LANL Reference

PROGRESS REPORT FOR THE PERIOD ENDING SEPTEMBER 30, 1991

TITLE: **"BIODEGRADATION OF EXPLOSIVES"**

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RESEARCH OBJECTIVES

It is the goal of the collaborating group at LANL to isolate the enzymes involved in the breakdown of TNT and related molecules, in order to perform a partial amino acid sequence analysis, synthesize gene primers based on the amino acid sequence, and use the PCR reaction to amplify the genes encoding these enzymes. A detailed understanding of the individual steps of the biotransformation reactions resulting in the breakdown of TNT, and related nitrobody-containing explosives, is a prerequisite for the identification of the involved enzyme systems. Therefore, the objective of the NMSU participants in this project is to isolate and identify the metabolites of TNT that are produced by cultures of ***Datura*** cells, and from cells of related plants in the **Solanaceae** plant family, in order to elucidate the metabolic pathways that lead from the parent molecule to its ultimate biotransformation products. In addition to providing the needed understanding of the enzymes involved in the biodegradation of the target chemicals, this study also will furnish information on the potentially toxic intermediates, or end products, that may be formed by the ***Datura*** cells, and/or cells from related plant that are members of the **Solanaceae** plant family, so that the efforts of genetic manipulation in the later parts of the program can be directed to maximize, minimize, or exclude the formation of such metabolites, as desired. Also, from a practical standpoint, it should be remembered that whatever takes place in tissue cultures usually occurs in whole plants, perhaps, with some quantitative differences.

SUMMARY

We are certain that the nitrobody-containing explosive, TNT, is not only rapidly taken up from the medium into the solanaceous plants ***Datura*** and ***Lycopersicon***, but that, once inside the plants, it is being metabolized into transformation products. Importantly, the transformation is not unique to ***Datura***. We are now fairly certain that one of the products is diamino-nitrobenzoic acid. We also believe that that we have identified the precursor to this compound,

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diamino-nitrobenzyl alcohol. The reduction of the nitro-groups to amino-groups also is significant. It would suggest that some of the additional metabolites that we can expect to find might be, among others, deaminated derivatives of this molecule. We are still proceeding with this analysis, although the work is proceeding a little more slowly than we would like. The fact that the renovation of Dr. Jackson's laboratory now has been completed will expedite these studies.

As indicated above, while waiting to continue our studies on *Datura* in Dr. Jackson's laboratory, we have been examining another member of the Solanaceae plant family, *Lycopersicon peruvianum*. Preliminary results indicate that tissue cultures from this plant, too, rapidly accumulate and metabolize TNT. We are seeing as many as 8 to 10 metabolites being accumulated and changing with time in this plant. This establishes that the phenomenon originally observed in *Datura innoxia* is not unique within the Solanaceae plant family.

MILESTONES

1. Verified that TNT is rapidly accumulated and metabolized by *Datura*. There is no evidence of glycosylation reactions.

2. Three analytes have now been identified; an amino-dinitro-toluene, a diamino-nitrobenzyl alcohol, and a diamino-nitrobenzoic acid. Other analytes are being elucidated.

3. As one would expect, the analyte quantity and patterns change with time. This should aid in elucidating the pathways involved.

4. An additional plant of the Solanaceae plant family, *Lycopersicon peruvianum*, rapidly accumulates and transforms TNT with much the same pattern as that observed for *Datura innoxia*. This might mean that all solanaceous plants utilize the same metabolic pathways for remediating xenobiotics of this type.

PROGRESS REPORT AND DISCUSSION

A) TNT Metabolism by Suspension Cultured Cells of *Lycopersicon* :
Experiments on ***Lycopersicon*** were initiated and are still in progress. Suspension cultured cells were grown using the same techniques as those used for ***Datura innoxia***. However, these cells were grown at NMSU in the Plant Genetics Laboratory. [Radiolabeled TNT was obtained by recovering ^{14}C-TNT from the medium of two ***Datura*** experiments from LANL which aborted]. Upon the inoculation of the suspension cell cultures of ***Lycopersicon*** with 134 mg/L of TNT (specific activity of ^{14}C-TNT 1.46 uCi/mmol), it was rapidly removed from the growth medium and metabolized, as described previously in the June quarterly report.

B) Isolation and Analysis of TNT Metabolites Produced by Suspension Cultured Cells of *Datura innoxia* and *Lycopersicon peruvianum*.
Samples of the metabolite fractions were recovered following preparative HPLC chromatograpy see **Figure 1**, Growth Medium and **Figure 2**, Cell Extract. The most polar of the metabolite fractions was analyzed by HPLC-mass spectrometry with thermospray interface and yielded a mass spectrum indicating the presence of a diamino-nitrobenzoic acid. None of the mass spectra obtained from this fraction by LC-MS shows any indication of conjugated metabolites. Subsequent GC-MS studies of the isolated metabolite fractions after methylation with diazomethane revealed the structure of three metabolites:

an amino-dinitrotoluene **(Figure 3)**
a diamino-nitrobenzyl alcohol **(Figure 4)**
and a diamino-nitrobenzoic acid **(Figure 5)**.

The mass spectra in figures 4 and 5 show the methylated derivatives of the plant metabolites. The pathway of biotransformation of TNT in ***Datura*** and ***Lycopersicon*** appears to be a combination of nitroreduction and stepwise oxidation of the methyl group. This requires the action of the following enzyme systems: Cytochrome P-450 is most likely involved both in nitroreduction and in the first oxidation step that converts the toluene methyl group to the benzyl alcohol. The following steps oxidizing the benzyl alcohol further to benzaldehyde and benzoic acid are then mediated by alcohol- and aldehyde dehydrogenases.
Recent mass spectral evidence for the presence of unsubstituted benzoic acid in extracts of ***Lycopersicon*** cells incubated with TNT indicates a mechanism of denitrification, probably deamination after the preceding reduction of the nitro-groups. This finding is, however, still somewhat tentative and requires further confirmation.
Of the metabolites found so far in our studies, the product of the very first step, an amino-dinitrotoluene was reported earlier as a plant metabolite of TNT by Palazzo and Legett (1986)*. The determination of the exact structures for all of the TNT metabolites in the ***Datura innoxia*** and in the ***Lycopersicon peruvianum*** cell suspension systems are presently in progress. It is important to find this process occurring in two solanaceous plants, because it shows that the process is not unique to ***Datura innoxia***.

*) Palazzo, A.J., and D.C. Leggett (1986) Effect and disposition of TNT in a terrestrial plant and validation of analytical methods, **USA Cold Regions Research and Engineering Laboratory (CRREL)**, Report 86-15.

FIGURE 1: RADIO-HPLC OF GROWTH MEDIUM
0.5, 2.5 AND 4.5 h AFTER TNT ADDITION

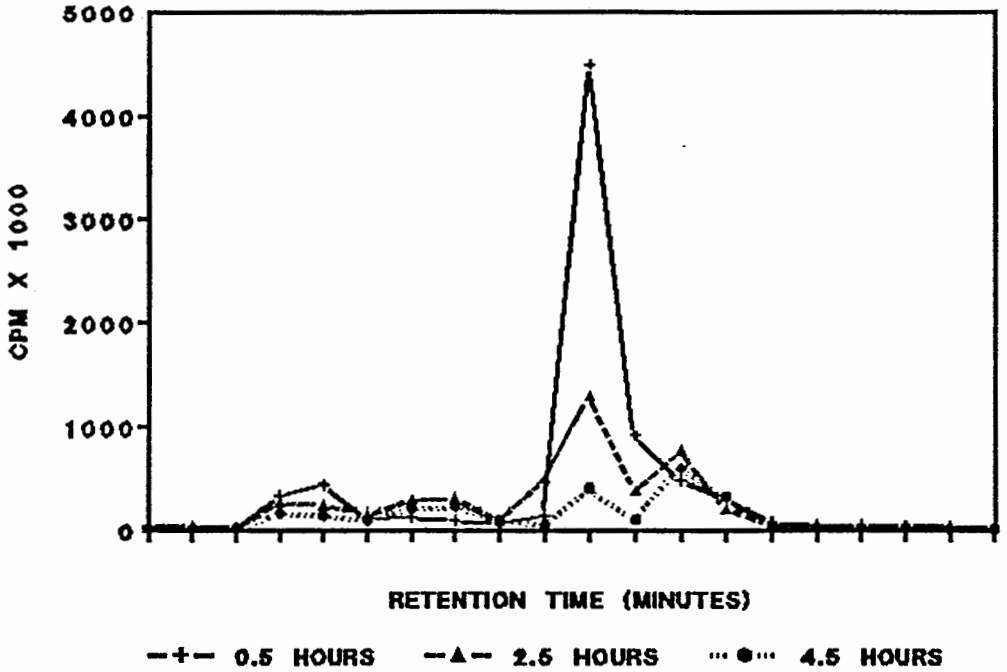
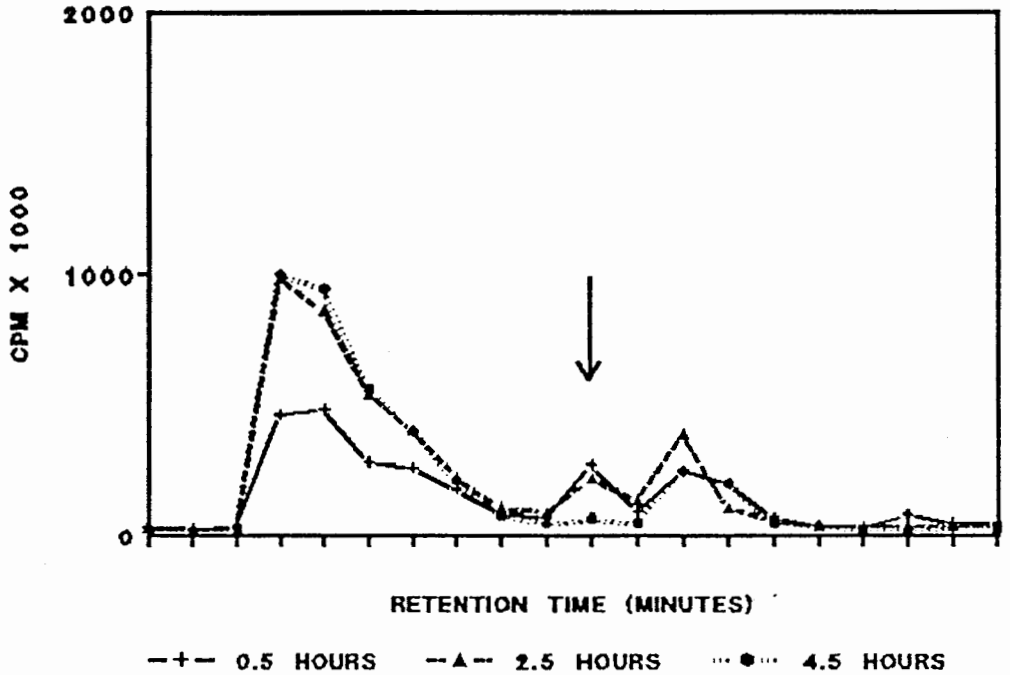


FIGURE 2: RADIO-HPLC OF CELL EXTRACT
0.5, 2.5 AND 4.5 h AFTER TNT ADDITION



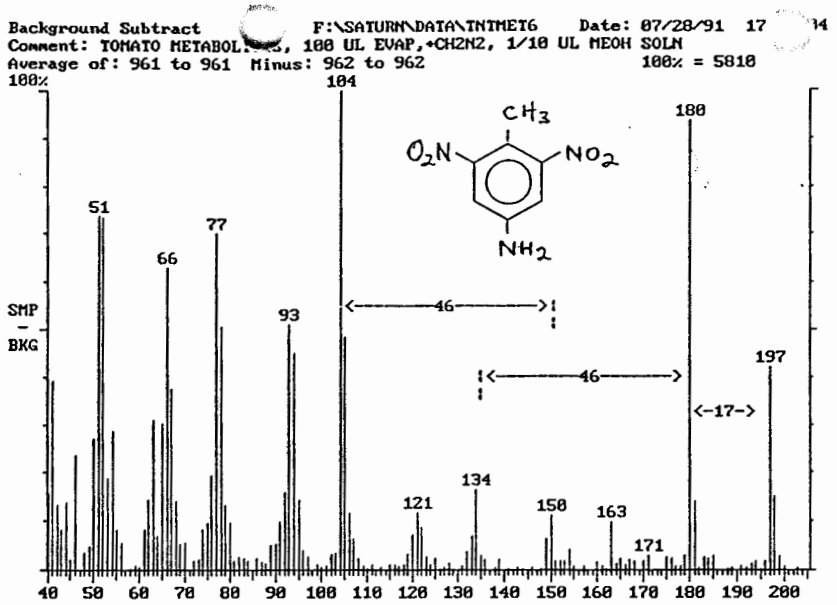


Figure 3

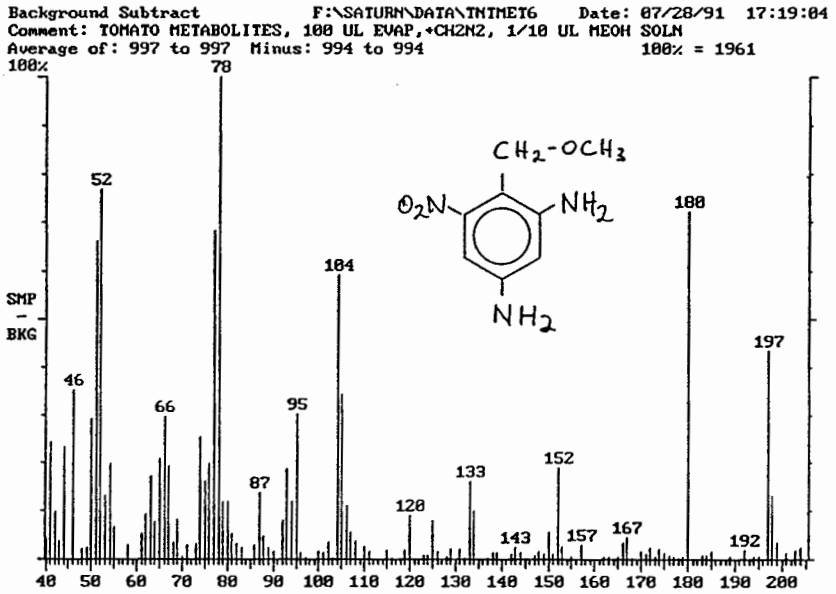


Figure 4

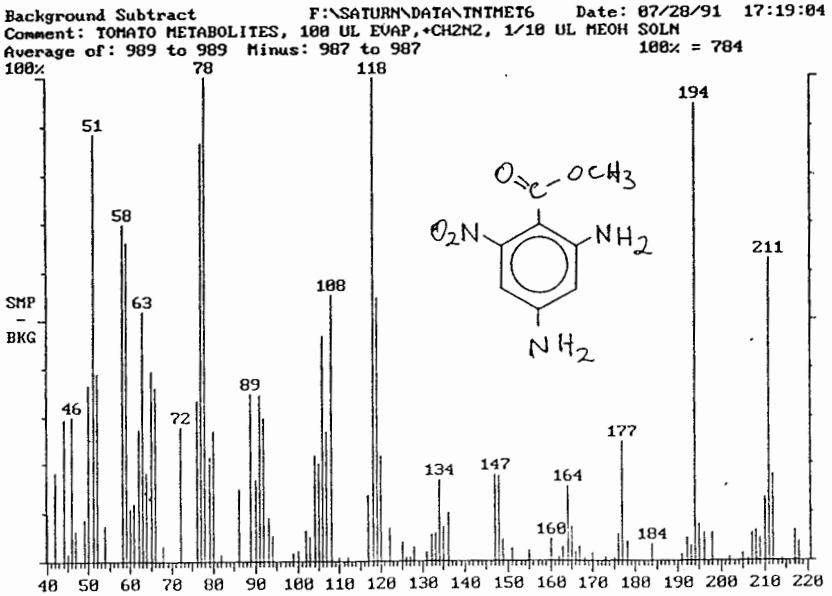


Figure 5