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AD-A251 876



REPORT DOCUMENTATION PAGE

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE SELECTED JUN 22 1992		1b. RESTRICTIVE MARKINGS	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited	
6a. NAME OF PERFORMING ORGANIZATION University of Illinois at U-C		5. MONITORING ORGANIZATION REPORT NUMBER(S) AFOSR-TR. 92-0560	
6b. OFFICE SYMBOL (If applicable)		7a. NAME OF MONITORING ORGANIZATION AFOSR/NL	
6c. ADDRESS (City, State and ZIP Code) c/o Grants and Contracts Office 105 Davenport House 809 S. Wright, Champaign, IL 61820		7b. ADDRESS (City, State and ZIP Code) Bolling Air Force Base, D.C. 20332-6448	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION AFOSR/NL		8b. OFFICE SYMBOL (If applicable)	
8c. ADDRESS (City, State and ZIP Code) Bolling Air Force Base, DC 20332-6448		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER AFOSR-91-0432	
11. TITLE (Include Security Classification) Mechanisms & Effects of Plant Activation of Chemicals in the Environment		10. SOURCE OF FUNDING NOS.	
		PROGRAM ELEMENT NO. 61102F	PROJECT NO. 2312
12. PERSONAL AUTHOR(S) Plewa, Michael J.		TASK NO. A4	WORK UNIT NO.
13a. TYPE OF REPORT Annual	13b. TIME COVERED FROM 91-09-30 TO 92-04-30	14. DATE OF REPORT (Yr., Mo., Day) 92-04-20	15. PAGE COUNT 12
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP		
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
<p>Plants can activate promutagens into stable mutagens and these genotoxic agents may be hazardous to the environment and to the public health. Using <i>m</i>-phenylenediamine (<i>m</i>-PDA) as an archetype compound we developed a working model for plant-activation. (1) The aromatic amine is transported into the plant cell, (2) intracellular peroxidase oxidizes the molecule (3) the metabolite is conjugated to a macromolecule, (4) the amine-conjugate is secreted into the extracellular medium, (5) the conjugate or a deconjugated plant-activated metabolite is absorbed by the <i>Salmonella</i> tester strains, (6) the plant-activated <i>N</i>-hydroxylated product is acetylated and deacetylated by the bacterial acetyl-CoA: <i>N</i>-hydroxyarylamine <i>O</i>-acetyltransferase, and (7) the deacetylation results in a highly reactive nitrenium ion that can react with or adduct to DNA. The relative potency of six plant-activated aromatic amines is 2-aminofluorene > benzidine > <i>m</i>-phenylenediamine > 4-aminobiphenyl > 2,4-diaminotoluene. 2-Naphthylamine was not plant-activated. From a structure-function perspective, there appears to be some correlation between free diamino functional groups. These structure-function relationships are expressed by the relative plant-activation potency of <i>m</i>-PDA and 2,4-diaminotoluene, and benzidine and 4-aminobiphenyl. The plant-activated products are stable, and are associated with a macromolecule (>300 kDa). The plant-activated product is further metabolized by cells with high acetyl-CoA: <i>N</i>-hydroxyarylamine <i>O</i>-acetyltransferase activity. The possibility exists that these plant-activated products may be formed into non-toxic proximal mutagens in intact plants that can be metabolized back into potent frameshift mutagens in organisms that consume the plants. This research suggests that plants may serve as a repository of proximal mutagenic metabolites of xenobiotics that may be transported through the food chain.</p>			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT. <input type="checkbox"/> OTIC USERS <input type="checkbox"/>		21. ABSTRACT SECURITY CLASSIFICATION approved for public release; distribution unlimited	
22a. NAME OF RESPONSIBLE INDIVIDUAL Dr. Jimmy Cornette		22b. TELEPHONE NUMBER (Include Area Code) 202-767-4278	22c. OFFICE SYMBOL NL

**THE MECHANISMS AND EFFECTS OF THE PLANT-ACTIVATION OF
CHEMICALS IN THE ENVIRONMENT
Grant N° AFOSR-91-0432**

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April 19, 1992

Interim Report for Period 30 September, 1991 – 30 April, 1992

Distribution Statement: Approved for public release; distribution unlimited

Prepared for:

**AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Bolling Air Force Base, DC 20332-6448**

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Directorate of Life and Environmental Sciences**

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92-15731


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1 INTRODUCTION

Plants can activate promutagens into stable mutagens and these genotoxic agents may be hazardous to the environment and to the public health. Plant systems have been widely employed in classical and environmental mutagenesis. However, the environmental and human health impact of plants exposed to environmental xenobiotics were not well recognized until the presence of pesticide contaminants in food supplies caused alarm. The capability of plants to bioconcentrate environmental agents and activate promutagens into toxic metabolites is significant when one realizes the immense diversity of xenobiotics to which plants are intentionally and unintentionally exposed. Finally, we all must be attentive to the effects that toxic agents may have on the biosphere and the grave global consequences that would result in a disruption in the carbon cycle.

Plant activation is the process by which a promutagen is metabolically transformed into a mutagen by a plant system. In mammalian systems the majority of enzymes participating in oxidative desulfuration, dealkylation, epoxidation, or ring hydroxylation involve cytochrome P-450-type monooxygenases. It is unknown if microsomal cytochrome P-450 in plants have enzymatic characteristics similar to those of mammalian liver. The optical and magnetic properties of plant cytochrome P-450 are similar to those of hepatic microsomes. Although limited data exist about the inducibility of plant cytochrome P-450, it is unknown if there is an equivalent inducible system to hepatic monooxygenases. Plant peroxidases catalyze the oxidation of a diverse class of xenobiotics. Peroxidases are ubiquitous in plants, however, only limited data are available that demonstrate their participation in the *in vivo* metabolism of foreign compounds.

The plant activation of *m*-phenylenediamine (*m*-PDA) has been well studied in our laboratory. *m*-PDA is activated into potent frameshift mutagens by a number of cultured plant cell species as detected by *Salmonella typhimurium* tester strains in the plant cell/microbe coinubation assay (Figure 1). The purpose of this report is to review our recent findings on the plant activation of aromatic amines and to present a working model of the biochemical mechanisms involved.

1.1 Plant Cell/Microbe Coinubation 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. The plant and microbial cells are coinubated 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 (Figure 2). Long-term plant cell suspension cultures of tobacco (*Nicotiana tabacum*), cell line TX1 were maintained in MX medium. *S. typhimurium* strains TA98 and YG1024 were separately used as the genetic indicator organisms. A TX1 cell culture was grown at 28°C to early stationary phase, and the cells were washed and sus-

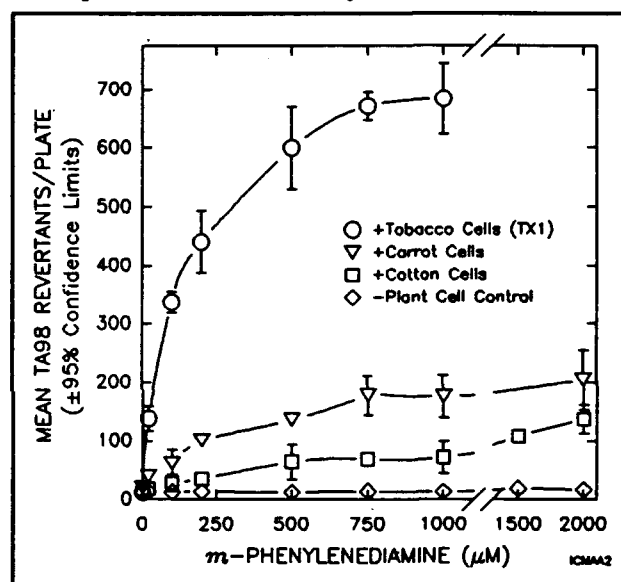


Figure 1 Activation of *m*-PDA by various plant cell species in the plant cell/microbe coinubation assay.

pended in MX^- medium. MX^- medium lacks plant growth hormone. The fresh weight of the plant cells was adjusted to 100 mg/ml, and the 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 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 $\leq 25 \mu\text{l}$ 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 $550 \mu\text{M}$ 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. 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. After incubation at 37°C for 24 to 36 h, the bacterial colonies were counted.

2 RESULTS AND DISCUSSION

2.1 Current Working Model

Our current model of the TX1 cell activation of *m*-PDA is on the data presented in this report and from our previous studies. The model (Figure 3) — 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 conjugated to a macromolecule, (R-NHOH-conjugate), (4) the amine-conjugate is secreted into the extracellular medium, (5) the conjugate or a deconjugated plant-

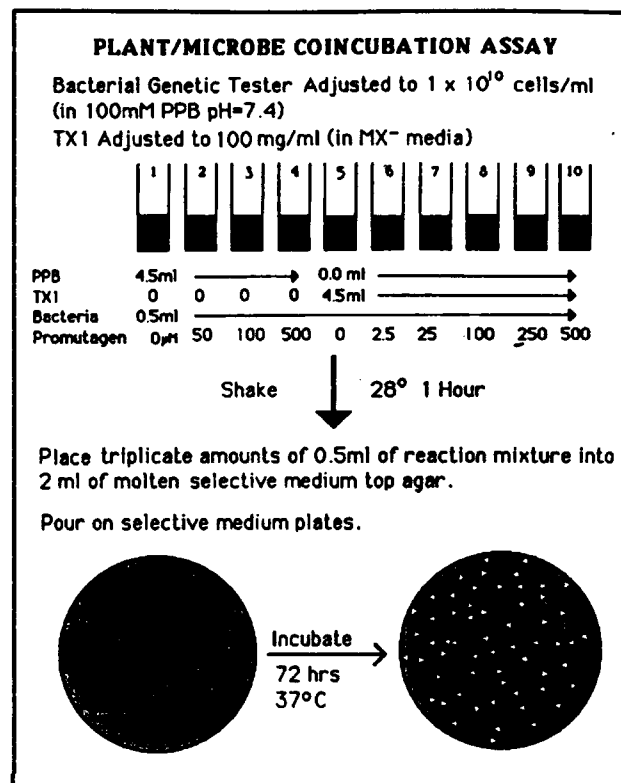


Figure 2 Diagram of the plant cell/microbe coincubation assay.

activated metabolite is absorbed by the *Salmonella* tester strains (TA98 or YG1024), (6) the plant-activated *N*-hydroxylated product is acetylated (R-NHO-COCH₃) and deacetylated by the bacterial acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase, and (7) the deacetylation results in a highly reactive nitrenium ion (R-NH⁺).

2.2 Involvement of TX1-Cell Peroxidase

In our model, the aromatic amine is *N*-hydroxylated by intracellular TX1-cell peroxidase (Figure 3, step 2). Support for this comes from our studies employing enzyme inhibitors. We studied seven inhibitors for their ability to affect the plant activation of 2-aminofluorene (2-AF) or *m*-PDA. The resolution of the plant cell/microbe coincubation assay is sufficiently high that the effect of μ M to mM concentrations of specific inhibitors was easily detected. We also determined if each inhibitor was a direct-acting mutagen, a plant-activated pro-mutagen, or a toxic agent to the cells. Our data indicated that plant cell peroxidases play a major role in the TX1 cell activation of *m*-PDA and 2-AF (Wagner et al., 1989; 1990).

An example of an inhibition analysis is presented in Figure 4. Diethyldithiocarbamate (DEDTC) in a concentration range of 25 μ M–50 mM inhibited the TX1 activation of 500 μ M *m*-PDA. Under these conditions the viability of TX1 or TA98 cells were not affected. DEDTC was not a direct-acting mutagen to TA98. A significant decline in activation occurred above 75 μ M DEDTC with 50% inhibition induced at approximately 1 mM. We discovered that DEDTC suppressed the TX1 cell activation of aromatic amines by inhibiting intracellular peroxidases (Plewa et al., 1991). Intact TX1 cells were exposed *in vivo* to DEDTC concentrations from 250 μ M–25 mM for 1 h. After the cells were washed, TX1 cell homogenates were prepared and both peroxidase activity and protein content were measured. Peroxidase activity was measured by determining the oxidation of guaiacol by observing the change in absorbance at 470 nm.

TX1 cells exposed to DEDTC express reduced peroxidase activities when normalized on a μ g protein basis. Figure 4 illustrates that within the range of 100 μ M–10 mM DEDTC the rate of inhibition

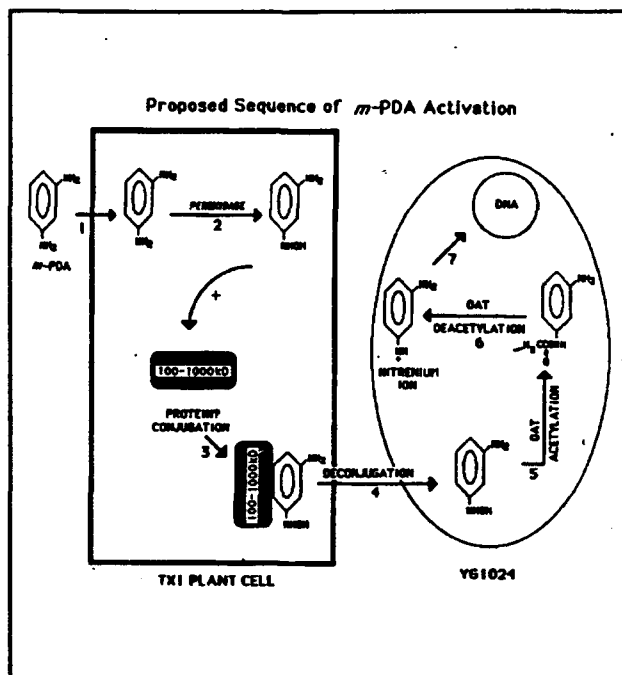


Figure 3 A hypothetical schematic of our model for the plant-activation of *m*-phenylenediamine.

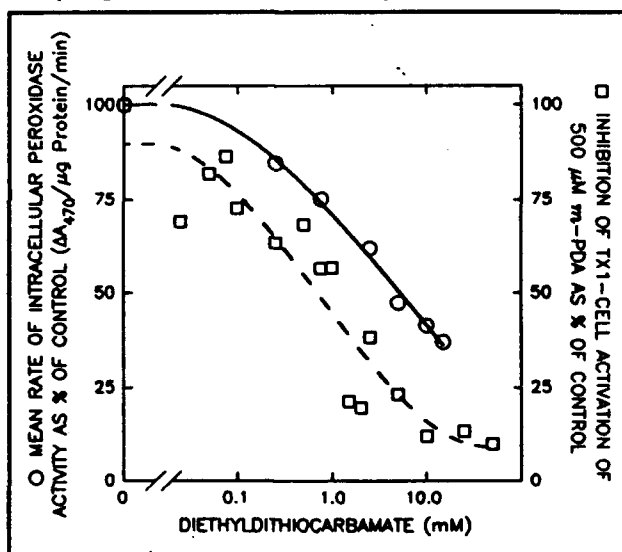


Figure 4 Comparison between the inhibition of TX1 cell peroxidase activity and the repression of TX1 cell activation of *m*-PDA as a function of diethyldithiocarbamate concentration.

of peroxidase (○) paralleled the rate of repression of TX1 cell activation of *m*-PDA (□). These data indicate that the activation of *m*-PDA by TX1 cells is directly dependent on the activity of TX1 cell peroxidase.

2.3 Isolation of the Plant-Activated *m*-PDA Product

For *m*-PDA the plant-activated product can be easily isolated from the extracellular medium. The activated promutagen is a very stable frameshift mutagen; medium recovered from treated TX1 cells have retained their mutagenic characteristics after 6 months of storage at 4°C. TX1 cells were treated with 500 μ M *m*-PDA for 3 h while shaking at 28°C. The cells were removed from the medium by centrifugation. The supernatant fluid was recovered and centrifuged at 100,000 \times g for 3 h at 4°C. Under these conditions large molecules >1,000 kDa should sediment out. The supernatant from the control and treated cells was assayed for mutagenicity. Reaction tubes contained concentrations of the ultracentrifuged supernatant that ranged from 0.5–10.0%, 1×10^9 YG1024 cells and 100 mM phosphate buffer, pH 7.4 to a total volume of 1 ml. The tubes were shaken for 1 h at 37°C. 500 μ l aliquots were added to supplemented molten top agar and poured onto VB plates. The plates were incubated for 72 h at 37°C and scored. The data presented in Figure 5 illustrate that the supernatant recovered from the treated cells contain a frameshift mutagen while the control supernatant is negative.

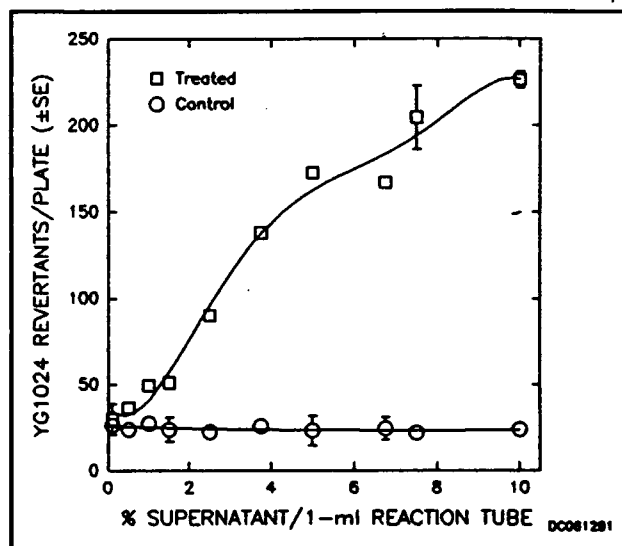


Figure 5 Mutagenicity of TX1 cell supernatants from control (untreated) cells and cells treated with 500 μ M *m*-PDA.

2.4 Conjugation of the Aromatic Amine Metabolite(s)

After the TX1-cell peroxidase-mediated metabolism of the aromatic amine, we propose that the product is conjugated to a macromolecule and transported into the extracellular medium (Figure 3, steps 3 and 4). In another series of experiments we used supernatants from control and *m*-PDA-treated TX1 cells. The supernatants contained molecules of $\leq 1,000$ kDa. These supernatant fluids were concentrated by being passed over an Amacron YM100 or XM300 ultrafiltration filter that had either a 100 kDa or 300 kDa cutoff. One liter of each supernatant type was concentrated 10 \times to a 100 ml retentate. Both the retentate and filtrate were collected. The YM100 and XM300 retentates contained molecules with

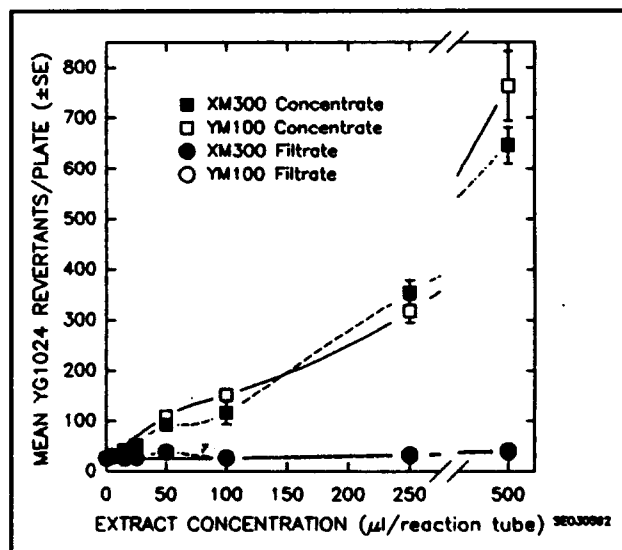


Figure 6 Mutagenicity of the YM100 and XM300 ultrafiltration retentates.

molecular weights from 100 kDa, 1,000 kDa and 300 kDa–1,000 kDa, respectively. The YM100 and XM300 filtrates isolated molecules <100 kDa and <300 kDa, respectively. These fractions from both control and treated cells were analyzed for mutagenicity using *S. typhimurium* strain YG1024 (Figure 6). Under preincubation conditions, μ l 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 72 h incubation at 37°C, the plates were scored for *his*⁺ revertants. The data indicate that only the YM100 and XM300 retentates from the *m*-PDA-treated TX1 cells were mutagenic; the other fractions from both the treated and control group were negative. The mutagen is associated with the treated TX1 cells in a fraction that includes molecules from 300 kDa–1,000 kDa. The molecular weight of the TX1 cell activated *m*-PDA mutagenic product is between 300 kDa and 1,000 kDa. Thus the plant activated product is associated with a large molecule. This type of association has not been observed with mammalian activation of aromatic amines.

2.5 HPLC Analysis for *m*-PDA in Ultracentrifuged Supernatant Fluids and XM300 Fractions from Treated TX1 Cells

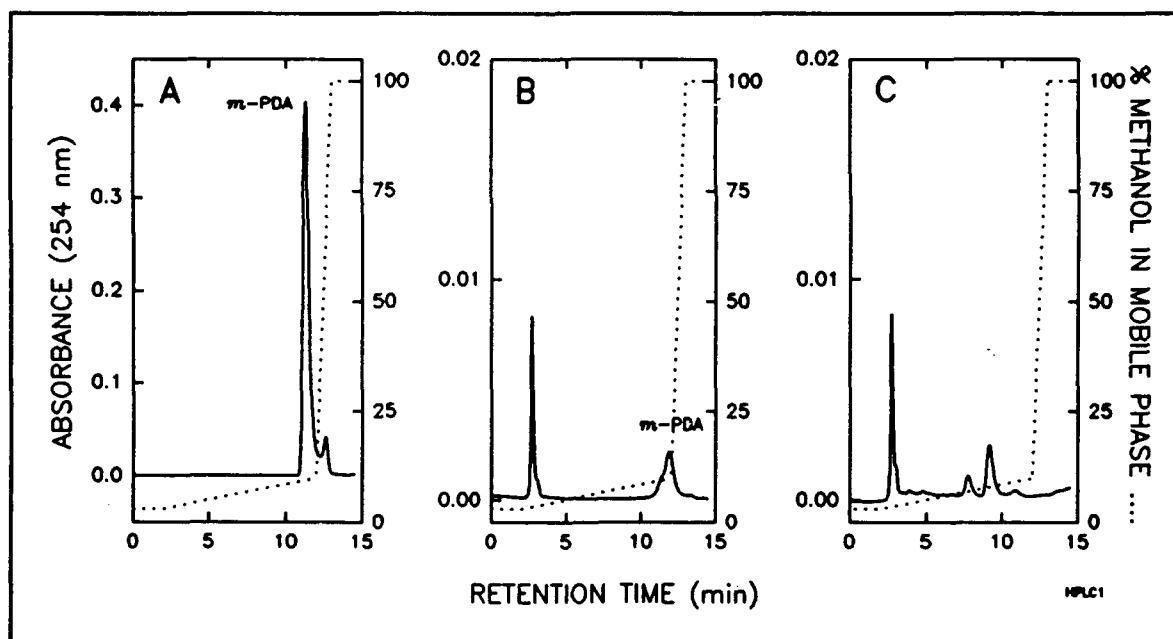


Figure 7 Panel A is a 10 μ l sample of a 10 mM *m*-PDA solution. Panel B is 10 μ l of the ultracentrifuged supernatant and panel C is 10 μ l of the XM300 retentate from treated TX1 cells.

To determine the amount of parent compound that was present in the mutagenic XM300 fraction we prepared a standard 10 mM solution of *m*-PDA. We also prepared samples of the ultracentrifuged supernatant and a XM300 retentate from TX1 cells treated with 500 μ M *m*-PDA (3 h at 28°C). A 10 μ l sample of each solution was analyzed with HPLC on a 5 μ m Ultrasphere C18 column (4.6 mm \times 150 mm) with a guard column. The mobile phase was 3–100% methanol with 50 mM phosphate buffer, 3 mM sodium heptane sulfonate, pH 6.1 with triethylamine (Figure 7). Panel A is a chromatograph of 10 μ l of a 10 mM solution of Sigma Chem. Co. reagent grade *m*-PDA. After TX1 cells were treated for 3 h with 500 μ M *m*-PDA, the cells were removed by centrifugation. The supernatant fluid was recovered and centrifuged at 100,000 \times g for 3 h. Panel

B is a chromatograph of 10 μl of the ultracentrifuged supernatant. A very small amount of *m*-PDA appears to be in the ultracentrifuged supernatant. This supernatant was passed over an Amacron XM300 ultrafiltration membrane and the retentate collected. Panel C is a chromatograph of a 10 μl sample of the XM300 retentate. Essentially all of the *m*-PDA is gone from this sample. Please note that the y-axis for Panels B and C have a $\sim 15\times$ increase in resolution as compared to Panel A. The unlabeled peaks in Figure 7 are unknown compounds or metabolites in the samples.

2.6 Acetylation/Deacetylation of the Mutagen-Macromolecule Conjugate

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-CoA: *N*-hydroxyarylamine *O*-acetyltransferase (OAT). One strain — YG1024 — expresses approximately $100\times$ higher *O*-acetyltransferase activity than TA98 and is exceedingly sensitive to arylhydroxylamines. These agents are activated metabolites of promutagenic nitroarenes and aromatic amines. Another TA98 derivative, TA98/1,8-DNP₆, contains a deletion of OAT and does not have any *O*-acetyltransferase activity. We compared the mutagenic response of YG1024, TA98 and TA98/1,8-DNP₆ to *m*-PDA with and without TX1 cell activation in the plant cell/microbe coinoculation assay. A concentration-response curve with 2.5–1,000 μM *m*-PDA is presented in Figure 8. *m*-PDA was not directly mutagenic to any bacterial tester strain, however, at all concentrations tested, strain YG1024 was approximately $3\times$ more sensitive to the TX1-activated *m*-PDA product. The data demonstrate that the plant-activated *m*-PDA metabolite is a substrate for the bacterial acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase. This suggests that the plant-activated mutagen has a *N*-hydroxyamino functional group (Figure 3, steps 5 and 6). 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 (Figure 3, step 7).

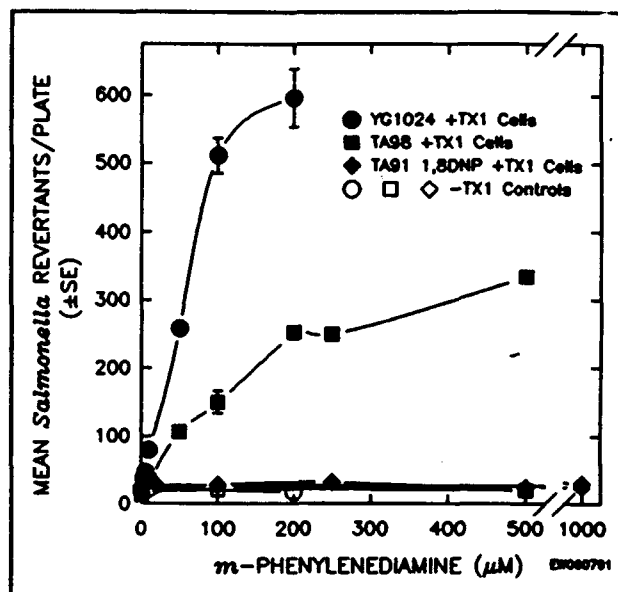


Figure 8 The effect of OAT expression on the mutagenicity of the high molecular weight fraction from supernatant derived from *m*-PDA-treated TX1 cells.

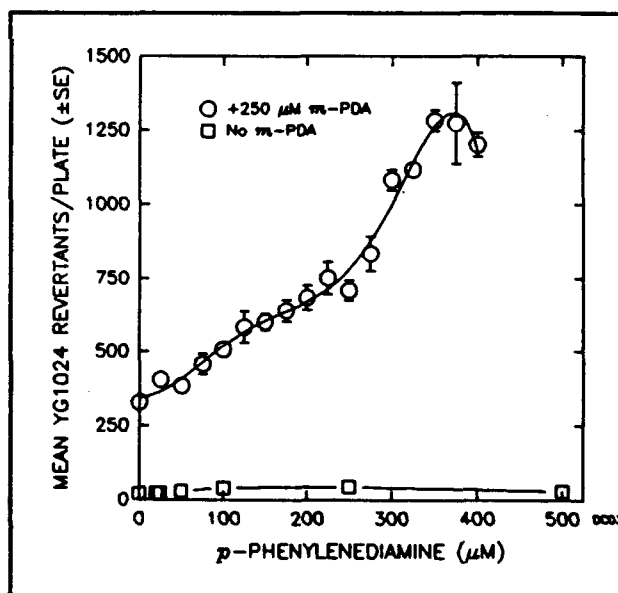


Figure 9 The effect of *p*-PDA on the plant-activation of 250 μM *m*-PDA.

2.7 Titration Experiments with *p*-Phenylenediamine

p-Phenylenediamine (*p*-PDA) is an isomer of *m*-PDA and it is not activated by TX1 cells. We tried to determine the relative affinity for *m*-PDA and the phase III high molecular weight conjugate by titrating *p*-PDA in plant cell/microbe coincubation reaction tubes that contained 250 μM *m*-PDA. We expected that we would observe a decline in the rate of plant activation of *m*-PDA if increasing concentrations of *p*-PDA would compete for the conjugating target molecule. As is illustrated in Figure 9, *p*-PDA was not plant-activated when analyzed in the coincubation assay in a concentration range from 25–500 μM (\square). However, when *p*-PDA was titrated against a fixed concentration of 250 μM *m*-PDA there was a concentration-response (\circ) with a direct increase in the mutagenic potency of the samples. These results were unexpected and may mean that there can be some sort of plant-mediated synergistic responses among these aromatic amines. Further experiments are planned to investigate this phenomenon during the next grant period.

2.8 Structure-Function Analysis of the Plant-Activation of Aromatic Amine Promutagens

Plant systems can activate promutagens into mutagenic forms and the biochemical basis of such transformations are now being uncovered (Wagner et al., 1989; 1990; Plewa et al., 1991). We developed the plant cell/microbe coincubation assay to investigate the plant activation of promutagens (Plewa et al., 1983; Plewa et al., 1988). In this assay, plant cells in suspension culture serve as the activating system and microbial cells serve as the genetic indicator organism.

The plant cell coincubation assay possesses great flexibility. Inhibitors of biochemical pathways can be introduced into the coincubation mixture of living plant and microbial cells with the promutagen. Alternatively, different microbial cells with different metabolic capacities can be employed. During the first grant period we expanded the aromatic amine substrates to include *m*-PDA, 2-aminofluorene, benzidine, 4-aminobiphenyl, 2,4-diaminotoluene and 2-naphthylamine (Figure 10).

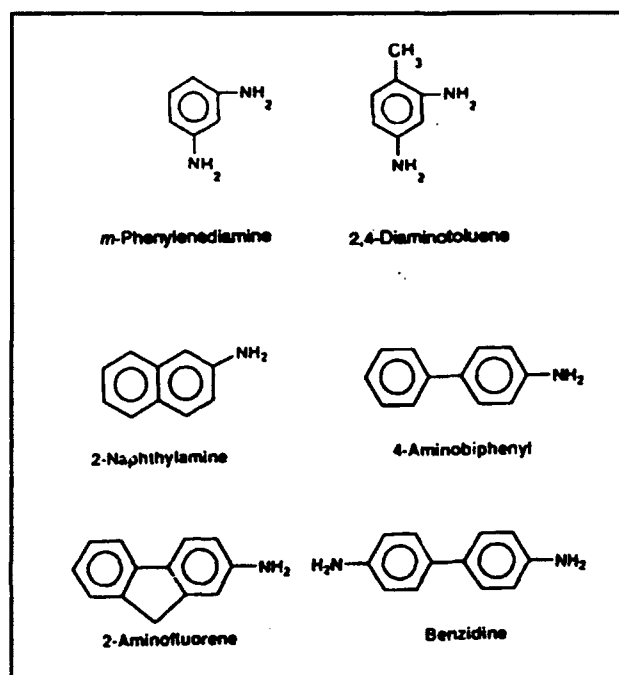


Figure 10 Aromatic amines analyzed in this study.

2.8.1 2-Aminofluorene

With the plant activation of 2-aminofluorene (2-AF), both YG1024 and TA98 exhibited an increase in mutation induction (Figure 11). YG1024 cells were exceedingly sensitive. The mutagenicity of the plant-activated 2-AF metabolite(s) was approximately 7 times higher in YG1024 than in TA98. TA98/1,8-DNP₆ was not affected by increasing 2-AF concentrations. These results are similar to those obtained with 2-AF and mammalian S9 activation where YG1024 was 30 times more sensitive than TA98 (Einistö et al., 1991).

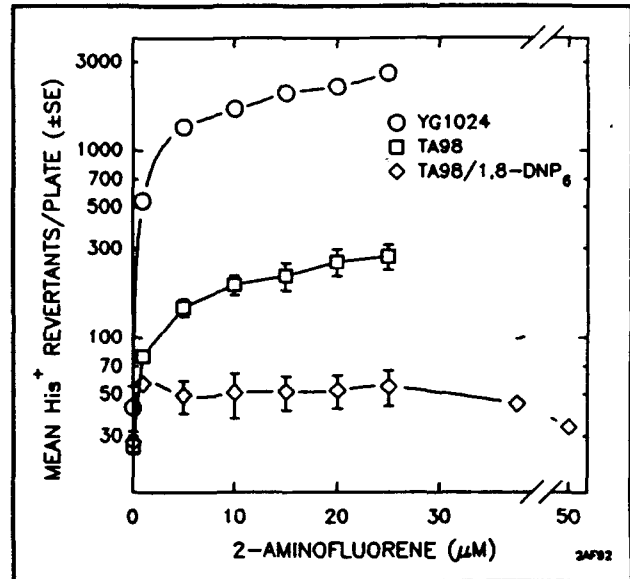


Figure 11 The plant activation of 2-aminofluorene assayed with *S. typhimurium* strains YG1024, TA98 and TA98/1,8-DNP₆.

2.8.2 Benzidine

Plant cell/microbe coinubation experiments were conducted with benzidine (Figure 12). There was a significant increase in YG1024 revertants with 0.05 μM benzidine. The increase in TA98 revertants was 5 - 10 times lower than YG1024. There was a slight increase in TA98/1,8-DNP₆ revertants at high concentrations of benzidine.

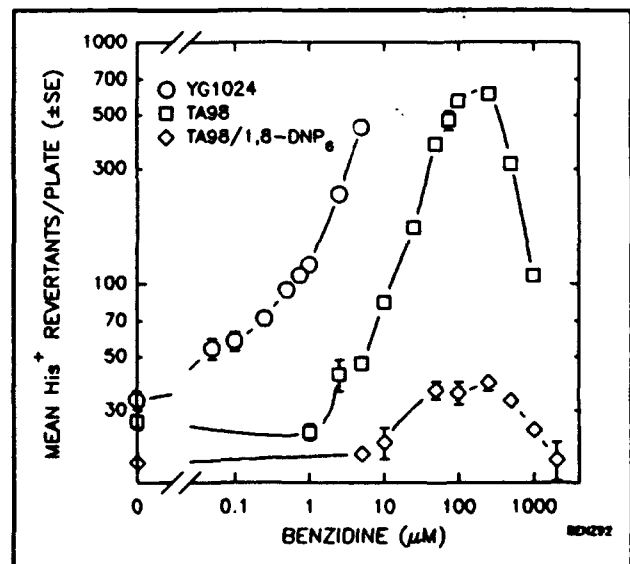


Figure 12 The plant activation of benzidine assayed with *S. typhimurium* strains YG1024, TA98 and TA98/1,8-DNP₆.

2.8.3 4-Aminobiphenyl

The plant activation of 4-aminobiphenyl was analyzed with these three strains (Figure 13). YG1024 was 4 – 7 times more sensitive than TA98. 4-Aminobiphenyl did not affect TA98/1,8-DNP₆. The removal of an amine group — as compared to benzene (Figures 10 & 12) — caused a significant reduction of the mutagenic potency of 4-aminobiphenyl.

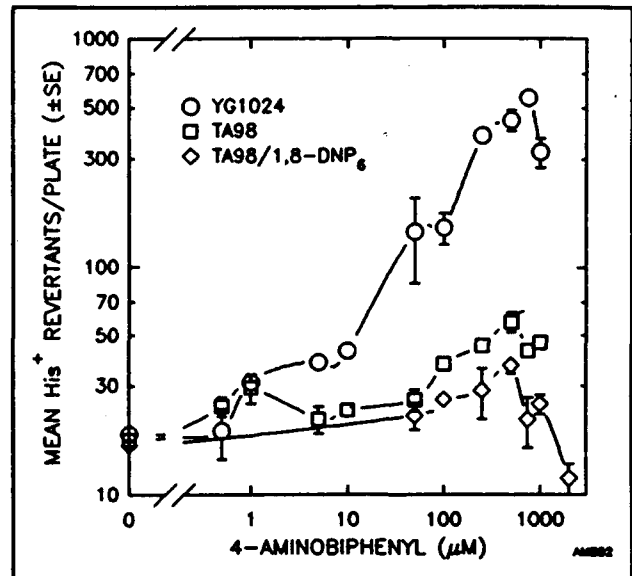


Figure 13 The plant activation of 4-aminobiphenyl assayed with *S. typhimurium* strains YG1024, TA98 and TA98/1,8-DNP₆.

2.8.4 2,4-Diaminotoluene

2,4-Diaminotoluene is very similar to *m*-PDA (Figure 10). High concentrations, 5 mM and greater, caused an increase in YG1024 revertants (Figure 14). A very slight increase in TA98 revertants was exhibited. No increase was observed in TA98/1,8-DNP₆. YG1024 was 1.5 – 2 times more sensitive than TA98. The mutagenicity of 2,4-diaminotoluene was compared in these strains using mammalian S9 activation (Cunningham and Matthews, 1990). The mutation rate was increased in YG1024 approximately 5 fold over that in TA98. The addition of a methyl group — as compared to *m*-PDA (Figure 8) — caused a significant reduction in the mutagenic potency of 2,4-diaminotoluene.

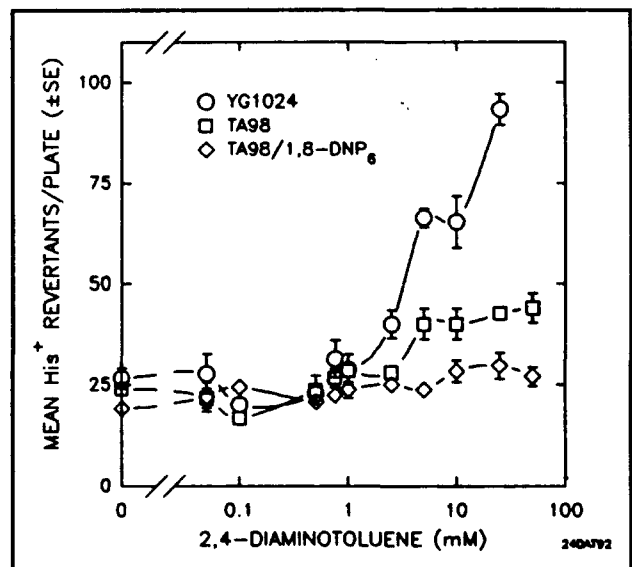


Figure 14 The plant activation of 2,4-diaminotoluene assayed with *S. typhimurium* strains YG1024, TA98 and TA98/1,8-DNP₆.

2.8.5 2-Naphthylamine

2-Naphthylamine was not activated by plant cells (Figure 15). There was no significant increase in YG1024 revertants. No significant increase in TA98 or TA98/1,8-DNP₆ revertants was observed. It is noted that 2-naphthylamine is a potent mammalian promutagen and procarcinogen.

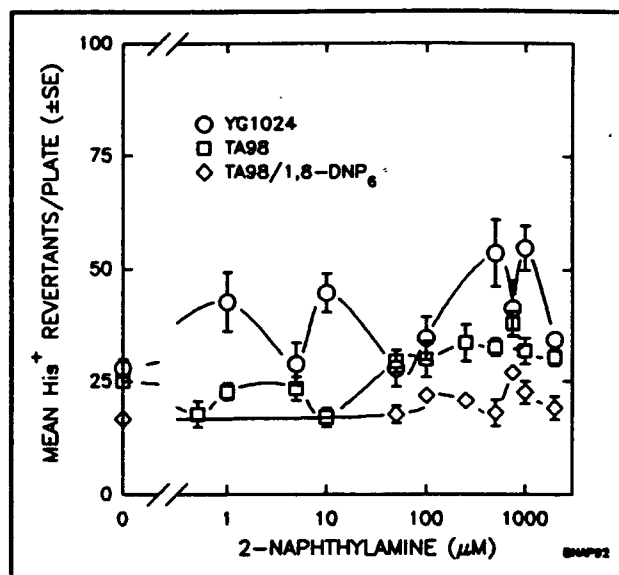


Figure 15 The plant activation of 2-naphthylamine assayed with *S. typhimurium* strains YG1024, TA98 and TA98/1,8-DNP₆.

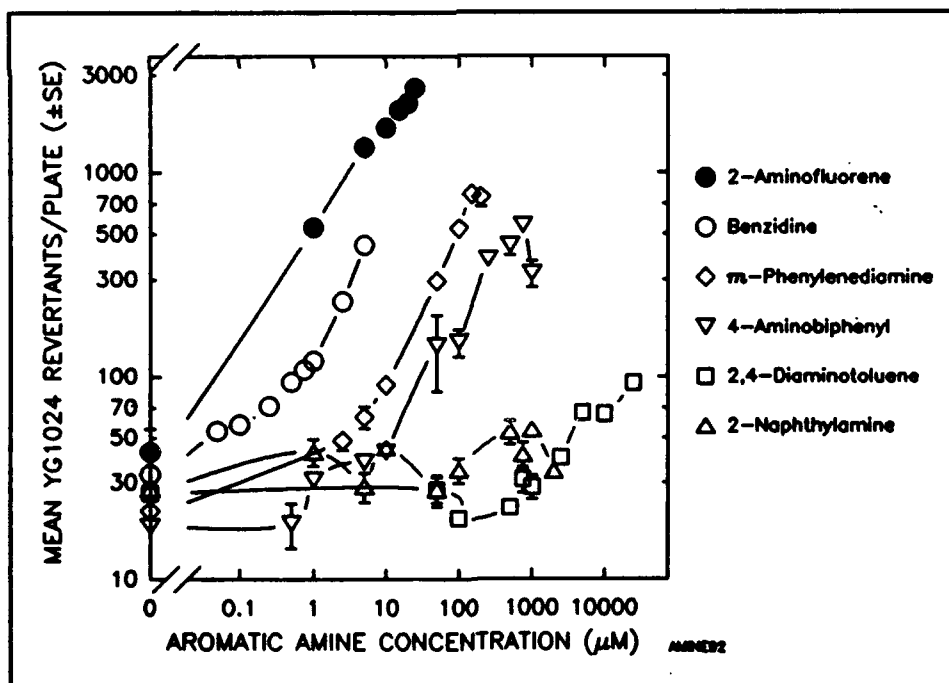


Figure 16 The relative potency of six aromatic amines activated by TX1 plant cells and assayed in *S. typhimurium* strain YG1024.

It is clearly evident that YG1024 is much more sensitive than TA98 to the plant-activated metabolite(s) of selected aromatic amines. The response of YG1024 with the plant activation of six aromatic amines is presented in Figure 16. This differential activation was in the order of 2-aminofluorene > benzidine > *m*-phenylenediamine > 4-aminobiphenyl > 2,4-diaminotoluene. 2-

Naphthylamine was not plant-activated. From a structure-function perspective, there appears to be some correlation between free diamino functional groups. This becomes evident if you consider the relative plant-activation potency of *m*-PDA and 2,4-diaminotoluene, and benzidine and 4-aminobiphenyl (Figure 16). Although the model presented here may not be specifically correct, it is a general account for the data on the plant-activation of aromatic amines. The plant-activated *m*-PDA product is stable, and is associated with a macromolecule. The plant-activated product is further metabolized by cells with high acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase activity. The possibility exists that these plant-activated products may be formed into non-toxic proximal mutagens in intact plants that can be metabolized back into potent frameshift mutagens in organisms that consume the plants. This research suggests that plants may serve as a repository of proximal mutagenic metabolites of xenobiotics that may be transported through the food chain.

3 SUMMARY

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REPORT OF INVENTIONS AND SUBCONTRACTS

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

FORM APPROVED
OMB NO. 0750-0046

1a. NAME OF CONTRACTOR/ SUBCONTRACTOR University of Illinois	c. CONTRACT NUMBER AFOSR-91-0432	3. TYPE OF REPORT (check one) <input checked="" type="checkbox"/> INTERIM <input type="checkbox"/> FINAL
b. ADDRESS (include Zip Code) 105 Davenport House 809 S. Wright, Champaign IL 61801	d. AWARD DATE (Y/N/M/D) 910930	4. REPORTING PERIOD (Y/N/M/D) FROM 910930 TO 920430

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None", so state)		d. ELECTION TO FILE PATENT APPLICATIONS		6. CONFIRMATION OF INSTRUMENT OR ASSIGNMENT (FORWARD TO CONTRACTING OFFICE)
a. NAME OF INVENTOR(S) (Last, First, M.I.)	b. TITLE OF INVENTION(S)	UNITED STATES		
		YES	NO	YES
none				
1. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR.				7. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED
ii. NAME OF INVENTOR (Last, First, M.I.)				8. FOREIGN COUNTRY OF PATENT APPLICATION
iii. NAME OF EMPLOYER				
iii. ADDRESS OF EMPLOYER (include Zip Code)				

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

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR. (If "None", so state)		d. "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	1. SUBCONTRACT DATES (Y/N/M)	AWARD ESTIMATED TO BE COMPLETED
a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (include Zip Code)	c. SUBCONTRACT NO.(S)	DATE (Y/N/M)			
			CLAUSE NO.	DATE (Y/N/M)		
none						

SECTION III - CERTIFICATION

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

8. 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.

9. TITLE _____ SIGNATURE OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL _____ DATE (Y/N/M) _____