al., 1977) and is a plant promutagen (Lhotka et al., 1987). Concentration-response curves for the TX1 cell activation of 2-AF (●) and *m*-PDA (■) are presented in Figure 2. The negative controls (○,□) consisted of reaction tubes that contained the promutagen in MX^- medium plus TA98 without plant cells. 2-AF is a more potent promutagen than *m*-PDA. As little as 25 nmol 2-AF/reaction tube caused a significant increase in mutant TA98 cells. Thus, the plant/cell microbe coincubation assay is a sensitive method to screen for plant-dependent promutagens.

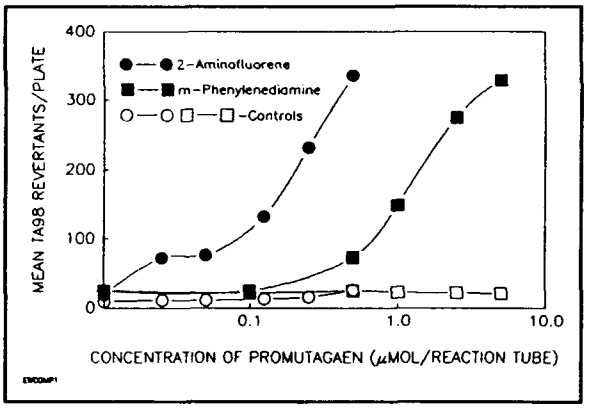

Figure 2 The activation of 2-AF and *m*-PDA by TX1 cells.

2.1.4 Activation of Aromatic Amine Promutagens by *Selenastrum capricornutum* Cells

Experiments were conducted to determine if *S. capricornutum* could activate 2-AF or *m*-PDA into a mutagen using *his*$^+$ reversion in *S. typhimurium* strain TA98 as the genetic endpoint. A series of reaction tubes were constructed containing either 4.5 ml of MP medium or 4.5 ml of the algal cell suspension. Concentrations of 2-AF ranging from 0-0.5 μmol/reaction tube in no more than 50 μl dimethylsulfoxide and concentrations of *m*-PDA ranging from 0-10 μmol/reaction tube in no more than 100 μl dimethylsulfoxide were introduced. Concurrent controls contained either potassium phosphate buffer, MP medium, algal cells with no 2-AF or *m*-PDA, algal cells with the highest concentration of DMSO with no promutagen, or heat-killed algal cells with 0.5 μmol 2-AF or 10 μmol *m*-PDA. The reaction tubes were then incubated and treated as described in §2.1.1 except the algae and 2-AF or *m*-PDA were pre-incubated for 48 hr at 20°C with shaking in gold light prior to the plant cell/microbe coincubation assay. In our experiments *S. capricornutum* did not activate *m*-PDA at concentrations similar to those used for the TX1 studies, although 2-AF was weakly activated. Both agents appeared to be non-toxic at the highest concentrations, based on microscopic observation of the algal cells after exposure to the chemicals. Under the conditions tested, it was concluded that the enzyme system(s) responsible for the activation of these chemicals in tobacco cells is either at low concentrations or inactive in the algal cells (Smith et al., 1989).

2.2 The Investigation of the Biochemical Mechanisms of Plant Activation by the Use of Specific Enzyme Inhibitors

We studied seven inhibitors for their ability to affect the plant activation of 2-AF or *m*-PDA (Table 1). We also determined if each inhibitor was a direct-acting mutagen or a plant-activated promutagen (Plewa et al., 1988; Wagner et al., 1989; 1990). The endpoint of viability was included in the experimental design to investigate if the inhibitor, alone or in combination with TX1 cells and/or the promutagen, was toxic to the activating system or to the TA98 cells (Figure 1). Viability is crucial for data interpretation; a typical inhibition curve with decreasing numbers of TA98 revertants with increasing "inhibitor" concentrations could be due to toxicity in the plant cells, toxicity in the bacterial cells, toxicity in both cell types or a true inhibition of plant cell activation. Thus, with viability as an endpoint, significant alteration of the reversion frequency of TA98 could be interpreted as a true amendment of TX1 cell activation, toxicity due to the inhibitor alone, or a toxic synergistic effect of the TX1 cells, inhibitor and promutagen (Plewa, 1991). The resolution of the plant cell/microbe coincubation assay is sufficiently high that the effect of μM to mM concentrations of specific inhibitors was easily detected.

Table 1. Inhibitors Used to Study the Mechanisms of the Plant-Activation of Aromatic Amine Promutagens.

Inhibitor	Action/Effect on Plant Activation	Reference
Acetaminophen	• A co-substrate for horseradish peroxidase and prostaglandin H synthase. Also can be metabolized by cytochrome P-450 isozymes.	Nelson et al., 1981; Harvison et al., 1988; Raucy et al., 1989
	• Concentrations above 2.5 mM or 10 mM inhibited the TX1 cell activation of *m*-PDA or 2-AF by TX1 cells, respectively.	Wagner et al., 1989; 1990
7,8-Benzoflavone	• Specific cytochrome P-448 inhibitor. Inhibited 2-aminofluorene-*N*-hydroxylase.	Ullrich et al., 1973; Razzouk et al., 1980
	• Did not inhibit the TX1 cell activation of *m*-PDA, however, low concentrations (10–250 μM) inhibited the plant activation of 2-AF.	Wagner et al., 1989; 1990
(+)-Catechin	• Antimutagen and antioxidant, and may function by binding to mutagenic metabolites and scavenging free radicals.	Conn, 1981; Nagabhushan and Bhide, 1988; Nagabhushan et al., 1988
	• Inhibited the TX1 cell activation of *m*-PDA at concentrations above 1 mM. In a concentration range from 25 μM–2.5 mM it enhanced the plant activation of 2-AF, but caused inhibition at higher concentrations.	Wagner et al., 1989; 1990
Diethyldithiocarbamate	• Metal chelator and reduced the concentration of cytochrome P-450 in mammals. Specific inhibitor of plant peroxidases.	Jensen et al., 1981; Hunter and Neal, 1975; Plewa et al., 1991
	• At concentrations above 75 μM inhibited the TX1 cell activation of *m*-PDA and 2-AF due to the inhibition of TX1 cell peroxidase.	Wagner et al., 1989; 1990; Plewa et al., 1991

Table 1. Inhibitors Used to Study the Mechanisms of the Plant-Activation of Aromatic Amine Promutagens.

Inhibitor	Action/Effect on Plant Activation	Reference
Methimazole	• A high-affinity flavin containing monooxygenase substrate. • Inhibited the TX1 cell activation of *m*-PDA but enhanced the plant activation of 2-AF.	Poulsen et al., 1974; Frederick et al., 1982 Wagner et al., 1989; 1990
Metyrapone	• Specific inhibitor of cytochrome P-450 in mammals and yeast. Inhibited 2-AF hydroxylase. • Did not inhibit the TX1 cell activation of *m*-PDA or 2-AF.	Goujon et al., 1972; Carratore et al., 1986; Razzouk et al., 1980 Wagner et al., 1989; 1990
Potassium cyanide	• Inhibited horseradish peroxidase and peroxidase-type monooxygenase activity. • Inhibited the TX1 cell activation of *m*-PDA but was refractory the activation of 2-AF.	Wise et al., 1983; Dennis and Kennedy, 1986 Wagner et al., 1989; 1990

2.2.1 Experiments With Diethyldithiocarbamate

Diethyldithiocarbamate (DEDTC, 25 μM-50 mM) was introduced into reaction tubes with plant cells, bacterial cells and 500 μM *m*-PDA (Figure 3). The inhibition of revertant TA98 colonies was a function of increased DEDTC concentration with 50% inhibition between 750 μM and 1 mM (●). At a concentration of 250 μM DEDTC a significant inhibition of the plant activation of *m*-PDA was noted. No decrease in the relative viability of the TX1 (□) or TA98 (△) cells attended the inhibition curve (Figure 3). In separate studies, DEDTC was titrated (50 μM-50 mM) in coincubation reaction tubes with plant cells, bacterial cells and a constant amount of 50 μM 2-AF (Figure 3). The inhibition of revertant TA98 colonies was a function of increased DEDTC concentration with 50% inhibition between 750 μM and 1 mM. At the lowest concentration of DEDTC (50 μM) a significant inhibition of 2-AF activation was noted (●). No consistent decrease in the relative viability of the TX1 (□) or TA98 (△) cells attended the inhibition curve. DEDTC was not mutagenic (○).

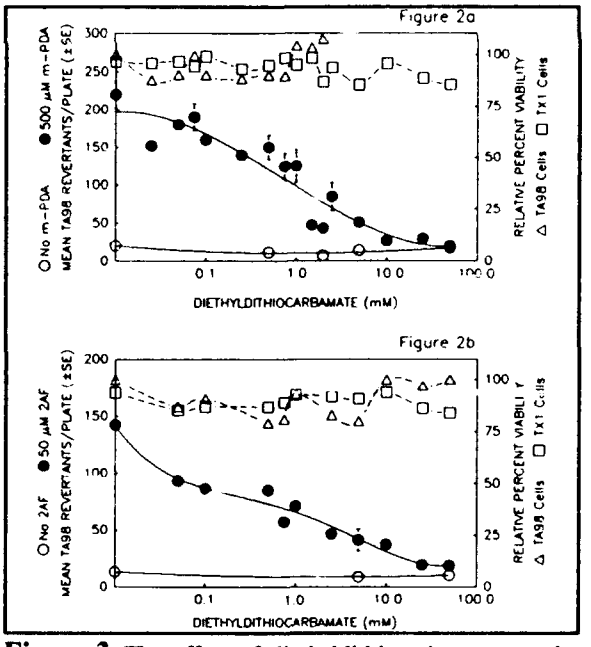

Figure 3 The effect of diethyldithiocarbamate on the TX1-cell activation of *m*-PDA and 2-AF.

2.2.2 Experiments With Metyrapone

Metyrapone is a specific cytochrome P-450 inhibitor in mammals and a weak inhibitor of 2-AF *N*-hydroxylase. Metyrapone at concentrations below 7.5 mM did not significantly inhibit the

activation of *m*-PDA (Figure 4). The activation of *m*-PDA was diminished by approximately 50% by 15 mM metyrapone (●). At metyrapone concentrations above 1 mM, toxicity to TX1 cells was expressed (□). The reduction of the activation of *m*-PDA by TX1 cells appears to be a function of metyrapone toxicity in the plant cells. Metyrapone at concentrations below 7.5 mM did not significantly inhibit the activation of 2-AF (Figure 4). The activation of 2-AF was diminished by approximately 50% by 15 mM metyrapone (●). However, at this concentration, toxicity was beginning to be expressed in the TX1 cells (□). The reduction of the activation of 2-AF appears to be due to toxicity in the plant cells. These data illustrate the necessity of monitoring viability when studying the inhibition of activation or mutagenesis. Metyrapone with no 2-AF at concentrations above 10 mM was not toxic to TA98 (△) or TX1 (□), and it was not mutagenic (○). These data indicate that metyrapone and 2-AF may interact synergistically to produce a toxin or that metyrapone may inhibit a step in the TX1 metabolism of 2-AF that results in a phytotoxic, nonmutagenic intermediate.

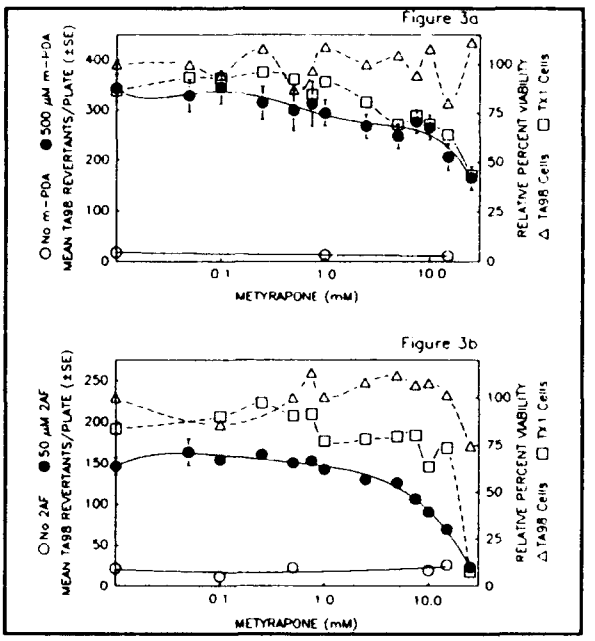

Figure 4 The effect of metyrapone on the TX1-cell activation of *m*-PDA and 2-AF.

2.2.3 Experiments With 7,8-Benzoflavone

The effect of 7,8-benzoflavone (BF) on the plant activation of *m*-PDA was investigated (Figure 5). There was no decrease in the number of TA98 revertants over the entire concentration range of 1 μM to 1.5 mM (●). BF is not an inhibitor of *m*-PDA activation by TX1 cells. The capacity of 7,8-benzoflavone (BF) to inhibit the activation of 2-AF was evaluated over a concentration range from 1 μM-2 mM (Figure 5). At concentrations above 1 μM, BF significantly reduced the mean number of TA98 revertants per plate (●). BF was not a direct acting mutagen and was not activated by plant cells (○). The inhibition curve exhibited a concentration-dependent decrease with increasing BF concentration. The viability of the TX1 (□) and TA98 (△) cells was not affected at concentrations below 750 μM. However, above this concentration the inhibitor was toxic to both cell types. An inhibition of TA98 reversion was observed without concomitant cellular toxicity from 10-250 μM BF. Thus BF at μM concentrations is an effective inhibitor of 2-AF activation by TX1 cells.

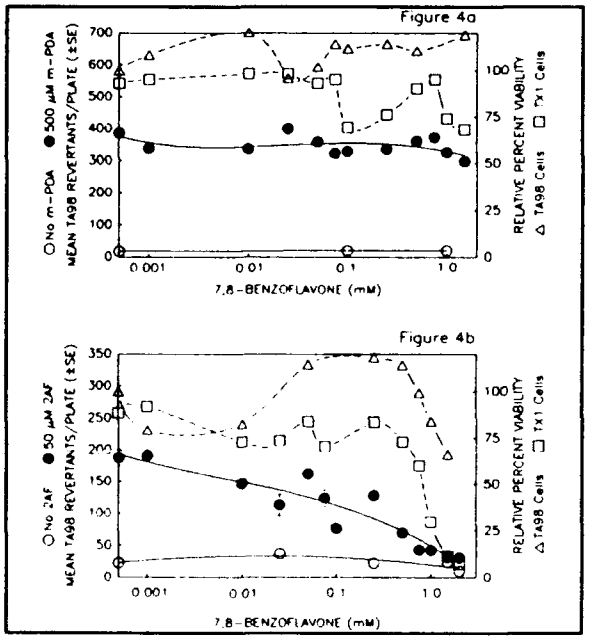

Figure 5 The effect of 7,8-benzoflavone on the TX1-cell activation of *m*-PDA and 2-AF.

2.2.4 Experiments With Potassium Cyanide

Potassium cyanide (KCN) is an inhibitor of plant peroxidases. The effect of 5 μM-5 mM KCN was studied in the activation of *m*-PDA (Figure 6). A significant inhibition of mutation induction was observed at KCN concentrations above 750 μM (●). There was not a corresponding decrease in viability in either cell type (□, △). Thus KCN inhibited *m*-PDA activation at nontoxic concentrations. In the experiments with 2-AF, KCN was toxic to the TX1 cells at concentrations above 1 mM (Figure 6, □). However, a significant enhancement in the activation of 2-AF was observed in some reaction tubes from 100 μM to 1 mM (●); within this concentration range, TX1 (□) and TA98 (△) viability was not affected. Prior to its toxic effects, KCN may be enhancing the plant activation of 2-AF. However, the great variability of the data and the broad range of cellular effects induced by KCN preclude a more detailed analysis with this agent.

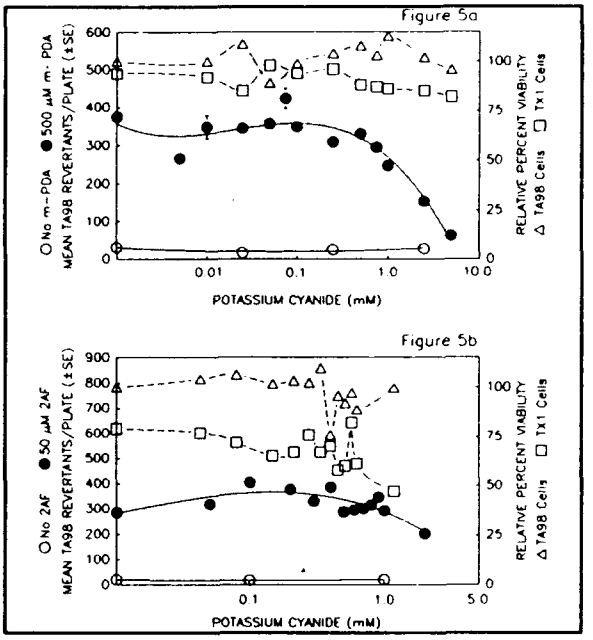

Figure 6 The effect of potassium cyanide on the TX1-cell activation of *m*-PDA and 2-AF.

2.2.5 Experiments With (+)-Catechin

(+)-Catechin was titrated against TX1 and TA98 cells with 500 μM *m*-PDA (Figure 7). There was no effect on the mean number of TA98 revertants at concentrations below 2.5 mM (●). From 2.5 mM -25 mM there was a concentration-dependent reduction in the number of revertants. (+)-Catechin concentrations above 10 mM were toxic to TX1 (□) and TA98 (△) cells. From 1-10 mM, (+)-catechin inhibited the plant activation of *m*-PDA without any toxicity. Concentrations of (+)-catechin from 25 μM-25 mM were titrated against TX1 and TA98 cells with 2-AF (Figure 7). From 25 μM-2.5 mM, (+)-catechin significantly enhanced the plant activation of 2-AF into a mutagen (○). The positive control (2-AF only) had 219.8 TA98 revertants per plate. The highest enhancement was induced with 750 μM (+)-catechin resulting in 408.3 TA98 revertants per plate. (+)-Catechin was not a direct-acting or a plant-activated mutagen and it was not toxic to the bacterial cells (△). The only toxicity to the plant cells was observed at 25 mM (□). (+)-Catechin concentrations above 5 mM significantly inhibited the activation of 2-AF. Monooxygenase enzymes that hydroxylate the ring carbons of 2-AF render the agent non-mutagenic ^t

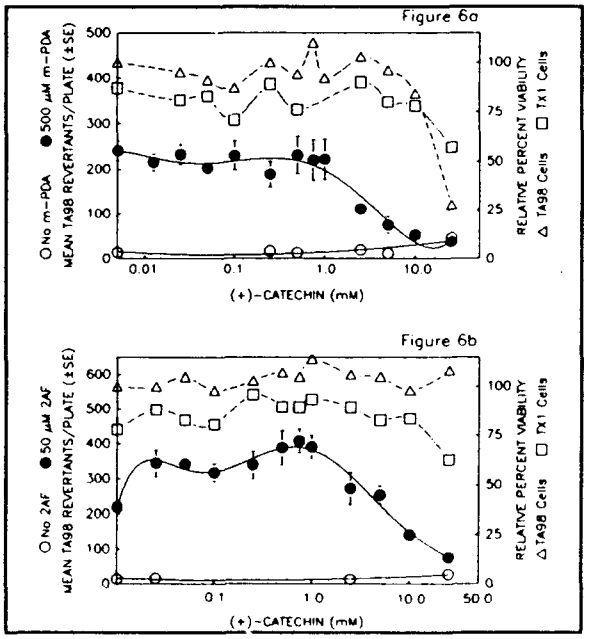

Figure 7 The effect of (+)-catechin on the TX1-cell activation of *m*-PDA and 2-AF.

low concentrations of (+)-catechin, *C*-hydroxylation might be preferentially inhibited resulting in an enhancement in the TA98 reversion frequency. At higher concentrations of (+)-catechin, the presumed *N*-hydroxylation of 2-AF may be inhibited with a decrease in the mutation frequency.

2.2.6 Experiments With Methimazole

Methimazole is a high-affinity flavin-containing monooxygenase substrate. Concentrations of methimazole from 50 μM to 25 mM were added to TX1 cells, TA98 cells and 500 μM *m*-PDA (Figure 8). Methimazole at concentrations above 2.5 mM inhibited the plant activation of *m*-PDA (●). No toxic effects to the TX1 (□) or TA98 (△) cells were observed. These data indicate that a flavin-containing monooxygenase may be involved in the plant activation of *m*-PDA. 50 μM to 25 mM methimazole was added to TX1 cells, TA98 cells and 50 μM 2-AF (Figure 8). Methimazole did not inhibit the plant activation of 2-AF (○). At concentrations above 100 μM it exhibited a significant enhancement of the mutagenic potency of 2-AF. This enhancement was much lower than that induced with (+)-catechin. Methimazole was not directly mutagenic (○) or toxic (□, △). These data suggest the same mechanism of action suggested for (+)-catechin with the inhibition of detoxification enzymes resulting in more 2-AF available for activation.

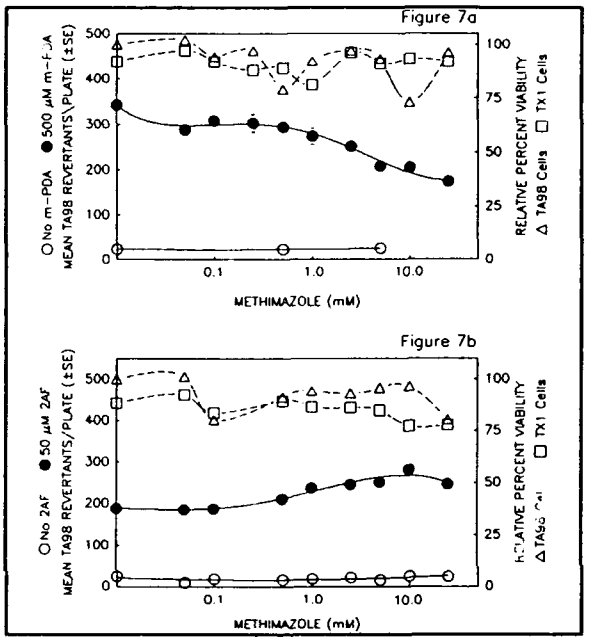

Figure 8 The effect of methimazole on the TX1-cell activation of *m*-PDA and 2-AF.

2.2.7 Experiments With Acetaminophen

Acetaminophen is a substrate and can act as a competitive inhibitor of peroxidase. Recently Raucy et al. (1989) reported that acetaminophen was also a substrate for cytochrome P-450 monooxygenase. With *m*-PDA as the promutagen, concentrations of acetaminophen above 2.5 mM caused a concentration-dependent decrease in the mutation frequency (Figure 9,●). There was no decrease in viability for either cell type (□, △). Concentrations of acetaminophen that significantly inhibited the activation of *m*-PDA by TX1 cells also inhibited the activity of cellular peroxidase in the same cells. These data suggest that tobacco cell peroxidases function as a major pathway for the plant activation of *m*-PDA. There was inhibition in the plant activation of 2-AF at concentrations of acetaminophen above 10 mM (●). Acetaminophen alone was not mutagenic (○) or toxic (□, △). These data suggest that tobacco cell peroxidase is also a pathway in the activation of 2-AF (Figure 9).

By using specific enzyme inhibitors with the plant cell/microbe coincubation assay we investigated the biochemical mechanisms of plant activation. The activation of *m*-PDA was inhibited by μM amounts of diethyldithiocarbamate and mM amounts of (+)-catechin, methimazole, potassium cyanide, and acetaminophen. There was no enhancement effect as observed with (+)-catechin and 2-AF. This may be due to the different biochemical pathways involved in activation. Inhibition by

methimazole indicates the presence of a FAD-dependent monooxygenase in the activation of *m*-PDA. The inhibition of the plant activation of *m*-PDA by potassium cyanide and acetaminophen indicates that peroxidases may function as a major pathway. The activation of 2-AF by TX1 cells was governed by an enzyme system(s) that was inhibited by μM amounts of diethyldithiocarbamate or 7,8-benzoflavone and mM amounts of acetaminophen. (+)-Catechin (at low concentrations) or methimazole enhanced the activation of 2-AF while higher concentrations of (+)-catechin were inhibitory. One pathway of the plant activation of 2-AF is via a cytochrome P-448-dependent *N*-hydroxylase. However, TX1 peroxidase may be the major pathway. The presence of a FAD-dependent monooxygenase in the activation of 2-AF was not detected. The experiments with metyrapone and potassium cyanide illustrate the importance of measuring not only the altered mutation induction frequency, but also the viability of the activating system and the genetic indicator organism to eliminate artifacts due to toxicity. Without viability as an endpoint, no study on the inhibition of mutagenesis or activation can be considered complete.

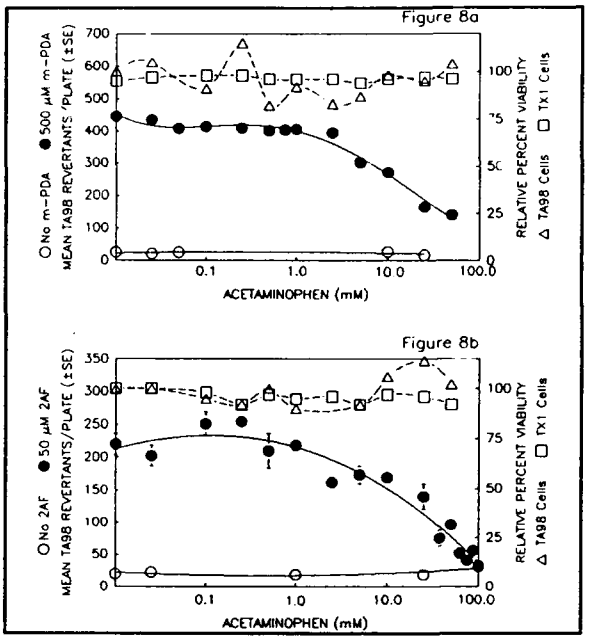

Figure 9 The effect of acetaminophen on the TX1-cell activation of *m*-PDA and 2-AF.

2.3 Investigation of the Mechanism of Inhibition by Specific Inhibitors

We investigated the biochemical mechanisms of the suppression of plant activation by diethyldithiocarbamate (DEDTC). DEDTC inhibited the metabolism of promutagens in both animal systems and plant systems (Gichner and Veleminsky, 1984; Gichner et al., 1988). We discovered that DEDTC inhibited the tobacco cell activation of 2-AF and *m*-PDA. By using specific enzyme inhibitors we reported indirect evidence that tobacco cell peroxidases were involved in the activation of these aromatic amines (Wagner et al., 1989; 1990). We determined that DEDTC suppressed the tobacco cell activation of aromatic amines by inhibiting cellular peroxidases (Plewa et al., 1990).

2.3.1 Plant Cells and Plant Cell Homogenates

For each experiment, TX1 cells from a 7-day culture were harvested, washed, and adjusted to 100 mg fresh weight/ml in MX^- medium. These cells were treated with DEDTC (0 - 25 mM) at 28°C for 1 h with shaking (150 rpm). After treatment the cells were centrifuged, the supernatant was decanted, and the cells were suspended in MX^- medium. Each cell suspension was homogenized with a PolyTron tissue disrupter for 45 sec at 4°C, and the cell debris was removed by centrifugation at 15,000 × g for 2 min. An aliquot of the supernatant was frozen at −80°C for later protein analysis. The other portion was kept on ice and immediately analyzed for peroxidase activity.

2.3.2 Assay for Protein Concentration

In order to investigate the biochemical mechanisms of plant activation, a growth curve was established for the TX1 cells. Several flasks were inoculated with 3 g each from a 7-day culture. At approximately 24-h intervals, fresh weight was measured from three 25-ml samples by drying the cells under vacuum and weighing the cells. These cells were then titered by suspending them to a final concentration of 100 mg/ml solution in distilled water. Protein was extracted from this solution by shearing the cells with a PolyTron homogenizer and centrifuging at 15,000 × g for 2 min to remove cellular debris. The supernatant was then assayed for protein content using the Bio-Rad protein assay. The growth curve revealed that TX1 cells remain in lag phase for 3-4 days followed by log growth for approximately 3-4 days, reaching stationary phase around day 7. The protein content of TX1 cells, however, did not coincide with their growth curve. Instead, the protein increased quickly with a maximum content during log phase followed by a sharp decrease back to base levels during stationary phase (Figure 10).

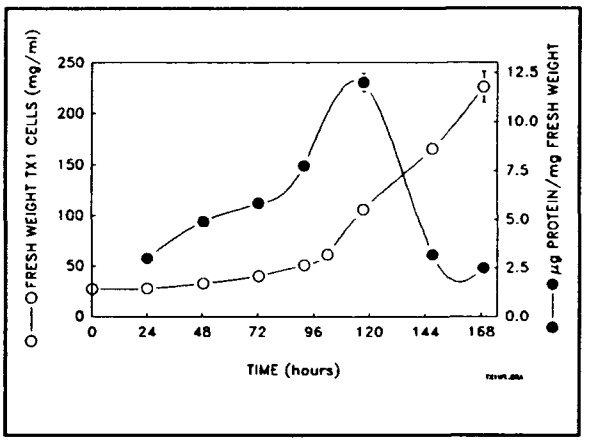

Figure 10 Relationship between the growth curve of TX1 cells and protein content.

2.3.3 Protein Content in Fresh Versus Frozen Cells

When conducting lengthy studies involving cell suspension cultures, it would be convenient to store samples for extended periods of time for later analysis. The Bio-Rad protein assay requires that a standard protein curve be run with every assay. Being able to freeze and store samples (TX1 cells) until the end of a study would lessen the amount of time, work, and supplies used. Because of the ease of preparation, 7-day TX1 cells were used. The fresh weight of the cells was titered to 100 mg/ml. The titered solution was divided into two samples. One sample was sheared with a PolyTron homogenizer, centrifuged at 10,000 × g for 2 min and the resulting supernatant was analyzed in a dose-dependent manner to determine protein content using the Bio-Rad assay. The other sample was frozen in a −80°C freezer for a minimum of 24 h before being thawed, sheared, centrifuged, and analyzed in the same manner. Less protein was extracted from the frozen cells than from the fresh preparation. This was contrary to our hypothesis that freezing and thawing cells would cause additional disruption of the cells and allow more protein to be extracted from frozen cells than from fresh cells. The protein content from the frozen cell preparations had much greater variance than did the fresh cell preparations. This suggests that freezing the cells prior to shearing, centrifuging, and measuring protein content not only reduces the amount of protein extracted, but is also an unreliable measure of the true value (Smith et al., 1989).

2.3.4 Protein Content in Fresh Versus Frozen Cell Homogenates

We extended our analysis of protein content in fresh and frozen TX1 cell homogenates. A 7 day old TX1 culture was titered to 100 mg/ml fresh weight, sheared, and centrifuged as in §2.3.2. This homogenate was divided into two samples. One sample was analyzed for protein content immediately using the Bio-Rad protein assay. The other sample was frozen in a −80°C freezer for a minimum

of 24 h before thawing and analyzing. Ten samples of 35 µl each were analyzed for protein content from both groups. Three independent experiments were conducted, and in all three cases, the protein content of the frozen cell homogenate did not significantly differ from that of fresh cells (Figure 11). In two of the three experiments, a portion of the initial titered cell suspension was removed prior to shearing, was frozen, thawed, sheared, centrifuged, and analyzed for protein content. In both cases, significantly less protein was extracted from the frozen cells, confirming our previous study (§2.3.3). We concluded that freezing the cell homogenate, as opposed to the whole cells, does not affect protein content of TX1 cell cultures (Smith et al., 1989).

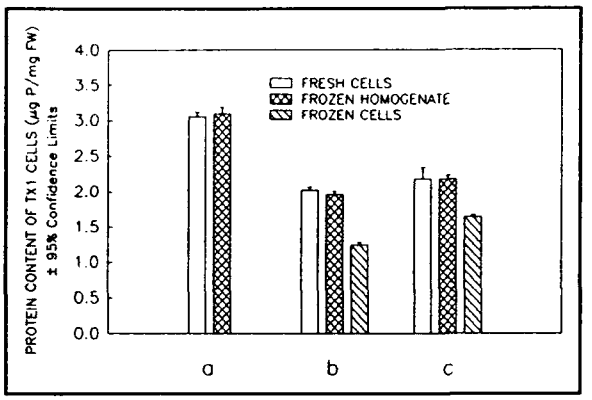

Figure 11 Comparison of protein content in 3 different 7-day TX1 cells preparations. *a*, *b*, and *c* represent data collected from three independent experiments

2.3.5 Determination of Peroxidase Activity

We measured peroxidase activity of the TX1 cells by determining the oxidation of guaiacol to tetraguaiacol by observing the change in absorbance at 470 nm (Maehly and Chance, 1954). Peroxidase activity was analyzed in a reaction volume of 3 ml containing 50 mM potassium phosphate buffer, pH 7.0, 100 µl of 0.3% H_2O_2, 1 ml of a 1% guaiacol solution, and 25 µl of the TX1 cell homogenate. The TX1 cell homogenate was prepared as in §2.3.1. The cuvettes used as blanks were identical except that MX^- medium was used instead of homogenate. Peroxidase activity was measured over a 5 min time period using a model 552A Perkin-Elmer double-beam spectrophotometer at 470 nm. Three independent replicates were conducted for each measurement within each experiment.

2.3.6 *In Vivo* Inhibition of TX1 Cell Peroxidase by Diethyldithiocarbamate

DEDTC inhibited the TX1 cell activation of *m*-PDA and 2-AF (Figure 3). A significant decline in activation occurred above 75 µM DEDTC with 50% inhibition between 1 and 1.5 mM. With both promutagens there was no decrease in the viability of either TX1 or TA98 cells.

Intact TX1 cells were exposed *in vivo* to DEDTC concentrations from 250 µM - 25 mM for 1 h in four separate experiments. TX1 cell homogenates were prepared and both peroxidase activity and protein content were measured. One experiment is presented in Figure 12. TX1 cells exposed to DEDTC express reduced peroxidase activities when normalized on a protein basis. The DEDTC concentrations which caused a 50% reduction in

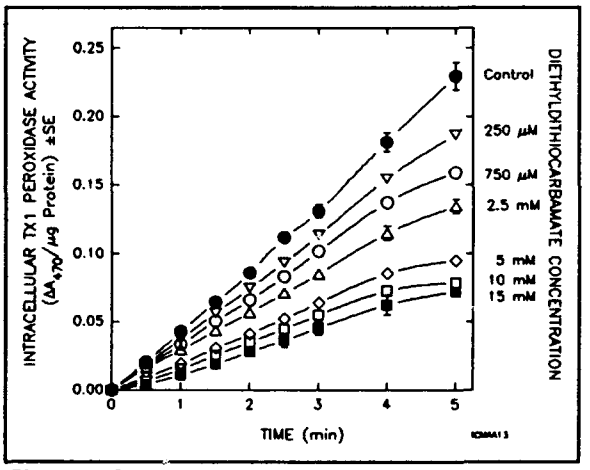

Figure 12 *In Vivo* inhibition of TX1 cell peroxidase by diethyldithiocarbamate.

TX1 cell peroxidase activity (750 μM - 2.5 mM) also caused a 50% inhibition of the TX1 cell activation of *m*-PDA and 2-AF.

2.3.7 *In Vitro* Inhibition of TX1 Cell Peroxidase and Horseradish Peroxidase by Diethyldithiocarbamate

The *in vitro* inhibition of TX1 cell peroxidase was determined by adding DEDTC (25 - 750 μM) directly to TX1 cell homogenates in five independent experiments. We previously determined that these concentrations of diethyldithiocarbamate did not directly react with the buffer, hydrogen peroxide and guaiacol solutions (data not shown). A concentration-dependent reduction in peroxidase activity was observed throughout the entire concentration range. The data from one experiment are presented in Figure 13. To confirm that DEDTC can inhibit peroxidase enzymes, two experiments were conducted using pure horseradish peroxidase. A concentration-dependent reduction in the activity of horseradish peroxidase was observed (data not shown).

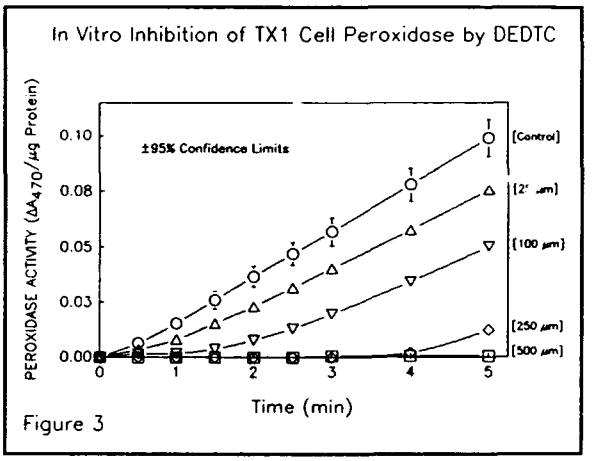

Figure 13 *In Vitro* inhibition of TX1 cell peroxidase by diethyldithiocarbamate.

2.3.8 Kinetics of TX1 Cell Peroxidase Inhibition

TX1 cells were titered and incubated with 750 μM or 25 mM diethyldithiocarbamate as well as concurrent controls in which the cells were handled identically except they were not exposed to the inhibitor. Separate TX1 cell homogenates were prepared under identical conditions. The peroxidase activities of triplicate samples of these homogenates were measured varying the concentration of substrate (H_2O_2). Velocity was calculated from the change in absorbance in the linear portion of the curve and ϵ_{470} = 26.6 mM^{-1} cm^{-1} for tetraguaiacol (Chance and Maehly, 1955). The mean V_{max} and K_m values for the above experiments are presented in Table 2. The data are represented in a Lineweaver-Burk plot (Figure 14). From three independent experiments, the K_m value for the control cells was 2.79 ± 0.50 mM. The K_m value for the cells treated with 750 μM and 25 mM DEDTC was 2.31 ± 0.27 mM and 2.65 ± 0.62 mM, respectively. The mean K_m value for all the groups was 2.58 ± 0.23 mM. The K_m values among the control and treated groups did not differ significantly while the V_{max} values were different (Table 2). These data indicate that DEDTC is a non-competitive inhibitor of TX1 cell peroxidase.

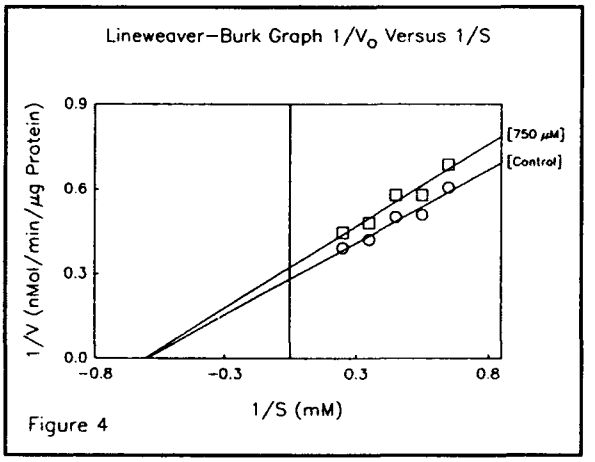

Figure 14 Lineweaver-Burk analysis of the inhibition of TX1-cell peroxidase by diethyldithiocarbamate.

From the above studies we concluded that DEDTC suppressed the tobacco cell activation of *m*-PDA and 2-AF. DEDTC was a potent inhibitor of tobacco cell peroxidase activity under *in vivo* and *in vitro* conditions. Kinetic experiments demonstrated that DEDTC was a non-competitive inhibitor of TX1 cell peroxidase. One mechanism for the antimutagenic effect of DEDTC was through its inhibition of cellular peroxidases (Plewa et al., 1990).

Table 2 V_{max} and K_m Values of TX1 Cell Peroxidase in Control Cells and in Cells Treated with Diethyldithiocarbamate

Treatment Group	Mean V_{max} ± SE[1] nmol Tetraguaiacol/min/μg Protein	Mean K_m ± SE mM
Control TX1 cells	4.02 ± 0.26	2.79 ± 0.50
TX1 cells treated with 750 μM diethyldithiocarbamate	3.41 ± 0.26	2.31 ± 0.27
TX1 cells treated with 25 mM diethyldithiocarbamate	2.12 ± 0.15	2.65 ± 0.62

[1]SE, standard error of the mean.

2.4 Use of *S. typhimurium* Strains with Enhanced Levels of *O*-Acetyltranferase

Recently Watanabe et al., (1990) developed *Salmonella* strains that were derivatives of TA98 and possess pYG219. pYG219 is a plasmid that contains a copy of the gene that encodes acetyl-Co A: *N*-hydroxyarylamine *O*-acetyltransferase (OAT). One strain — YG1024 — expresses approximately

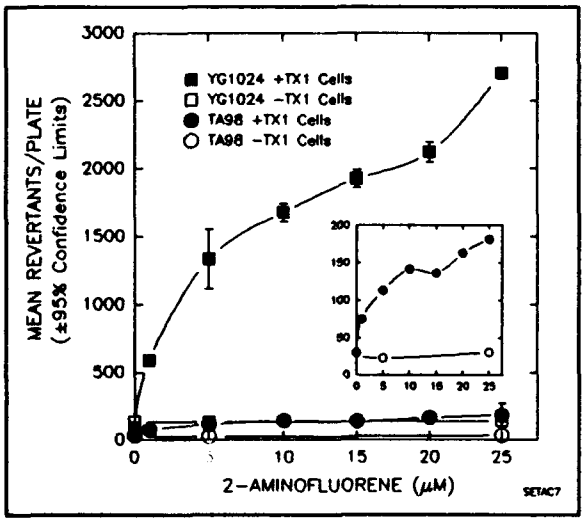

Figure 15 Comparison of the concentration-response curves of TX1 cell-activated 2-AF in *S. typhimurium* strains TA98 (insert) and YG1024.

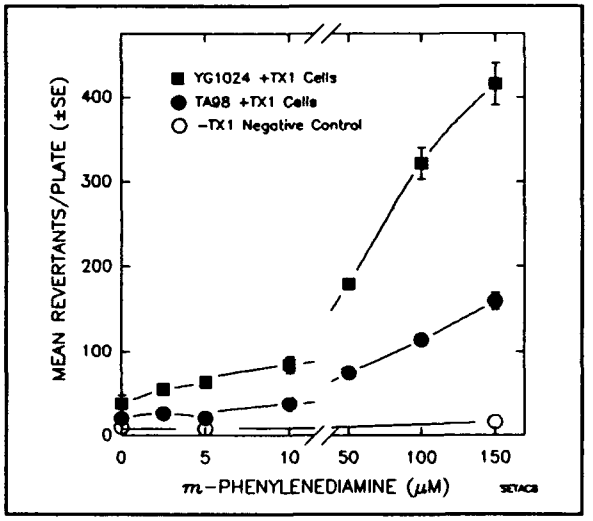

Figure 16 Comparison of the concentration-response curves of TX1 cell-activated *m*-PDA in *S. typhimurium* strains TA98 and YG1024.

100× higher *O*-acetyltransferase activity than TA98 and is exceedingly sensitive to arylhydroxylamines. These agents are activated metabolites of promutagenic nitroarenes and aromatic amines. We compared the mutagenic response of TA98 and YG1024 to 2-AF and *m*-PDA with and without TX1 cell activation in the plant cell/microbe coincubation assay. In a concentration range of 1–25 μM 2-AF, both TA98 (○) and YG1024 (□) were negative without TX1-activation (Figure 15). However, in the reaction tubes with TX1 cells the YG1024 cells (■) were approximately 15× more responsive than TA98 (●) to the plant-activated 2AF metabolite. Concentration-response curves with 2.5–150 μM *m*-PDA were compared for TA98 and YG1024 with and without TX1-activated *m*-PDA (Figure 16). *m*-PDA was not directly mutagenic to either bacterial tester strain (○), however, with 150 μM *m*-PDA, the TX1-activated product elicited a 2.5× increase in revertants/plate in YG1024 (■) as compared to TA98 (●). The data demonstrate that the metabolite is a substrate for the bacterial acetyl-Co A: *N*-hydroxyarylamine *O*-acetyltransferase (OAT) (Figures 15 and 16). This suggests that the plant-activated mutagen has a *N*-hydroxyamino functional group. We propose that acetylation followed by deacetylation of the plant-activated product causes the formation of a highly reactive aromatic nitrenium ion which can adduct to DNA and induce genetic damage.

2.5 Isolation of the Plant-Activated 2-AF and *m*-PDA Products

TX1 cells were treated with 500 μM *m*-PDA for 3 h while shaking at 28°C and the cells removed by centrifugation. Concurrent control cells were not treated with *m*-PDA. The supernatant fluid from each group was lyophilized at −70°C to approximately 20% of the original volume. The samples were filter sterilized and assayed for mutagenic activity using TA98 under preincubation conditions (Maron and Ames, 1983). No mutagenic response was seen in the supernatant from untreated cells. Additionally the supernatant from the treated cells retained its mutagenic properties for extended periods of time.

2.5.1 Ultrafiltration

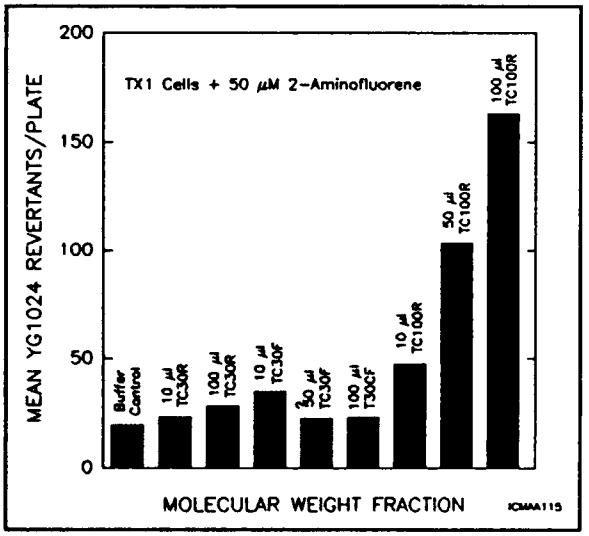

Figure 17 Isolation, partial purification by ultrafiltration, and mutagenic analysis of the TX1 cell-activated 2-AF product.

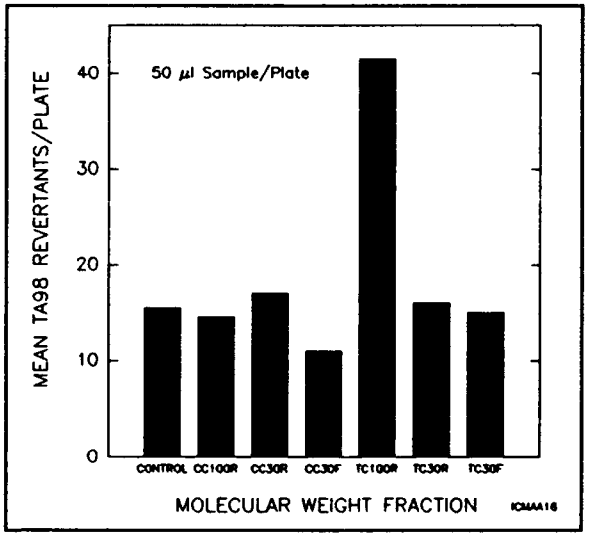

Figure 18 Isolation, partial purification by ultrafiltration, and mutagenic analysis of the TX1 cell-activated *m*-PDA product.

In another series of experiments we isolated supernatants from control and *m*-PDA-treated TX1 cells. After the cells were harvested the medium was centrifuged at 100,000 × g for 3 h at 4°C. The resulting supernatant fluids contained molecules of ≤1,000 kd. These supernatant fluids were not concentrated by lyophilization but were passed over Centricon ultrafiltration columns. Centricon C-100 and C-30 ultrafiltration centrifugal micro-concentrators have molecular weight cut-off values at 100,000 and 30,000 daltons, respectively. The supernatant was passed over the C-100 column at 1,000 × g for 1 h and both the retentate and filtrate were collected. The C-100 filtrate was then passed over a C-30 column at 5,000 × g for 1 h and the retentate and filtrate were collected. The C-100 retentate contained molecules with molecular weights from 1,000 kd – 100 kd, the C-30 retentate isolated molecules from 100 kd – 30 kd, and the C-30 filtrate contained molecules ≤30 kd. These fractions from both control and treated cells were analyzed for mutagenicity with TA98 cells (Figures 17-18). Under preincubation conditions, 50 µl samples were exposed to 5 × 10^8 cells for 1 h at 37°C while shaking. Into each reaction tube, 2 ml of supplemented molten top agar was added and poured onto VB plates. After 48–72 h incubation at 37°C, the plates were scored for TA98 *his*$^+$ revertants. The data indicate that only the C-100 retentate from the *m*-PDA-treated TX1 cells was mutagenic (TC100R); the other fractions from both the treated and control group were negative. Thus, the mutagen is associated with the treated TX1 cells in a fraction that includes molecules from 1,000 kd – 100 kd.

2.6 Current Working Model

We developed a model of the TX1-cell activation of aromatic amines based on the data presented in this paper and from our previous studies. The model (Figure 11) — albeit simplistic and incomplete — integrates our data into a mechanistic framework and serves as a foundation for new experimental designs. The model has seven components. They are, (1) the aromatic amine (R–NH_2) is transported into the plant (TX1) cell, (2) TX1 intracellular peroxidase oxidizes the molecule (R–NHOH), (3) the metabolite is polymerized or conjugated to a macromolecule (R–NHOH-conjugate), (4) the amine-conjugate is secreted into the extracellular medium, (5) the conjugate is absorbed by the bacterial tester strain (TA98), (6) the molecule may be deconjugated and is acetylated (R–NHO–$COCH_3$) and deacetylated by the bacterial acetyl-Co A: *N*-hydroxyarylamine *O*-acetyltransferase, and (7) the deacetylation results in a highly reactive nitrenium ion (R–NH^+) (Sandermann, 1988).

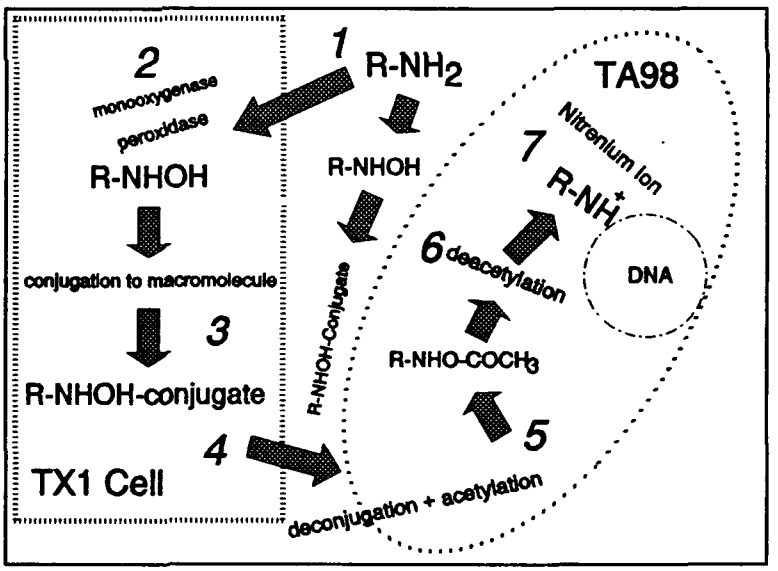

Figure 19 The current model for the plant-activation of promutagenic aromatic amines. The model has seven components.

3 LIST OF PUBLICATIONS GENERATED BY GRANT Nº AFOSR-88-0336 (September 15. 1988 – September 30, 1991)

3.1 Published Abstracts (Peer Reviewed)

Wagner, E.D., M.M. Verdier and M.J. Plewa. 1989. Comparison of the plant cell activation of two promutagens using enzyme inhibitors. Environ. Molecular Mutagenesis 14:211, Suppl. 15.

Smith, S.R., M.M. Verdier, E.D. Wagner and M.J. Plewa. 1989. Protein content of tobacco cells in relation to the plant activation of *m*-phenylenediamine and 2-aminofluorene. Environ. Molecular Mutagenesis 14:188-189, Suppl. 15

Plewa, M.J. 1989. The activation of promutagens by plant cell systems. (Invited symposium lecture to the Fifth International Conference on Environmental Mutagens). Environ. Molecular Mutagenesis 14:154, Suppl. 15

Wagner, E.D., S.R. Smith, K. Hajek and M.J. Plewa. 1990. The mechanism of the antimutagenic effect of diethyldithiocarbamate in the plant activation of promutagens. Environ. Molecular Mutagenesis 15:62, Suppl. 17.

Plewa, M.J. and E.D. Wagner. 1990. The biochemical basis of the activation of promutagens by plant cell systems. Environ. Molecular Mutagenesis 15:48, Suppl. 17.

Wagner, E.J., S.R. Smith, F. Hoff and M.J. Plewa. 1991. The inhibitory effects of 8-methoxypsoralen and vanadate in the plant activation of two promutagenic aromatic amines. Environ. Molecular Mutagenesis 17:75, Supp. 19.

Seo, K.Y. and M.J. Plewa. 1991. Partial isolation of the mutagenic product of *m*-phenylenediamine after activation by tobacco cells. Environ. Molecular Mutagenesis 17:67, Supp. 19.

3.2 Published Papers (Peer Reviewed)

Wagner, E.D., J.M. Gentile, and M.J. Plewa. 1989. Effect of specific monooxygenase and oxidase inhibitors on the activation of 2-aminofluorene by plant cells. Mutation Research 216:163-178.

Plewa, M.J. 1990. Activation of promutagens by plant cell systems. *In* Mendelsohn M.L. and R.J. Albertini (Eds). Mutation and the Environment: Environmental Genotoxicity, Risk and Modulation. Wiley-Liss, New York, 385 pp.

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Smith, S.R., E.D. Wagner and M.J. Plewa. 1991. Antimutagenic effects of 8-methoxypsoralen and vanadate on the plant activation of aromatic amines. In preparation.

Plewa, M.J., E.D. Wagner, S.R. Smith and K.Y. Seo. 1991. Biochemical and mutagenic characterization of plant-activated aromatic amines. J. Environ. Tox. Chem. SETAC AFOSR Review Paper. Submitted.

Plewa, M.J. 1991. Blocking the plant activation of promutagenic aromatic amines by peroxidase inhibitors. Invited paper: Proceedings of the Third International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis, Lucca, Italy. Plenem Press, New York. IN PRESS.

4 LIST OF PROFESSIONAL PERSONNEL

4.1 Professional Staff

Ms. Elizabeth D. Wagner, M.S., Associate Research Biologist, Institute for Environmental Studies, University of Illinois at Urbana-Champaign.

Ms. Shannon Smith, M.S., Project Assistant, Institute for Environmental Studies, University of Illinois at Urbana-Champaign.

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Ms. Kathryn L. Hajek, School of Life Sciences, University of Illinois at Urbana-Champaign.

Ms. Lucie Dobrovolny, School of Life Sciences, University of Illinois at Urbana-Champaign.

Ms. Joan Riley, School of Life Sciences, University of Illinois at Urbana-Champaign.

Mr. David Cortez, School of Life Sciences, University of Illinois at Urbana-Champaign.

4.4 Degrees Awarded

Ms. Mary M. Verdier, B.S. May 1989. Senior Research Paper Title, "Inhibitors Affecting the Plant Activation of *m*-Phenylenediamine."

Ms. Kathryn L. Hajek, B.S. May 1990, Senior Thesis Title, "The Effect of Diethyldithiocarbamate on Plant Cell Peroxidase."

Ms. Lucie Dobrovolny, B.S. May 1991, Senior Thesis Title, "The Relationship Between Intracellular Peroxidase Activity and Protein Content Throughout the Growth Curve of Cultured Tobacco Cells."

Ms. Elizabeth D. Wagner, M.S. May 1989, Thesis Title, "Effects of Specific Monooxygenase and Oxidase Inhibitors on the Activation of 2-Aminofluorene by Plant Cells," 96 pp.

Ms. Shannon Smith, M.S. May 1990, Thesis Title, "Studies with *Nicotiana tabacum* and *Selenastrum capricornutum* that Lead to the Biochemical Mechanisms of the Plant Activation of *m*-Phenylenediamine and 2-Aminofluorene," 87 pp.

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REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

OMB No. 0704-0046

1a. NAME OF CONTRACTOR/ SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	e. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
University of Illinois	AFOSR-88-0336		AFOSR-88-0336	[ ] INTERIM [X] FINAL
b. ADDRESS *(include Zip Code)*	d. AWARD DATE *(YYMMDD)*	b. ADDRESS *(include Zip Code)*	d. AWARD DATE (YYMMDD)	4. REPORTING PERIOD (YYMMDD)
105 Davenport House 809 S Wright, Champaign 61820	880915		880915	FROM 880915 TO 910930

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None", so state)*

a. NAME OF INVENTOR(S) *(Last, First, M.I.)*	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS				e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER	
			UNITED STATES		FOREIGN			
			YES	NO	YES	NO	YES	NO
none								

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR

i. NAME OF INVENTOR *(Last, First, M.I.)*	i. NAME OF INVENTOR *(Last, First, M.I.)*
ii. NAME OF EMPLOYER	ii. NAME OF EMPLOYER
iii. ADDRESS OF EMPLOYER *(include Zip Code)*	iii. ADDRESS OF EMPLOYER *(include Zip Code)*

g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED

i. TITLE OF INVENTION	ii. FOREIGN COUNTRIES OF PATENT APPLICATION

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None", so state)*

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS *(include Zip Code)*	c. SUBCONTRACT NO.(S)	d. DAR "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD)	
			CLAUSE NO.	DATE *(YYMM)*		AWARD	ESTIMATED COMPLETION
none							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if [ ] Small Business or [X] Non Profit organization.)* *(Check appropriate box)*

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, M.I.)*

b. TITLE

c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

SIGNATURE OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL — DATE (YYMMDD)

DD FORM 882

EDITION OF 1 OCT 75 IS OBSOLETE