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# TNT METABOLITES IN ANIMAL TISSUES

## Quarterly Technical Progress Report (No. 2)

## January 1, 1989, to March 31, 1989

L. R. Shugart

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ENVIRONMENTAL SCIENCES DIVISION

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Date Published - - April 1989

Prepared for

U.S. Army Biomedical Research & Development Laboratories
Fort Detrick, MD 21701-5010

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Contract No.:

U.S. Army no. 88PP8866
Interagency Agreement no. 1016-B073-A1

Contract Title:

**TNT METABOLITES IN ANIMAL TISSUES**

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Report Date:

April 1, 1989

Type of Report:

Quarterly Technical Progress Report (No. 2)
January 1, 1988 to March 31, 1988

Contracting Officer's Technical Representative:

Dr. David H. Rosenblatt
U.S. Army Biomedical Research & Development Laboratories
ATTN: SGRD-UBZ-C
Fort Detrick, MD  21701-5010

## I. PURPOSE AND SCOPE OF RESEARCH EFFORT

The overall objectives of this project are: (1) to provide quantitative analytical procedures for the analysis of TNT and at least eight of its metabolites in animal tissues, and (2) to obtain representative samples of tissues from animals from designated Army sites and to determine the presence or absence of TNT and its metabolites in these samples. The study is divided into two Phases corresponding to the stated overall objectives of the project.

## II. OVERALL PROGRESS

### 1. PHASE I.

a. Task 1: Procurement of authentic metabolites of TNT.

(1). Metabolite synthesis and purification. The work related to the preparation of the TNT metabolites for an animal tissue study has been successfully completed in this report period. In the last five months, a total of ten TNT metabolites have been either purchased or synthesized. The following seven compounds have been purchased from an outside vendor (Aldrich) or obtained in SARM purity from the U. S. Army: 2,4,6-trinitrotoluene; 2,4,6-trinitrobenzoic acid; 2-amino-4,6-dinitrotoluene; 4-amino-2,6-dinitrotoluene; 2,4-diamino-6-dinitrotoluene; 2,6-diamino-4-nitrotoluene; and 1,3,5-trinitrobenzene. The purity of each compound was checked using high-pressure liquid chromatography (HPLC) on a C-18 reversed phase column. With the exception of 2,4,6-trinitrobenzoic acid, the purity of each purchased compound was found to be more than 99%.

The commercially-available 2,4,6-trinitrobenzoic acid was only 60-70% pure. Further purification was performed using HPLC; 100 mg of this compound was isolated. The structure of the product was deduced from its positive acidic character (extractable by sodium hydroxide solution) and its infra-red spectrum (carboxyl group plainly visible). This analyte is not stable in aqueous solution, and it autodecarboxylates easily to 1,3,5-trinitrobenzene.

The 4-hydroxylamino-2,6-dinitrotoluene has been synthesized according to Nielsen et al (1979). It was purified using a preparative scale silica column, and also by preparative TLC. The structural determination was based upon mass spectrometry. About 100 mg of pure 4-hydroxylamino-2,6-dinitrotoluene was prepared.

Our attempts, as well as those of Sitzmann (1974), to prepare 2-hydroxyamino-4,6-dinitrotoluene by the above method or by the method of McGookin et al (1940) were unsuccessful. Further literature surveys questioned whether this compound had ever been truly synthesized. We noted some confusion in the literature between the 2- and 4- isomers. Nielson et al (op. cit.) was only able to report a speculated NMR spectrum of 2-

hydroxylamino-4,6-dinitrotoluene in 4-hydroxylamino-2,6-dinitrotoluene reaction mixture. All of these observations suggest that 2-hydroxylamino-4,6-dinitrotoluene really has never been synthesized. A great effort for obtaining this never-made compound may be needed if it appears to be an important intermediate or stable product of TNT metabolism.

The 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (not an originally scheduled compound, but one which may be important to determine) has been synthesized easily according to Sitzmann (1974). The product was recrystallized twice from benzene, yielding 50 mg of purified compound.

While 2,4,6-trinitrobenzyl alcohol may be prepared using several different reaction methods, the best is that reported by Gauguly (1925). We modified this procedure somewhat to accommodate our existing laboratory facility. The synthesis procedure is a two-step reaction with an overall yield of 12%, described as follows:

(1) Bromination of 2,4,6-trinitrotoluene with liquid bromine at 150-160 C for 20 hours yields 20% 2,4,6-trinitrobenzyl bromide.

(2) Hydrolysis of 2,4,6-trinitrobenzyl bromide at 115 C for three hours yields 60% 2,4,6-trinitrobenzyl alcohol.

Both the intermediate and final products were purified by HPLC, and the bromination and hydrolysis, respectively, were confirmed by mass spectrometry. About 100 mg of purified 2,4,6-trinitro- benzyl alcohol was obtained.

The amount of each of ten compounds either purchased or synthesized is sufficient for supporting the activities described in Phase I, Task 2, Development of Analytical Procedures (see below). After the important and detectable TNT metabolites are determined, the syntheses should be scaled-up as required.

We have also shipped ca. 100 mg aliquots of each of three phenylenediamine isomers, the two diamino-mononitrotoluene isomers, and 2,4,6-trinitrobenzoic acid to Dr. Dominic Cataldo (Pacific Northwest Laboratory) at the request of Dr. Wayne Mitchell.

(2). <u>Enzymatic</u> <u>Synthesis</u>. Work has begun using an S9 preparation for the <u>in</u> <u>vitro</u> synthesis of TNT metabolites. Initial findings, based on HPLC profiles, using non-radiolabelled TNT as a substrate, show four major metabolites of TNT; the 2-amino and 4-amino dinitrotoluenes, and the 2,4- and 2,6-diamino nitrotoluenes. These results will be verified using $^{14}$C-TNT, however, it appears that no unusual metabolite will be obtained using this approach. In addition, the metabolites that are generated are obtainable commercially.

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Gauguly, Chem. Berichte, 708 (1925)
McGookin et al, J. Soc. Chem. Ind., **<u>59</u>**:92-94 (1940)
Nielsen et al, J. Org. Chem., **<u>44</u>**:2499-2504 (1979)
Sitzmann, J. Chem. Eng. Data, **<u>19</u>**: 179-181 (1974)

b. Task 2: Development of analytical procedures.

## General Considerations

Two liquid chromatographic instruments were used to characterize the standard compounds fully and to optimize the separation of the ten test analytes. The diode array detector of the Hewlett-Packard 1090 Liquid Chromatograph permitted complete UV absorbance spectra (190-500 nm) of each compound to be recorded "on the fly". Most of the method optimization work was performed on the Beckman Model 334 Liquid Chromatograph, which features a fixed loop injector (nominal values 10 through 250 uL) and a single-wavelength UV detector (254 nm).

Examination of the analyte UV spectra demonstrated that many of the analytes are weak absorbers. This observation was expected because the usual chromophore is a single aromatic ring with one or more nitro substituents. A monitoring wavelength of ca. 230 nm was considered optimal; however 254 nm would be acceptable.

## Optimization of the HPLC Separation Method

Four different types of columns employing two different pairs of eluents were investigated for their suitability in this work. Brief summaries of both the column and eluents employed and relevant observations for each case are given on the following pages.

### Silica with THF/Hexane Eluent.

A normal phase separation featuring an RSil silica column (250 x 4.6 mm i.d., 5 micron particle diameter, Alltech Associates, Deerfield, IL, with Brownlee "NewGuard" silica guard column cartridge, Brownlee Labs, Inc., Santa Clara, CA) and a UV-grade tetrahydrofuran (THF)/hexane eluent (15% THF for 10 min, increase linearly to 65% THF over 55 min) successfully resolved five of the ten test analytes. The exceptions were TNT (a), TNBA, and Az, which eluted with the solvent front and were separated at less than 25% valley. The remaining two compounds, TNB and TNBAlc, were not investigated.

We did not pursue the normal phase separation further for several reasons. First, even UV-grade THF possesses sufficient absorbance at 254 nm to render high-sensitivity measurements impossible under significant gradient conditions. As the concentration of THF in the eluent increases, so does its absorbance. As a result, the absorbance of small peaks becomes obscured by the baseline ramp. Second, in order to achieve the desired

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(a) TNB = 1,3,5-trinitrobenzene; TNBA = 2,4,6-trinitrobenzoic acid; TNBAlc = 2,4,6-trinitrobenzyl-1-ol; 26DA-4NT = 2,6-diamino-4-nitrotoluene; 24DA-6NT = 2,4-diamino-6-nitrotoluene; TNT = 2,4,6-trinitrotoluene; 4HyA26-DNT = 4-hydroxylamine, 2,6-dinitrotoluene; 2A46-DNT = 2-amino-4,6-dinitrotoluene; 4A26-DNT = 4-amino-2,6-dinitrotoluene; Az = 2,2′, 6,6′-tetranitro-4,4′-azoxytoluene sensitivity of 1 ug/mL for each analyte, it would be necessary to inject a large sample volume consisting mainly of THF, which would be needed to dissolve the test analytes. Such an injection would likely disrupt whatever resolution was obtained at the beginning of the program, where the eluent is largely hexane. The same problems would doubtless apply to the injection of real tissue samples as well. During the development of the separation method, a maximum injection volume did not exceed 10 uL.

### Econosphere C8 with Methanol/Water Gradient.

A partial resolution of nine test compounds was achieved when they were eluted from an Econosphere C8 (Octyl) column (150 x 4.6 mm i.d., 5 micron particle diameter, Alltech Associates, Deerfield, IL, equipped with a dry-packed guard column, Upchurch Scientific, Inc., Oak Harbor, WA) using a methanol/water gradient (15% methanol for 30 min, then increase to 90% methanol over 40 min). Two serious coelution problems were observed: TNB / TNBA and TNT / 4A26-DNT / 4HyA26DNT. TNBAlc was not tested; however, it would probably coelute with TNB and TNBA.

The coelution problems were sufficiently severe to render this particular separation unusable, and we did not pursue it further.

### Econosphere C8 with Acetonitrile/Water Gradient.

A more satisfactory method resulted when acetonitrile was substituted for methanol in the system described above. The gradient program started with 20% acetonitrile (hold for 30 min), changed linearly to 30% acetonitrile over five minutes (hold at 30% for 20 min), then ramped linearly again to 90% acetonitrile over 25 min. When a mixture of all analytes except TNBAlc was injected onto the column, all species except TNB and TNBA were resolved cleanly. TNBAlc would probably coelute with the latter two species.

This method was certainly more workable than that employing a methanol/water gradient, but it required an intermediate "hold" step in the middle of the program, and therefore was not a straightforward procedure. Furthermore, the baseline absorbance did increase with increasing acetonitrile concentration, albeit not as steeply as with UV grade THF/hexane. We pursued separation procedures using other columns rather than stay with this procedure.

### Zorbax C18 with Methanol/Water Gradient.

A partial separation of a nine-component test mixture (1 ug/mL in all components except TNBAlc) was achieved by eluting a 250 uL aliquot of the standard through a Zorbax ODS column (250 x 4.6 mm i.d., 5 micron particle diameter, MAC-MOD Analytical, Inc., Chadds Ford, PA, equipped with a dry-packed guard column, Upchurch Scientific) using a methanol/water gradient (35% methanol for 10 min, then increase linearly to 85% methanol over 60 min). The resulting chromatogram was similar to that produced using the Econosphere C8 column described above. TNB and TNBA coeluted, as did TNT and 4HyA26-DNT. The two isomers 2A46-DNT and 4A26-DNT were just barely resolved, and quantitation of either one would be unreliable.

The coelution problems were sufficiently severe to render this particular separation unusable, and we did not pursue it further.

Zorbax C18 with Acetonitrile/Water Gradient.

The separation of a 250 uL of a ten-component test mixture (1 ug/mL in all components, water matrix) was evaluated using the Zorbax ODS column and guard column described above, but employing a different solvent system. The analytes were eluted using an acetonitrile/water program, in which the concentration of acetonitrile was initially held at 20% for 10 minutes, then increased linearly over 60 minutes to 75% (hold for 10 minutes).

The resulting chromatogram, shown in Figure 1, clearly shows nine peaks for the ten analytes. TNB and TNBA coeluted (peaks d and e in the figure), while the two monoamine-dinitro isomers (peaks f and g) were resolved at less than 25% valley. Reliable quantitation of the latter analyte pair would not be feasible. On the other hand, a difficult pair of analytes, TNT and 4Hy26-DNT, were separated with almost baseline resolution.

Figure 1 also demonstrates that all species can be detected easily at the 1 ug/mL level (25 ng injected), and that all species with the exception of 24DA-6NT could probably be detected at 0.1 ug/mL (25 ng injected). The detection limit of the aforementioned analyte is probably ca. 0.3 ug/mL (75 ng injected).

This analytical procedure would be acceptable if further research demonstrated that TNB, TNBA, and the two monoamine-dinitro isomers were present in negligible concentrations, and that the most important peaks would be the two diamine-mononitro isomers, TNT, 4Hy26-DNT, and TNBAlc.

Supelco LC-CN with Methanol/Water Gradient.

Another analytical separation with useful features employed a Supelco LC-CN column (250 x 4.6 mm i.d., 5 micron particle size, Supelco, Inc., Bellefonte, PA, equipped with a Brownlee "NewGuard" cyano guard column cartridge, Brownlee Labs, Inc., Santa Clara, CA) and a methanol/water gradient (3% methanol for 30 min, then increase the methanol concentration to 85% in 10 min and hold at 85% for 10 min). Under these conditions, all analytes except Az elute with 3% methanol. The methanol concentration is then increased rapidly to remove Az. A typical chromatogram of a 250 uL aliquot of a ten-component mixture (water matrix, all analytes present at 1 ug/mL, 250 ng injected) is shown in Figure 2.

Because the ten selected analytes are inherently poor UV absorbers, the most convenient way to achieve the desired sensitivity was to increase the sample volume taken. In one experiment, the sample volume was increased from 10 uL to 250 uL without significant loss of resolution of most analytes, as shown in Figure 2. The exceptions were 26DA-4NT / 24DA-

ORNL-DWG 89-9278

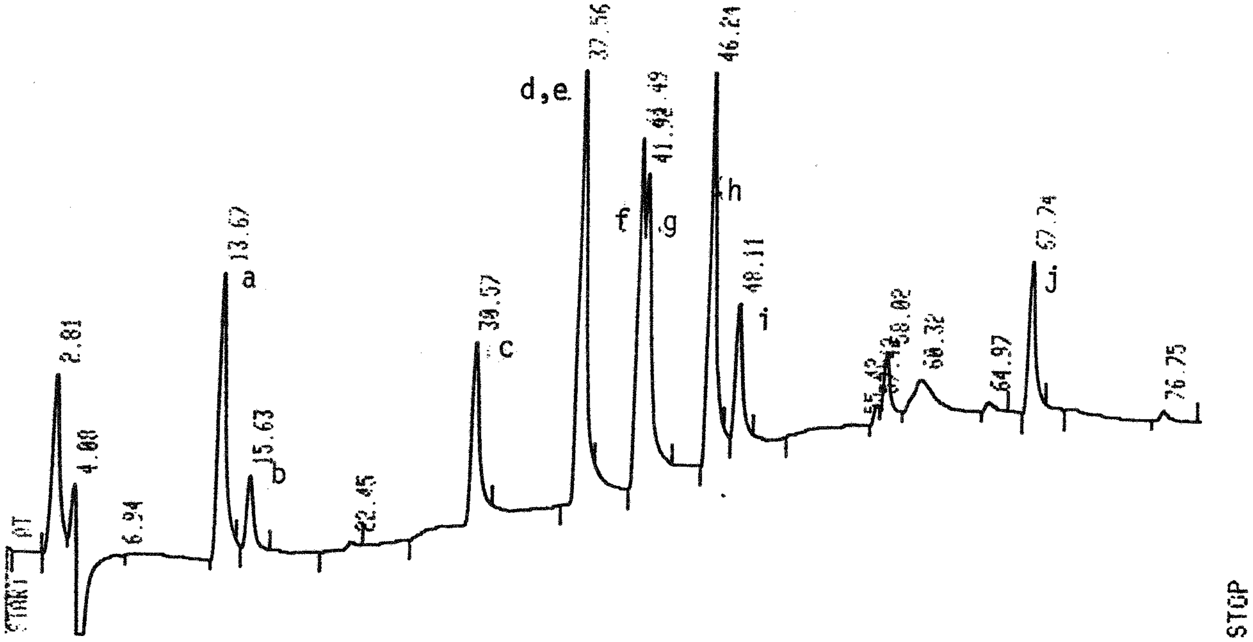

Figure 1. Separation of a Ten-Component Mixture of TNT Metabolites at the 1 ug/mL Level Using a Zorbax ODS 250 x 4.6 mm Column. Eluent program: Acetonitrile/Water, as described in text. Injection volume: 250 uL of water matrix.

a = 2,6-diamino-4-nitrotoluene; b = 2,4-diamino-6-nitrotoluene; c = 2,4,6-trinitrobenzyl alcohol; d = 1,3,5-trinitrobenzene; e = 2,4,6-trinitrobenzoic acid; f = 2-amino-4,6-dinitrotoluene; g = 4-amino-2,6-dinitrotoluene; h = 2,4,6-trinitrotoluene (TNT); i = 4-hydroxylamine, 2,6-dinitrotoluene; j = 2,2′,6,6′-tetranitro- 4,4′-azoxytoluene

ORNL-DWG 89-9279

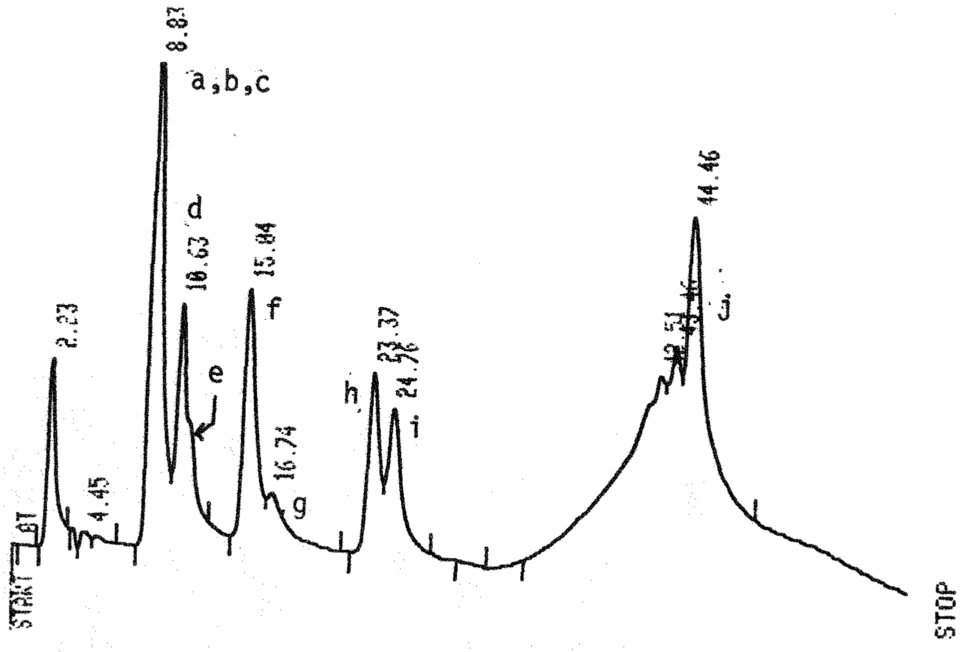

Figure 2. Separation of a Ten-Component Mixture of TNT Metabolites at the 1 ug/mL Level Using a Supelco LC-CN 250 x 4.6 mm Column. Eluent program: Methanol/Water, as described in text. Injection volume: 250 uL of water matrix.

a = 1,3,5-trinitrobenzene; b = 2,4,6-trinitrobenzoic acid; c = 2,4,6-trinitrobenzyl alcohol; d = 2,6-diamino-4-nitrotoluene; e = 2,4-diamino-6-nitrotoluene; f = 2,4,6-trinitrotoluene (TNT); g = 4-hydroxylamino-2,6-dinitrotoluene; h = 2-amino-4,6-dinitro- toluene; i = 4-amino-2,6-dinitrotoluene; j = 2,2′,6,6′-tetranitro-4,4′-azoxytoluene

6NT and TNT / 4HyA26DNT. The three compounds TNB, TNBA, and TNBAlc, coelute as the first peak in the chromatogram.

Water must be the principal solvent (>95%) of the samples to insure proper compatibility with the eluent. Under these conditions, the analytes 26DA-4NT, TNT, 2A46-DNT, 4A26-DNT, and Az may be detected individually and routinely at 1 ug/mL (250 ng injected) with a detection limit of ca. 0.1 ug/mL (25 ng injected) for these five species, as shown in Figure 2. The three aforementioned TNB species are observed as an aggregate (nonresolved) peak with a detection of ca. 0.050 ug/mL (probably each can be detected individually with a detection limit of 0.1 ug/mL, or 25 ng injected).

This particular analytical method should be considered the procedure of choice if further research indicates that the principal metabolites are the monoamino-dinitro isomers, since these species are separated at better than 50% valley and can be identified and quantitated reliably.

The possible resolution of the TNT metabolites using the Supelco LC-CN column was suggested by the work of Jenkins and Walsh (1987), who used both a Supelco LC-18 (octadecyl reversed-phase) column with a 50/50 (vol/vol) methanol/water eluent to separate a mixture of munitions and the Supelco LC-CN (cyano) column (same eluent) to confirm the identity of their analytes. The authors commented that retention on the LC-18 column appeared to follow normal hydrophobic behavior, with the retention order predictable from the octanol-water partition coefficients of the analytes, while there appeared to be a specific interaction between the nitrile function of the LC-CN column and the nitramine function of the analytes. The authors added that a specific interaction between the nitrile function and the nitramines would also explain the 20 to 30-fold increase in solubility for these compounds compared to methanol.

c. Task 3: Analysis of metabolites in laboratory animals.

Scheduled to commence April, 1989. (see Section IV below)

2. PHASE II.

Scheduled to commence April, 1989.

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T. F. Jenkins and M. E. Walsh, "Development of an Analytical Method for Explosive Residues in Soil", CRREL Report 87-7, U. S. Army Toxic and Hazardous Materials Agency, Report AMXTH-TE-FR-86102, June, 1987.

## III. PROBLEM AREAS

We have observed that 2,4,6-trinitrobenzoic acid and its autodecarboxylation product 1,3,5-trinitrobenzene cannot be separated using either Supelco LC-CN, Zorbax ODS, or Econosphere C8, as documented in the progress for Phase I, Task 2, but can be separated readily using an Adsorbosphere HS C18 column (Alltech Associates, Deerfield, IL, 250 x 4.6 mm) with an acetonitrile/ water, 50/50 (vol/vol) eluent. Adsorbosphere HS C18 is comparable to Zorbax ODS in terms of particle shape (spherical), specific surface area (350 $m^2/g$), and carbon loading (20%). We suggest that there may be subtle differences in the silica surface and/or endcapping procedures which yield a different selectivity. We will compare the performance of the Adsorbosphere HS C18 column with that of Zorbax ODS for all of the test metabolites in the next reporting period.

## IV. WORK TO BE PERFORMED DURING NEXT QUARTER

### 1. PHASE 1.

a. Task 1. The procurement of TNT and its metabolites is essentially complete as described. Additional quantities will be purchased or synthesized as needed.

Enzymatic synthesis of TNT metabolites will be attempted using preparations spiked with $^{14}$C-TNT. Separation of synthesized metabolites will be by HPLC. Preliminary identification will be by relative retention times compared to standards.

b. Task 2. The HPLC capability to identify TNT and its metabolites is in place and will be used in the completion of Task 1 (see above) as well as the verification of a suitable extraction procedure that is being developed as part of Task 3.

c. Task 3. Several procedures were tested for the extraction of TNT and its metabolites from spiked animal tissues (liver and muscle). The procedure that appears to produce the highest recoveries from these tissues with the minimum co-extraction of UV-interfering materials consists of: homogenization in water/$CH_3CN$ followed by solid phase extraction using a C18 SepPak. The HPLC techniques described in Task 2 will be used to verify the extraction efficiencies found for TNT and its various metabolites from 10 grams each of rabbit and deer tissue spiked with 1 microgram of each compound.

The dosing of small laboratory animals with radiolabelled TNT will be initiated as outlined in the Technical Proposal.

### 2. PHASE 2.

a. Task 4. Sampling of deer, rabbit, and quail from the U.S. Department of Energy reservation at the Oak Ridge National Laboratory will be initiated. Storage of suitable tissues obtained from these animals will be at liquid nitrogen temperature.

A preliminary meeting has been scheduled with Cody Jackson and Dennis Jones of ATSDR/CDC Atlanta, GA for April 19, 1989 to discuss the sampling of animals at an appropriate Army Site as described in the Technical Proposal.

3. OTHER.

a. A meeting with the Technical Representative, Dr. David Rosenblatt, to discuss the progress of this work, has been scheduled for Monday, May 8, 1989 at the Oak Ridge National Laboratory.

b. Distribution of metabolites. TNT metabolites (about 100 milligram quantities) will be made available, upon request, to authorized representatives of U.S. Army Biomedical Research & Development Laboratories.

## V. ADMINISTRATIVE COMMENTS

This report was prepared according to the instructions found in the U.S. Army Medical Bioengineering Research & Development Laboratory document entitled "Contractor Reporting Requirements for the Health Effects Research Division" dated March 1984.

## VI. GANTT CHART

Attached.

## VII. COST SUMMARY REPORT

1. PROJECT COST SUMMARY

Attached.

2. COST SUMMARY GRAPH

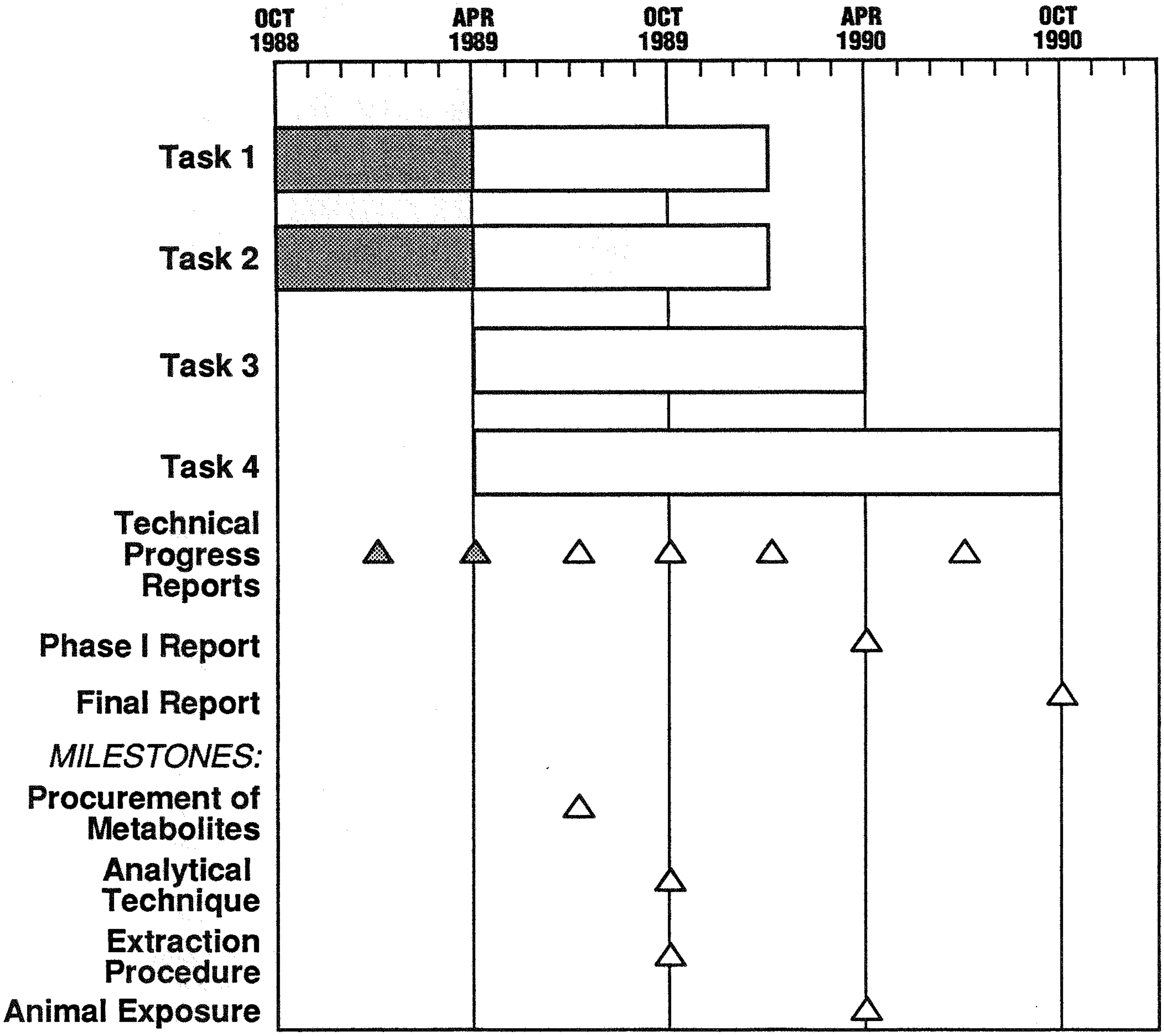
Attached.

L. R. Shugart

L. R. Shugart, March 31, 1989

ORNL-DWG 89M-1510

**Project Title: TNT Metabolites in Animal Tissues**
**Contract No.: U.S. Army 88PP8866**
**IAG 1016-B073-A1**
**Reporting Period: January 1, 1989 to March 31, 1989**
**Performing Organization: Oak Ridge National Laboratory**
**Principal Investigator: L. R. Shugart**
**Date: April 1, 1989**



# PROJECT COST SUMMARY

Project Title/No.: TNT Metabolites in Animal Tissues/U.S. Army #88PP8866
Organization/PI: Oak Ridge National Laboratory/L. R. Shugart
Reporting Period: January 1, 1989 to March 31, 1989

Total Allocation: $114,553
91,079
$205,632

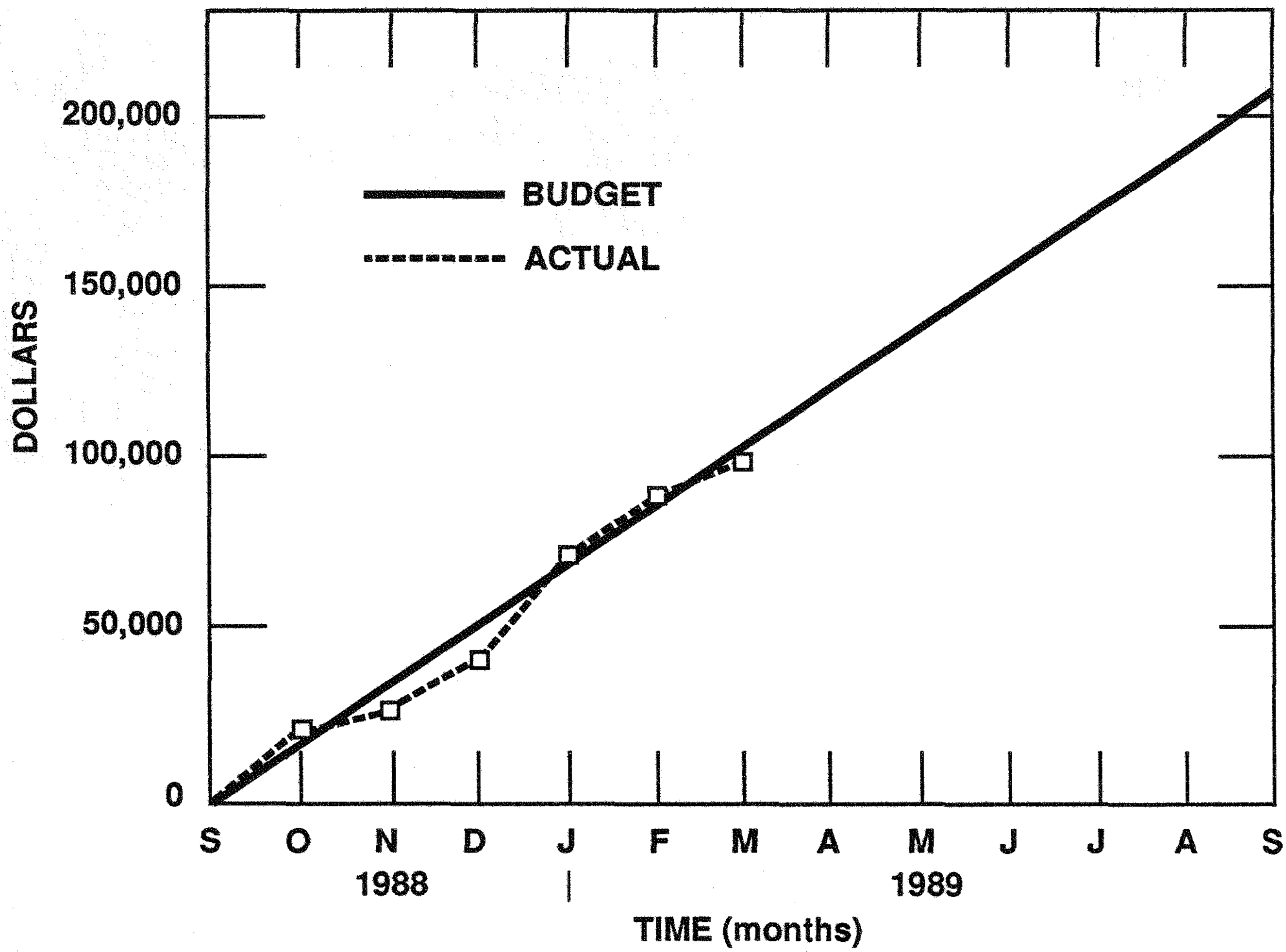
| | Monthly Expenditures | | | Cumulative Expenditures | | | Available |
|---|---|---|---|---|---|---|---|
| | Budget | Actual | Variance | Budget | Actual | Variance | Balance |
| | | | | | | | 205,632 |
| Oct | 17,136 | 17,641 | + 505 | 17,136 | 17,641 | + 505 | 187,991 |
| Nov | 17,136 | 8,906 | -8,230 | 34,272 | 26,547 | -7,725 | 179,085 |
| Dec | 17,136 | 16,092 | -1,044 | 51,408 | 42,639 | -8,769 | 162,993 |
| Jan | 17,136 | 28,446 | +11,310 | 68,544 | 71,085 | +2,541 | 134,547 |
| Feb | 17,136 | 18,301 | +1,165 | 85,680 | 89,386 | +3,706 | 116,246 |
| Mar | 17,136 | 10,322 | -6,814 | 102,816 | 99,708 | -3,108 | 105,924 |
| Apr | 17,136 | | | 119,952 | | | |
| May | 17,136 | | | 137,088 | | | |
| Jun | 17,136 | | | 154,224 | | | |
| Jul | 17,136 | | | 171,360 | | | |
| Aug | 17,136 | | | 188,496 | | | |
| Sep | 17,136 | | | 205,632 | | | |

Note: Cost data as of end of each month.
Note: This report was prepared on 3/31/89.

ORNL-DWG 89M-1509

# Cost Summary Graph

**Project Title / No.: TNT Metabolites in Animal Tissues / U.S. Army No. 88PP8866**
**Organization / PI: Oak Ridge National Lab / L. R. Shugart**
**Reporting Period: January 1, 1989 to March 31, 1989**
**Total Allocation: $205,632**



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