



The contents of this report are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such commercial products.

The findings of this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.



PRINTED ON RECYCLED PAPER

# **Explosives Conjugation Products in Remediation Matrices: Interim Report**

by J. C. Pennington, M. E. Honeycutt, A. S. Jarvis,  
V. A. McFarland, D. Gunnison, H. Fredrickson

U.S. Army Corps of Engineers  
Waterways Experiment Station  
3909 Halls Ferry Road  
Vicksburg, MS 39180-6199

A. Z. Li, K. A. Marx  
Department of Chemistry  
University of Massachusetts Lowell  
Lowell, MA 01854

P. G. Thorne, D. C. Leggett  
U.S. Army Cold Regions Research and Engineering Laboratory  
Hanover, NH 03755-1290

D. R. Felt, C. A. Hayes, B. E. Porter  
ASCI Corporation  
1365 Beverly Road  
McLean, VA 22101

C. H. Allersmeier, J. Walker, D. L. Kaplan  
Biotechnology Division  
U.S. Army Natick Research, Development and Engineering Center  
Natick, MA 01760-5020

K. A. Thorn  
U.S. Department of Interior Geological Survey  
Box 25046 Federal Center  
Denver, CO 80225-0046

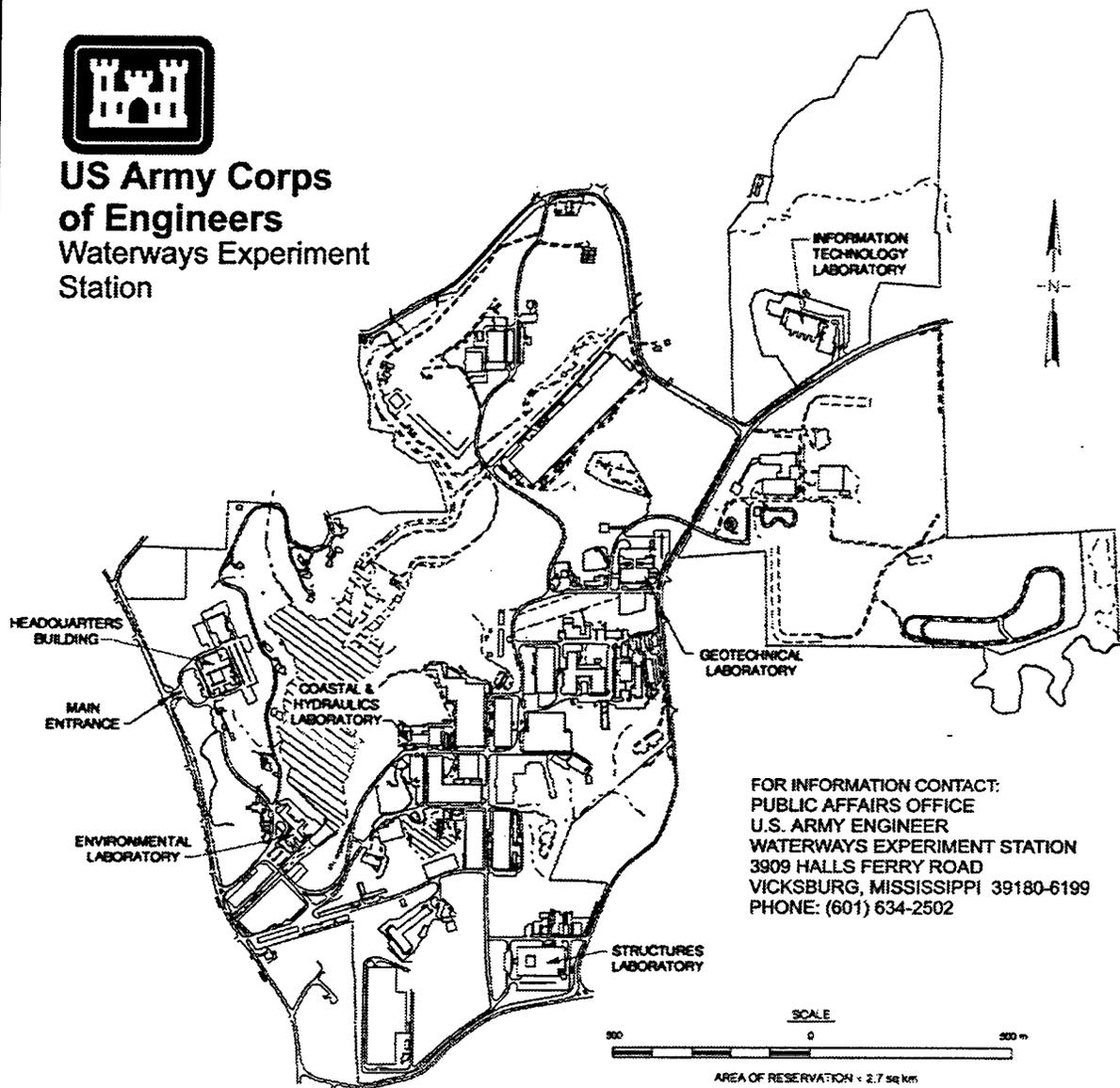
Final report

Approved for public release; distribution is unlimited

DTIC QUALITY INSPECTED 3



**US Army Corps  
of Engineers**  
Waterways Experiment  
Station



**Waterways Experiment Station Cataloging-in-Publication Data**

Explosives conjugation products in remediation matrices : interim report / by J.C. Pennington  
... [et al.] ; prepared for U.S. Army Corps of Engineers.

156 p. : ill. ; 28 cm. -- (Technical report ; SERDP-97-7)

Includes bibliographic references.

1. Soil remediation. 2. Nitrotoluene. 3. Humic acid. I. Pennington, Judith C. II. United States Army. Corps of Engineers. III. U.S. Army Engineer Waterways Experiment Station. IV. Strategic Environmental Research and Development Program (U.S.) V. Series: Technical report (U.S. Army Engineer Waterways Experiment Station) ; SERDP-97-7. TA7 W34 no. SERDP-97-7

# Contents

---

Preface .....	iv
Introduction .....	1
Summary of Results .....	3
1—Cytotoxicity and Mutagenicity of 2,4,6-Trinitrotoluene (TNT) and Its Metabolites .....	7
2—Trinitrotoluene and Metabolites Binding to Humic Acid .....	14
3—Hydrolytic Release of Bound Residues From Composted TNT-Contaminated Soil .....	30
4—Microbial Degradation of Conjugated Fractions .....	52
5—Analysis of Coupling Affinities Among TNT Degradation Products and Humic Acid Based on Surface Plasmon Resonance .....	61
Appendix A: BIOCore Programs and Data .....	A1

SF298

# Preface

---

The report herein was prepared by the Environmental Laboratory (EL) of the U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, in association with the U.S. Army Cold Regions Research and Engineering Laboratory (CRREL), Hanover, NH; the Biotechnology Division, U.S. Army Natick Research, Development and Engineering Center, Natick, MA; the Department of Chemistry, University of Massachusetts Lowell, MA; U.S. Geological Survey, Arvada, CO; and ASci Corporation, McLean, VA. The research was sponsored by the Strategic Environmental Research and Development Program (SERDP), Arlington, VA. Dr. John Harrison was Executive Director, SERDP. The Principal Investigator was Dr. Judith C. Pennington, EL.

The chapters of this report were authored by the following:

- Chapter 1 M. E. Honeycutt, A. S. Jarvis, and V. A. McFarland, WES.
- Chapter 2 A. Z. Li and K. A. Marx, University of Massachusetts Lowell, and J. Walker and D. L. Kaplan, Natick.
- Chapter 3 P. G. Thome and D. C. Leggett, CRREL.
- Chapter 4 D. R. Felt, C. A. Hayes, and B. E. Porter, ASci Corporation, and D. Gunnison and H. Fredrickson, WES.
- Chapter 5 C. H. Allersmeier, Natick.

This report was reviewed by Drs. James M. Brannon and William Davis, EL. The study was conducted under the direct supervision of Dr. Richard E. Price, Chief, Environmental Processes and Effects Branch, EL, and under the general supervision of Mr. Donald L. Robey, Chief, Environmental Processes and Effects Division, EL, and Dr. John Harrison, Director, EL.

At the time of publication of this report, Director of WES was Dr. Robert W. Whalin.

This report should be cited as follows:

Pennington, J. C., Honeycutt, M. E., Li, A. Z., Thorne, P. G., Felt, D. R., Allersmeier, C. H., Jarvis, A. S., Marx, K. A., Leggett, D. C., Hayes, C. A., McFarland, V. A., Walker, J., Porter, B. E., Kaplan, D. L., Gunnison, D., Fredrickson, H., and Thorn, K. A. (1997). "Explosives Conjugation Products in Remediation Matrices," Technical Report SERDP-97-7, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

# Introduction

---

## Background

Research from several sources indicates that 2,4,6-trinitrotoluene (TNT) becomes immobilized in soil and compost systems. Some of the earliest evidence for immobilization of TNT in soils came when radiolabeled TNT and 4-amino-2,6-dinitrotoluene (4A) were added to soils for a plant uptake study (Pennington 1988). Comparisons of percent recoveries of  $^{14}\text{C}$  by solvent (acetone) extraction and from a complete combustion method revealed that an average of four times as much of the added radioactivity was recovered by combustion. Solvent extraction failed to remove the immobilized residues of TNT, or of 4A. These results indicate that standard analytical techniques, which require solvent extraction of the TNT and its transformation products, fail to reveal a significant quantity of immobilized contaminant.

Evidence for immobilization of TNT in compost resulted from a study in which radiolabeled TNT was added to soil prior to composting (Pennington et al. 1995). After 20 days, the compost was extracted into ether followed by acetonitrile, then fractionated into cellulose, fulvic acid, humic acid, and humin. Less than 20 percent of the added radioactivity was solvent extractable. Since TNT is much less soluble in water than in these organic solvents, this result suggests that mobilization of TNT or of its products by water in the environment is limited. More than 30 percent of the added radioactivity was associated with the cellulose fraction, and more than 20 percent was associated with the humin. Cellulose is a biodegradable component of organic matter that may release the contaminant in some form, but humin is extremely recalcitrant to further degradation in the environment.

Results of these studies indicate that immobilization of TNT and/or its transformation products is a significant fate process. An understanding of the nature and mechanisms of immobilization may be very important to the development of effective treatment technologies. Furthermore, if the extent of immobilization in the environment approaches the extent observed in the laboratory, immobilization may exceed adsorption and degradation processes in importance as a contaminant fate process. The information generated by this research may be used to develop natural attenuation of explosives in soils and

groundwater, to assess the ultimate fate of explosives in compost, and to estimate the bioavailability of explosives in biotreatment systems.

## Objectives

The broad objectives of this study were to determine the nature and mechanism of the interactions between TNT and soils components, the long-term stability, biodegradability, and toxicity of the products of the interactions, and to develop methods for detecting products of the interactions. Specific objectives of each study are presented within each chapter.

## Approach

To understand the nature and mechanisms of the interactions between TNT and soil, two compartments of the soil were studied—clays and organic matter. Adsorption of TNT to several homoionic clays were examined in batch tests (report pending). Association of TNT with several fractions of the organic matter of TNT-contaminated soil and composted soil were investigated. These included solvent-extractable contaminant and contaminant associated with the cellulose, humic acid, fulvic acid, and humin fractions of soil and compost. Associations with these fractions were studied using batch tests and dialysis techniques (Chapter 2). Surface plasmon resonance was also investigated as a possible method for studying the kinetics and characteristics of interactions (Chapter 5). Microbial degradability of TNT and products of TNT interaction with these compartments were investigated with enzyme studies (ongoing study, Chapter 4). Toxicity was investigated with the AMES and Mutatox assays (Chapter 1). Detection of certain products of TNT interactions with the nonsolvent-extractable fractions of compost was achieved by acid/base hydrolysis (Chapter 3). Other ongoing studies having pending reports include earthworm bioassays for assessing toxicity of compost and nuclear magnetic resonance (NMR) analysis of TNT and TNT transformation products with surrogate functional groups of humic acids.

# Summary of Results

---

## Toxicology

This study was undertaken to examine the *in vitro* cytotoxicity and mutagenicity of TNT and several of its reduction products. Cytotoxicity assays were conducted using two cultured cell lines, Reuber H35 H4IIE rat hepatoma cells and Chinese Hamster Ovary-K1(CHO) cells. Mutagenicity of the compounds was assessed using Ames assay and Mutatox assay. Results indicated that TNT, 4-hydroxylamino-2,6-dinitrotoluene (4-OHA), 4,4',6,6',-tetranitro-2,2'-azoxytoluene (2,2'-azoxy), and 2,2',6,6',-tetranitro-4,4'-azoxytoluene (4,4'azoxy) are equally cytotoxic to H4IIE and CHO cells. Two other TNT transformation products, 4-amino-2,6-dinitrotoluene (4-A) and 2-amino-4,6-dinitrotoluene (2-A), were cytotoxic to H4IIE cells, but not to CHO cells. Another transformation product, 2,4-diamino-6-nitrotoluene (2,4-DANT), was noncytotoxic to either cell type. These results indicate that the transformation products of TNT mentioned above are as cytotoxic as, or only moderately less cytotoxic than, TNT *in vitro*. Results of Ames assays indicated that TNT with and without metabolic activation was slightly mutagenic to strain TA98, but not mutagenic to strain TA100. Only 2-A was slightly mutagenic to strain TA98 without activation. Both 2A and 2,2'-azoxy exhibited mutagenicity in strain TA100 with and without activation, while 4-A was slightly mutagenic without activation. In Mutatox assays, TNT was mutagenic without S9 activation; 2A, 4A, and 2,4-DANT were mutagenic both with and without S9 activation. One significant generalization of these results is that the two most commonly detected transformation products of TNT, 2-A and 4-A, are equally or only slightly less toxic than the parent compound.

## Dialysis Partitioning

These studies were conducted to quantify the adsorption of TNT and several of its transformation products to humic acid. Equilibrium dialysis was used to determine the effects of contaminant concentration, humic acid concentration, pH, and ionic strength on the formation kinetics and on extent of interaction. Results indicated that TNT, 2,6-diamino-4-nitrotoluene (2,6DAmNT), and 2-A bind to humic acid slowly. As the concentration of humic acid increased, the extent of binding of the two compounds decreased. As pH

increased, the extent of binding increased. Nearly a twofold increase in binding of TNT was observed for a fivefold increase in ionic strength. A linear model best fit the 2,6DAmNT isotherm data, while a Langmuir model best fit the TNT data. The maximum binding density of TNT for humic acid was 6 to 30  $\mu\text{M}$  TNT per  $\mu\text{M}$  humic acid.

## Hydrolytic Release

The objectives of this study were to differentiate between bound and unbound explosives and their transformation products in composted soil, to determine time course of metabolite evolution and binding during composting, and to evaluate various hydrolysis methods for assaying bound products. Compost samples were subjected to two hydrolysis procedures; acid (50 percent aqueous sulfuric acid) and base (0.5 M sodium hydroxide) followed by acid (concentrated sulfuric acid). Results indicated that significant quantities of identifiable aminodinitrotoluenes and diaminonitrotoluenes were released after solvent-extracted residues were subjected to acid or base hydrolysis. However, no TNT was released. The concentration of hydrolyzable metabolites reached a maximum at 10 days of composting, then decreased through 40 days. This study produced a method for removing and identifying a significant portion of the immobilized products of TNT.

## Microbial Degradation

An important question concerning the remediation technologies in which immobilization products are formed is long-term stability of the product. To address this concern, the biodegradability of immobilized products must be examined. This study has two objectives. The first objective is to determine the ability of several common enzymes to degrade immobilization products. These enzymes are typically active in degradation of aromatic contaminants and are common in microorganisms. The second objective is to evaluate the ability of soil microflora to mineralize radiolabeled TNT that has been immobilized in soil and/or compost. Results to date on the first objective show that none of the three enzymes, polyphenol oxidase, laccase, or polyphenol oxidase, exhibited activity against TNT. Results for the second objective to date show active enrichment cultures on the following fractions of compost: acetonitrile extract, cellulose, fulvic acid, humic acid, and humin. These studies continue. Enrichment cultures growing on fractions from radiolabeled compost exhibited mineralization ( $^{14}\text{CO}_2$ ) from cellulose and humic acid fractions. Further studies include examination of physiology and biochemistry of the interactions occurring between the microorganisms and the TNT-substrate products.

## Surface Plasmon Resonance

The biospecific Interaction Analysis System presents a method by which coupling affinity between molecules can be assessed. This is an extremely sensitive method for investigating molecular interactions at the surface of a sensor chip using *surface plasmon resonance* (SPR) to analyze the concentration of the biomolecules attached to the chip surface. The rationale was to bind the smaller molecules of TNT (or transformation products) to the surface matrix of the chip and introduce the larger humic acid molecules over the surface. If the humic acid interacted with the TNT, the kinetics could be followed by minute changes in weight to which the instrument is extremely sensitive. Since this is state-of-the-art technology requiring adaptation to procedures of this study, great care was taken to validate performance and execution of tests. Results indicated that neither TNT nor two of its transformation products, 4-A and 2,6DAmNT, could be immobilized on the chip surface. Various modifications of pH, concentration and flow rate were tried without success. Binding of the humic acid, rather than the explosive, was also explored without success. Apparently, binding of a substrate to the chip material is chemical class specific. Protein experiments confirmed that the system functions well with "biospecific" compounds, but not with all other classes of organic compounds.

## Covalent Binding

Recent research using liquid and solid state  $^{15}\text{N}$ -NMR has demonstrated that aniline binds covalently to isolated humic substances and to the organic matter in whole soils and peat (Thorn, Arterburn, and Mikita 1992; Thorn et al. 1996). Aniline reacts with isolated fulvic and humic acid in the presence and absence of phenoloxidase enzymes or metal catalysts. In the absence of catalysts, aniline undergoes a complex series of nucleophilic addition reactions with quinones and other carbonyl groups to form both heterocyclic and nonheterocyclic condensation products. In the presence of the enzyme or metal catalyst, aniline undergoes free radical coupling reactions together with nucleophilic addition reactions with the humic substances. Since common reductive products of TNT, i.e., 2-A, 4-A, 2,4-DANT, 2,6DAmNT, chemically resemble aniline in functionality groups, these reductive products may mimic the coupling reactions of aniline with humic acid.

The objectives of this study are (a) to determine covalent binding of 4-A, 2-A, and 2,4-DANT with model carbonyl compounds, with humic substances, and with whole compost and soil, and (b) to determine the chemical lability of the bound amino residues. All objectives will be approached using solid and/or liquid phase  $^{15}\text{N}$ -NMR. To demonstrate binding to model carbonyl compounds, the  $^{15}\text{N}$ -NMR shifts (liquid phase NMR) of condensation products of amines with, e.g., 4-methylcatechol, 1,4-benzoquinone, and glucose will be measured. The spectra of product mixtures from amine reactions will also be measured. Reference fulvic and humic acid (from the International Humic

Substances Society) will be reacted with TNT reduction products without catalysts (liquid and solid phase NMR). Organic matter fractions of compost will be extracted and reacted with TNT reduction products. The products will be analyzed by liquid and solid state NMR. Time series interactions ranging from weeks to months will be conducted to determine interactions between reduction products and both compost and surface soils. To determine the chemical lability of bound residues, attempts will be made to exchange the bound residues with other amines or release the residues by hydrolytic enzymes and microbial degradation. Results will provide a chemical definition of a significant immobilization process of TNT and TNT transformation products.

## References

- Pennington, J. C. (1988). "Plant uptake of 2,4,6-trinitrotoluene, 4-amino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene using  $^{14}\text{C}$ -labeled and unlabeled compounds," Technical Report EL-88-20, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.
- Pennington, J. C., Hayes, C. A., Myers, K. F., Ochman, M., Gunnison, D., Felt, D. R., and McCormick, E. F. (1995). "Fate of 2,4,6-trinitrotoluene in a simulated compost system," *Chemosphere* 30, 429-38.
- Thom, K. A., Arterburn, J. B., and Mikita, M. A. (1992). " $^{15}\text{N}$  and  $^{13}\text{C}$  NMR investigation of hydroxylamine-derivatized humic substances," *Environmental Science and Technology* 26(1), 107-16.
- Thom, K. A., Pettigrew, P. J., Goldenberg, W. S., and Weber, E. J. (1996). "Covalent binding of aniline to humic substances. 2.  $^{15}\text{N}$  NMR studies of nucleophilic addition reactions," *Environmental Science and Technology* 30(9), 2764-75.

# 1 Cytotoxicity and Mutagenicity of 2,4,6-Trinitrotoluene (TNT) and Its Metabolites<sup>1</sup>

---

## Introduction

The Army is investigating technologies for remediating TNT-contaminated soils including composting, which has been shown to substantially (>98 percent) reduce the amount of extractable TNT from soils and is substantially cheaper than methods such as incineration and disposal at hazardous waste landfills (Pennington et al. 1995; Griest et al. 1995). The pathway of microbial reduction has been well studied (Kaplan and Kaplan 1982). In this process, the nitro substituents on the two and/or four positions are reduced to hydroxylamines, which are further reduced to either amines or azoxy compounds. These reduction products then bind to soil components, becoming unextractable. Very little TNT mineralizes into carbon dioxide, and the long-term stability of TNT degradation product-soil complexes is not well studied, raising the question of whether the TNT degradation products will become available over time for uptake by fish, wildlife, or humans. While an extensive database for toxic effects on TNT does exist, little research has been conducted to date on the toxicity of its degradation products. Such information is extremely important since, in cases where these reduction products are produced, site remediation could actually create a more hazardous situation than previously existed.

This study was undertaken to examine the *in vitro* cytotoxicity and mutagenicity of TNT and several of its reduction products/metabolites. Cytotoxicity assays were conducted using two cultured cell lines, Reuber H35 H4IIE rat hepatoma cells and Chinese Hamster, Ovary-K1 (CHO) cells.

---

<sup>1</sup> Published as the following journal article: Honeycutt, M. E., Jarvis, A. S., and McFarland, V. A., (1996). "Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites," *Ecotoxicology and Environmental Safety* 35, 282-287.

Mutagenicity of the compounds was assessed using the Ames assay (Maron and Ames 1983) and the Mutatox assay.

## Materials and Methods

Reuber H35 H4IIE rat hepatoma cells (ATCC, Rockville, MD) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with vitamins, essential and nonessential amino acids, and 10-percent fetal bovine serum (FBS). CHO cells (ATCC, Rockville, MD) were grown in Ham's F-12 supplemented with 10-percent FBS. Cell stocks were grown in 75-cm<sup>2</sup> culture flasks at 37 °C in a humidified 5-percent CO<sub>2</sub> incubator and dissociated with 0.25-percent trypsin ethylenediaminetetraacetate (EDTA) in phosphate buffered saline (PBS). Experiments were performed in 24 well plates, seeded at a density of 100,000 cells per well in 2 ml of the previous medium, and allowed to incubate 18-24 hr.

Chemicals for cytotoxicity testing were dissolved in dimethylsulfoxide (DMSO) at their limits of solubility, then serially diluted tenfold over at least four orders of magnitude to conduct range-finding studies. Three replicate wells were dosed with 5 µL of each test solution at each concentration and incubated 24 hr as described above. The DMSO at this concentration (0.25 percent) was not cytotoxic. The growth medium was removed at the end of the incubation period, and the cells were washed with PBS and assayed for viability. A definitive cytotoxicity assay was then conducted for each compound using seven equally spaced log-dose exposure concentrations derived from the range-finding study. Six replicate wells of cells were each dosed, incubated, washed, and assayed as in the range-finding studies.

Immediately after treatment, all cells were incubated for 45 min with 0.25 ml 2 µM calcein AM in PBS. Calcein AM is absorbed by live cells and converted through intracellular esterase activity to the intensely fluorescent calcein. Calcein fluorescence was determined using a Dynatech Fluorite 1000 fluorescence multiwell plate reader (Dynatech Corporation, Chantilly, VA) equipped with 490 nm excitation and 516 nm emission filters. LC<sub>50</sub>s and 95-percent prediction limits were calculated for each compound by first testing for lack of fit in a regression model using SAS and plotting the linearized data for the best fitting regression model. The LC<sub>50</sub>s and 95-percent prediction limits were then read from the graphs as the exposure concentrations corresponding to the regression lines and prediction limits at a point equal to one-half the mean observed fluorescence in the control.

The Ames plate incorporation test was performed as described in Maron and Ames (1983) with and without S9 metabolic activation using five dose levels of each compound. The positive control for testing bacterial strain TA100 was sodium azide (2mg/plate) and for TA98 was 2,4,7-trinitro-9-fluorenone (2 µg/plate). Benzo [a]pyrene (B[a]P) (0.2 µg/plate) was used as a positive control to check the activity of the S9 in each series of tests with each bacterial strain. A mutagenic response was considered positive when (a) two

consecutive doses had an average number of revertants equal to at least twice the solvent control for that experiment, or at least twice those in the last nontoxic dose, and (b) demonstrated a linear dose response (modified twofold rule) (Chu et al. 1981). The slope of the linear portion of the dose response curves, including the zero dose, was determined by least squares analysis.

Mutatox tests were conducted with and without metabolic activation using standard protocols from Microbics Corporation. The data were interpreted using the modified twofold rule (Chu et al. 1981). B[a]P was used as an S9 metabolic activation positive control, and phenol was used as a direct-acting positive control.

## Results

TNT was equally cytotoxic to H4IIE cells and CHO cells (Table 1). The LC<sub>50</sub> values, expressed as micrograms per milliliter medium, for H4IIE and CHO cells were 4 µg/ml versus 24 µg/ml, respectively, with overlapping 95-percent prediction intervals. Four microbial reduction products studied, 4-OHA, 2-A, 2,2'-Azoxy, and 4,4'-Azoxy, were equally cytotoxic to both H4IIE and CHO cells with LC<sub>50</sub> in the 3- to 18-µg/ml range and were not significantly different from TNT cytotoxicity in both cell lines. 2-A and 4-A were cytotoxic to H4IIE cells with LC<sub>50</sub>s of 18 and 66 µg/ml, but were not cytotoxic to CHO cells. 2,4-Diamino-6-nitrotoluene (2,4-DANT) was noncytotoxic to either H4IIE or CHO cells. These data agree with data from another study which examined the *in vitro* cytotoxicity of three of the seven compounds used in the present study to H4IIE cells (Wellington and Mitchell 1991). The authors, using neutral red uptake, found NR<sub>50</sub>, i.e., LC<sub>50</sub>, values of 8 (5-10) (±1 σ), 89 (70-108), and 74 (59-90) µg/ml for TNT, 2-A, and 4-A, respectively.

Compound	H4IIE		CHO	
	LC <sub>50</sub> , µg/ml media	95% Prediction Interval	LC <sub>50</sub> , µg/ml media	95% Prediction Interval
TNT	4	2 - 18	24	6 - 53
4-OHA	6	2 - 13	4	1 - 10
4-A	66	50 - 82	>250	NA
2-A	18	3 - 108	>250	NA
2,4-DANT	>250	NA	>250	NA
2,2'-Azoxy	13	8 - 21	14	1 - 52
4,4'-Azoxy	4	1 - 14	3	1 - 8

Note: Na = not applicable.

In the Ames assay, TNT was slightly mutagenic in strain TA98 both with and without metabolic activation, while exhibiting no mutagenicity in strain TA100 with or without activation (Table 2). Of the metabolites, only 2-A exhibited slight mutagenicity in the Ames assay. This was in the TA98 strain without activation. 2-A and 2,2'-Azoxy exhibited mutagenicity in strain TA100 both with and without activation, while 4-A was slightly mutagenic in TA100 without activation.

**Table 2**  
**Mutagenicity of TNT and Its Metabolites in the Ames Assay and Mutatox**

Compound	Mutatox		Ames Assay			
	w/ S9	w/o S9	TA98 w/ S9	TA98 w/o S9	TA100 w/ S9	TA100 w/o S9
TNT	-	0.25 - 0.50 <sup>a</sup>	260 (36) <sup>b</sup>	409 (34)	-	-
2-A	1 - 3.9	3.9 - 15.6	-	270 (13)	513 (5)	402 (88)
4-A	0.5 - 7.8	2 - 15.6	-	-	-	218 (28)
2,4-DANT	15.6 - 62.5	7.8 - 31.6	-	-	-	-
2,2'-Azoxy	*	*	-	-	1769 (253)	1460 (253)
4,4'-Azoxy	*	*	-	-	-	-

Note: - = No mutagenic response; \* = inconclusive results.

<sup>a</sup>Range of mutagenic response in micrograms.

<sup>b</sup>Number of revertants per milligram of compound (standard deviation).

In the Mutatox assay, TNT was mutagenic only without S9 activation, while 2-A, 4-A, and 2,4-DANT were mutagenic both with and without S9 activation (Table 2). Mutagenic response profiles for the two azoxy compounds assayed using Mutatox elicited irregular response profiles, possible indicating their instability.

## Discussion

During microbial or mammalian metabolism, TNT is first reduced to 2-OHA and/or 4-OHA, then further reduced to either 2-A, 4-A, or 2,4-DANT. In microbes, the hydroxy intermediates may also be dimerized into azoxy compounds (2,2'-Azoxy or 4,4'-Azoxy) (Yinon 1990). From the cytotoxicity data in this research, one may deduce that either nitro groups occupying the 2, 4, and 6 position of the ring or a hydroxylamino group occupying the 2 or 4 position of the ring are responsible for the inherent toxicity of TNT and its metabolites. Both TNT and 4-OHA are cytotoxic to both H4IIE and CHO cells. 2-A and 4-A were cytotoxic to H4IIE cells and not to CHO cells. This implies that 2-A and 4-A were further metabolized, probably to 2-amino-4-hydroxylamino-6-nitrotoluene and 4-amino-2-hydroxylamino-6-nitrotoluene,

respectively, as intermediates before being further metabolized to 2,4-DANT. 2,4-DANT was noncytotoxic to either cell type, an indication that 2,4-DANT is probably not further metabolized, i.e., at the 6-nitro position. Indeed, 2-A, 4-A, and 2,4-DANT are the most common mammalian metabolites and 2,4,6-triaminotoluene has not been isolated in metabolism studies (Yinon 1990).

In trying to assess the environmental hazard of TNT and its by-products formed during manufacture, mutagenicity of these compounds has been well explored in the literature with varying results. In comparing Ames assay data from this study with other data found in the literature for TNT, 2-A, and 4-A, none of the studies agreed completely on whether the respective compounds are mutagenic with tester strains TA98 and TA100, either with or without metabolic activation. These erratic results, coupled with the lack of effect on *in vivo* and *in vivo* unscheduled DNA synthesis and on *in vivo* micronucleus formation, strongly suggest that these compounds do not pose a serious genotoxic threat.

However, TNT has been shown to be a serious environmental contaminant. Interest in the toxicity of TNT to aquatic species has arisen because the manufacture of TNT produced large amounts of wastewater containing TNT and by-products, which were discharge into streams or holding lagoons (Gordon and Hartley 1992). TNT levels of 5 mg/L are lethal to fish (Yinon 1990). Drzyzga et al. (1995) examined cytotoxicity of TNT and a series of related compounds in the luminescent bacterium *Vibrio fischeri* NRRL-B-1177. Of the same compounds that were examined in the present study, the authors grouped TNT and 4-A in the same toxicity classification, "very toxic to aquatic organisms," while 2-A and 2,4-DANT were grouped together in a lower toxicity classification, "toxic to aquatic organisms." This pattern of toxicity has been demonstrated in other studies with aquatic species as well. Johnson et al. (1994) found that TNT, 2-A, and 4-A were equally toxic to *Daphnia magna*, and that TNT and 4-A were equally toxic to planaria (*Dugesia dorotocephala*), while 2-A was slightly less toxic to planaria. A pattern emerges from the above studies and the present study of the 2- and 4-amino substituted dinitrotoluenes being equally or slightly less toxic than the parent TNT. These data contrast with the earlier study of Won, DiSalvo, and Ng (1976), who found that the metabolites studied in this and the above studies were nontoxic to green algae (*Selenastrum capricornutum*), copepods (*Tigriopus californicus*), and oyster larvae (*Crassostrea gigas*).

Composting has been advocated as an economical method of remediating TNT-contaminated soils and does significantly reduce TNT levels. However, this study and other have demonstrated that some of the degradation products of TNT formed during composting (2-A, 4-OHA, 4-A, 2,2'-Azoxy, 4,4'-Azoxy) are as cytotoxic as or only moderately less cytotoxic than TNT *in vitro*. In light of the cytotoxicity of the microbial reduction products that are formed during composting, the proportion of each metabolite formed and the bioavailability of these metabolites is central in determining the hazard of the finished compost. Greist et al. (1995) composted soils contaminated with 2,326 mg TNT/kg, 327 mg 4-A/kg, and 170 mg 2-A/kg for 40 days, reducing

the acetonitrile extractable concentrations to 2.84 mg TNT/kg, 2.56 mg 4-A/kg, and <1.6 mg 2-A/kg in nonaerated windrows. Other research has shown that during composting significant quantities of degradation products that are formed are not extractable nor leachable under laboratory conditions. Nor is the TNT converted to volatile organic compounds or carbon dioxide (Pennington et al. 1995). Apparently, the metabolites form conjugates with soil components, suggesting that they may not be bioavailable for eliciting harmful effects in the environment. However, long-term laboratory and field studies have not been conducted to assess whether these metabolites may become bioavailable over time through the processes of soil weathering, UV degradation, or acid deposition. Therefore, while composting may reduce the levels of TNT in the finished compost, the hazard associated with TNT-contaminated soils is probably lower, but still uncertain.

The concept of bioavailability of TNT metabolites from compost material is one that must be scrutinized in order to adequately assess the efficacy of composting for reducing the hazard associated with TNT-contaminated soils. In addressing the concept, both the acute and chronic effects of TNT and its degradation products and their fate in composting should be further elucidated.

## References

- Chu, K. C., Patel, K. M., Lin A. H., Tarone, R. E., Linhart, M. S., and Dunkel, V. C. (1981). "Evaluating statistical analyses and reproducibility of microbial mutagenicity assays," *Mutat. Res.* 85, 119-32.
- Drzyzga, O., Gorontzy, T., Schmidt, A., Blotvogel, K. H. (1995). "Toxicity of explosives and related compounds to the luminescent bacterium *Vibrio fischeri* NRRL-B11177," *Arch. Environ. Contam. Toxicol.* 28, 229-35.
- Gordon, L., and Hartley, W. R. (1992). "2,4,6-Trinitrotoluene (TNT)," *Drinking water health advisory: Munitions*. W. C. Roberts and W. R. Hartley, ed., Lewis Publishers, Boca Raton, FL, 327-98.
- Griest, W. H., Tyndall, R. L., Stewart, A. J., Caton, J. E., Vass, A. A., Ho, C. H., and Caldwell, W. M. (1955). "Chemical characterization and toxicological testing of windrow composts from explosives-contaminated sediments," *Environ. Toxicol. Chem.* 14, 51-59.
- Johnson, L. R., Davenport, R., Balbach, H., and Schaeffer, D. J. (1994). "Phototoxicity 3. Comparative toxicity of trinitrotoluene and aminodinitrotoluenes to *Daphnia magna*, *Dugesia dorotocephala*, and sheep erythrocytes," *Ecotoxicol. Environ. Safety* 27, 34-49.
- Kaplan, D. L., and Kaplan, A. M. (1982). "Thermophilic biotransformation of 2,4,6-trinitrotoluene under simulated composting conditions," *Appl. Env. Micro.* 44, 757-60.

- Maron, D. M., and Ames, B. N. (1983). "Revised methods for the *Salmonella* mutagenicity test," *Mutat. Res.* 113, 173-215.
- Pennington, J. C., Hayes, C. A., Myers, K. F., Ochman, M., Gunnison, D., Felt, D. R., and McCormick, E. F. (1995). "Fate of 2,4,6-trinitrotoluene in a simulated compost system," *Chemosphere* 30, 429-38.
- Wellington, D. R., and Mitchell, W. R. (1991). "*In vitro* cytotoxicity of certain munition nitroaromatic compounds," *Chemosphere* 23, 363-73.
- Won, W. D., DiSalvo, L. H., and Ng, J. (1976). "Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites," *Appl. Environ. Microbiol.* 31, 576-80.
- Yinon, J. (1990). *Toxicity and metabolism of explosives*. CRC Press, Boston, MA.

## 2 Trinitrotoluene and Metabolites Binding to Humic Acid<sup>1</sup>

---

### Introduction

Explosives residues in soil and most of their transformation products are all environmental concerns. The energetic nitroaromatic compounds were used as munitions for many decades. Since they are long-lived in the soil, they represent a significant contamination problem at many military sites in the United States and worldwide as well. The major munitions contaminant species trinitrotoluene (TNT), which along with its chemical breakdown products (Kaplan 1996; Kaplan 1992; Walter and Kaplan 1992), such as 2,6DAmNT, represents the class of compounds that are the focus of this study. However, the interactions between these nitroaromatic compounds and the complex soil matrix have not been extensively studied (Pennington and Patrick 1990; Bryniok et al. 1993).

The humic substance, of which humic acid (HA) is a component, constitute up to 80 percent of the organic matter in most inorganic soils. They are formed from the chemical and microbial degradation of both animal and plant material. Humic substances interact with a number of small ligands, including metal ions and organics such as toxic pollutants (Schnitzer 1986). HA is considered to be a flexible, linear polyelectrolyte at low concentrations (<3.5 g/L), above a certain pH (pH > 3.5), and at moderate ionic strength (<50 mM) (Schnitzer 1986; Ghosh and Schnitzer 1980). Beyond these conditions, HAs behave like rigid, uncharged colloids (Schnitzer 1986; Ghosh and Schnitzer 1980). Experiments were carried out under conditions that are within the boundaries where HA behaves as a linear polyelectrolyte. Thus, the configuration of HA is strongly affected by its concentration, pH, and ionic strength (Schnitzer 1986; Ghosh and Schnitzer 1980; Tsutsuki and

---

<sup>1</sup> Published as the following journal article: Li, A. Z., Marx, K. A., Walker, J., and Kaplan, D. L. (1997). "Trinitrotoluene and metabolites binding to humic acid," *Environmental Science and Technology* 31, 584-589.

Kuwatsuka 1984; Cornel, Summers, and Roberts 1986), and the binding phenomena of small ligands to humic substances are likely to resemble those of polyelectrolyte binding, which have received considerable research attention.

The goal of this research is to determine the dependence of binding of organo-nitro compounds on such factors as pH, ionic strength, HA concentration, ligand concentration, and binding kinetics using equilibrium dialysis and quantifying changes by reversed phase high performance liquid chromatography (RP-HPLC). Based on the experimental results, the nature of the binding mechanism of TNT and 2,6DAmNT were characterized to HA where HA is used as a model soil matrix.

## Experimental

### Materials

HA was purchased from Aldrich Chemical Co. Milwaukee, WI. Ultrafiltration of HA was performed in distilled and deionized water using the Stirred Cell (Spectrum Co.) and a membrane of molecular weight cutoff 5,000 daltons (Spectrum Co.) to fractionate the HA into molecular weights greater than and less than 5,000, respectively. The higher molecular weight fraction was used to prepare stock HA solutions. TNT (Sigma Chemical Co.) was purified and recrystallized by hot HCl. The 2,6-diamino-4-nitrotoluene (2,6DAmNT) was purchased from Aldrich Chemical Co. and 2-amino-4,6-dinitrotoluene (2AmDNT) obtained from the U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS. These chemicals were shown to be pure by HPLC ( $\geq 99$  percent). Sodium phosphate and potassium phosphate (Sigma) were used for buffers in all the binding experiments. The desired pH level and ionic strength of potassium phosphate buffer were made from the dilution of the mixture of low pH (4.5) and high pH (9.5) concentrated phosphate solutions (0.2 M). In experiments where sodium phosphate buffer was used, the low pH of sodium phosphate was adjusted by using HCl. Stock solutions of the nitroaromatic ligand were made in phosphate buffer. The stock solution concentrations (TNT and 2,6DAmNT were 72 ppm, and 2AmDNT was 42.5 ppm) were limited by the solubility of the compounds in the above aqueous buffers at desired ionic strength and pH.

### Instrumentation

The Dianorm Equilibrium Dialysis apparatus (Weder, Schildknecht, and Kesselring 1971), and the Diachema dialysis membranes (MWC 5,000) were purchased from Avestin, Inc., Ottawa, Canada, and used for separating the free and bound ligand. Two sizes of dialysis cell, Macro-5 with Q-factor 2.3 and Macro-1 with Q-factor 4.5, were used. A Waters RP-HPLC was used to provide separation and quantitation of free ligand TNT and its breakdown products (Jenkins et al. 1989) in the binding isotherm experiments. The RP-HPLC system was equipped with a solvent pump (model 510), a Rheodyne injector

(model 7725) with a 100- $\mu$ L loop, a fixed ultraviolet (UV) wavelength detector (model 441), and an HP integrator (model 3390A). The UV detector was set at 254 nm. A Waters reverse phase Nova-pak radial C<sub>8</sub> column (10 cm by 8 mm by 4  $\mu$ m) was used for the separations. A 100- $\mu$ L aliquot of analyte was injected into the mobile phase, which was a mixture of methanol and water (50:50; V:V) with a flow rate of 1.5 ml/min.

### Equilibrium method

Equilibria were studied at varying concentrations of the ligand as a function of pH, ionic strength, time, and HA concentration. To minimize the possible degradation of TNT and avoid changes in ligand structure unrelated to the HA, each solution (HA, nitroaromatic ligand, and buffer) was freshly filtered before reaction using 0.45- $\mu$ m centrifuge filters. The samples of complexes made from HA and nitroaromatic ligand were incubated with stirring in screw-top glass vials in the dark for about 48 hr before the dialysis. The equilibrium dialysis was performed in a two-compartment cell where a semipermeable membrane separated the Teflon dialysis cell into two chambers. The dialysis membranes were conditioned in at least three changes of distilled water, then in experimental buffer, before inserting into the cell. The dialysis cells were thoroughly cleaned by a methanol-water mixture (70:30), followed by boiling and cold distilled water washes. For each cell one chamber was loaded with phosphate buffer and the other chamber was filled with an incubated complex of HA and nitroaromatic ligand in phosphate buffer. The cells were rotated at a rate of 10 rpm to ensure thorough but gentle mixing. After equilibrium was reached, the concentration of the free ligand is equal on both sides of the membrane, while the bound ligand and HA will be retained only in one chamber. Since the transport of the ligand through the membrane is a function of temperature and ligand concentration, the equilibrium time of diffusion was experimentally determined for the two types of cells. Dialysis usually was run for 150 min for the Macro-5 cell (3.4-ml working volume) and 70 min for the Macro-1 cell (0.8-ml working volume). Figure 1 illustrated the kinetics of reaching equilibrium in the two-compartment Macro-1 dialysis cell. An aliquot of the collected free ligand in aqueous buffer was immediately mixed with an equal volume of acetonitrile and filtered by 0.45- $\mu$ m glass fiber filters. These samples were stored in glass vials at 4 °C until analyzed by HPLC. All the experiments, including incubation, dialysis, and HPLC analysis, were performed at room temperature, around 23 °C.

Based on the measured concentration of free ligand  $C_{L(f)}$  and the known initial ligand concentration  $C_{L(0)}$ , the ratio of bound ligand concentration

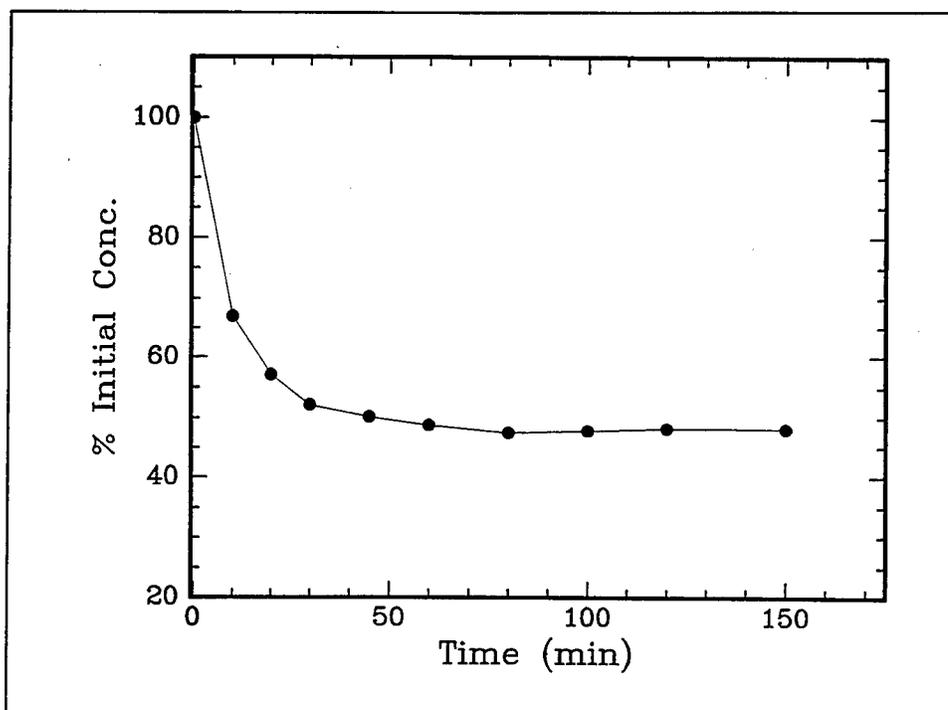


Figure 1. Determination of the equilibrium of diffusion of 2,6-diamino-4-nitrotoluene (2,6DAmNT) performed in a Macro-1S with a working volume 1.0 ml, each half cell at an initial concentration of 48  $\mu\text{M}$  in 5 mM sodium phosphate buffer pH 7.1 (Membrane diachema 10.14 disks, M. W. cutoff 5,000)

$C_{L(b)}$  to the known HA concentration was calculated using the following relationship:

$$C_{L(b)} = C_{L(0)} - 2 C_{L(f)} \quad (1)$$

or

$$C_{L(b)} = C_{L(0)} - 2 C_{L(f)} - C_{L(m)} \quad (2)$$

where  $C_{L(m)}$  is the concentration of ligand bound to the membrane that was determined experimentally. Extensive control samples (no HA) were also run providing the ligand concentration references: (a) the initial ligand concentration  $C_{L(0)}$  without dialysis); (b) permeate ligand concentration  $C_{L(P)}$  (in the dialysis chamber after crossing membrane); (c) retentate ligand concentration  $C_{L(R)}$  (in the other dialysis chamber after crossing membrane). When the controls  $C_{L(P)}$  and  $C_{L(R)}$  were found to be equivalent, equilibrium had been reached across the membrane, and the experimental data were considered valid. Also,  $C_{L(m)}$  could be estimated from the controls based on Equation 2 where

$C_{L(b)} = 0$ , and then  $C_{L(m)} = C_{L(O)} - C_{L(P)} - C_{L(R)}$ . In the experiments,  $C_{L(m)}$  was quite small and could be ignored when the dialysis membranes were conditioned as in the measurements.

## Results

TNT and its intermediates 2AmDNT and 2,6DAmNT all bound to HA under experimental conditions. The binding kinetics and equilibrium binding levels of TNT, 2AmDNT, are described to HA as a function of ionic strength, pH, HA concentration, and nitroaromatic concentration. Since HA is charged polymer (Hayes and Hines 1986), relatively low concentration of HA and relatively high buffer ionic strengths were designed for the isotherm experiments to minimize the Donnan effect (Cantour and Schimmel 1980), which results in an asymmetry of the free ligand concentration and solution pH across the membrane. For the data presented, the concentrations of bound ligand were normalized to HA concentrations in units of milligrams/gram HA and micromolar per micromolar HA. Although 5,000 daltons was used for the HA molecular weight, it is actually very polydisperse (Beckett 1987).

## Kinetics

The binding kinetics of TNT are shown in Figure 2 displayed as bound TNT normalized either by its initial concentration or the HA concentration. It should be noted that the uptake of TNT by HA is very slow with a low level of binding observed by 8 hr. Binding took place until well beyond 20 hr, and then the uptake was close to constant from 24 to 48 hr. This is in quantitative agreement with the known behavior of TNT in soil (Pennington and Patrick 1990). The uptake of 2,6DAmNT by HA, presented in Figure 3, also is a slow process and was similar to the kinetics of TNT (Figure 2). However, TNT binding continues to slowly increase after 2 days, while 2,6DAmNT remains at an constant level. Based on these observations, 48 hr was chosen as the standard incubation time for subsequent equilibrium binding experiments.

## Models

Since the mechanisms of interaction between HA and nitroaromatics are complicated and not well understood, the terms "association" and "association coefficient" (Carter and Suffet 1982) rather than "binding coefficient" or "absorption coefficient" will be used in the chapter. The binding data were fit either to a linear model or to one of the following two nonlinear models: the

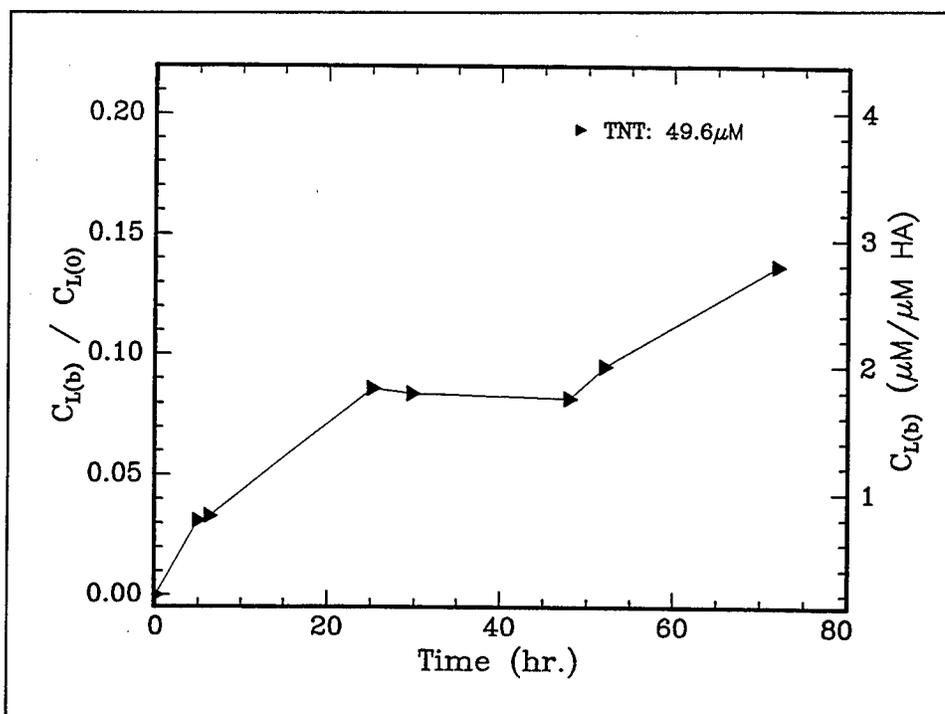


Figure 2. Measurement of the binding kinetics of TNT to HA at initial concentration of 49.6  $\mu\text{M}$  and HA 2.5  $\mu\text{M}$ , in 10 mM sodium phosphate buffer, pH 7.1

Langmuir Isotherm and the Freundlich Isotherm (Pennington and Patrick 1990; Weber 1972):

Langmuir model

$$C_{L(B)} = C_{L(B)\text{max}} k C_{L(f)} / (1 + k C_{L(f)}) \quad (3)$$

Freundlich model

$$C_{L(B)} = k_f C_{L(f)}^{1/n} \quad (4)$$

Linear model

$$C_{L(B)} = K_1 C_{L(f)} + d \quad (5)$$

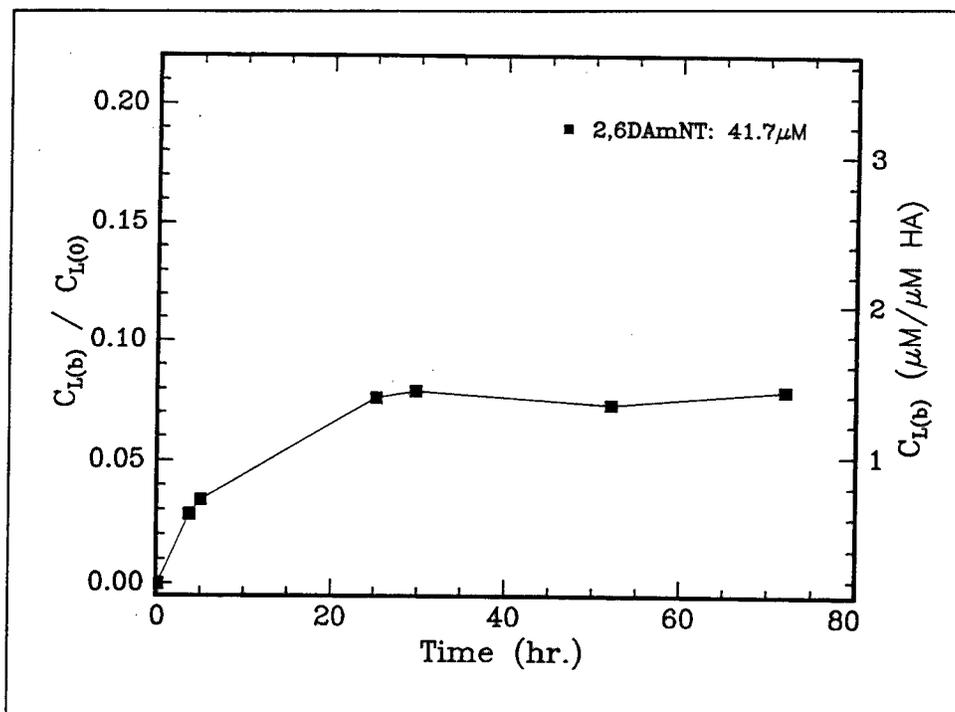


Figure 3. Measurement of the binding kinetics of 2,6DAmNT to HA at initial concentration of 41.7  $\mu\text{M}$  and HA 2.5  $\mu\text{M}$ , in 10 mM sodium phosphate buffer, pH 7.1

where  $C_{L(0)}$  refers to the free ligand concentration in  $\mu\text{M}$ , and  $C_{L(B)}$  refers to the normalized bound ligand concentration in  $\mu\text{M} / (\mu\text{M HA})$ . In the Langmuir model,  $k$  is the Langmuir constant in  $(\mu\text{M})^{-1}$  and  $C_{L(B)max}$  is the association capacity parameter in  $\mu\text{M} / (\mu\text{M HA})$ . In the Freundlich model,  $n$  is the Freundlich characteristic constant and  $K_f$  the association coefficient  $(\mu\text{M HA})^{-1}$ . In the linear model,  $k_f$  is the association coefficient  $(\mu\text{M HA})^{-1}$  and  $d$  the offset. The two nonlinear models were originally introduced to quantify adsorption phenomena. Here the two models are adopted to describe the binding behavior of chemicals to HA. The two nonlinear models were fit by a program written in the Mathematica language (Wolfram 1991).

In Table 1, four sets of TNT binding data are fit by Langmuir, Freundlich, and linear models, respectively. The goodness of fit is measured by  $\chi^2 = \sum_i |F_i - f_i|^2$  where  $F_i$  is the value of the  $i_{th}$  data point, and  $f_i$  is the value obtained from the fit. The best fit is the one that minimizes  $\chi^2$ . Figure 4 shows a comparison fitting of all three models for one set of TNT binding data (Table 1, line 4 fits). The Langmuir model fit the curve the best.

Table 1 Model Fitting Comparison of TNT Binding to Humic Acid										
Experiment Condition		Model								
		Langmuir			Freundlich			Linear		
pH	I. S., mM	$\chi^2$	$C_{L(B)max}$	$k$	$\chi^2$	$k_f$	$n$	$\chi^2$	$k_1$	$d$
4.6	10	0.0701	17.1	0.00343	0.0726	0.0667	1.07	0.0787	0.0516	0.0331
6.8	10	0.0465	29.0	0.00273	0.0544	0.0844	1.04	0.0603	0.0727	0.0144
7.1	4	0.0456	6.39	0.0240	0.621	0.210	1.33	0.859	0.0880	0.189
7.1	20	0.355	11.4	0.0187	0.467	0.280	1.26	0.729	0.137	0.241

Note:  $\chi^2 = \sum_i |F_i - f_i|^2$ , the goodness of fit, where  $F_i$  is the value of the  $i_{th}$  data point, and  $f_i$  is the value obtained from the fit. The best fit is the one minimize  $\chi^2$ .  
 $k$ : Langmuir constant ( $1/\mu\text{M}$ );  $C_{L(B)max}$ : association capacity parameter ( $\mu\text{M}/\mu\text{M HA}$ );  $n$ : Freundlich characteristic constant;  
 $k_f$ : association coefficient ( $1/\mu\text{M HA}$ );  $k_1$ : association coefficient ( $1/\mu\text{M HA}$ );  $d$ : offset.

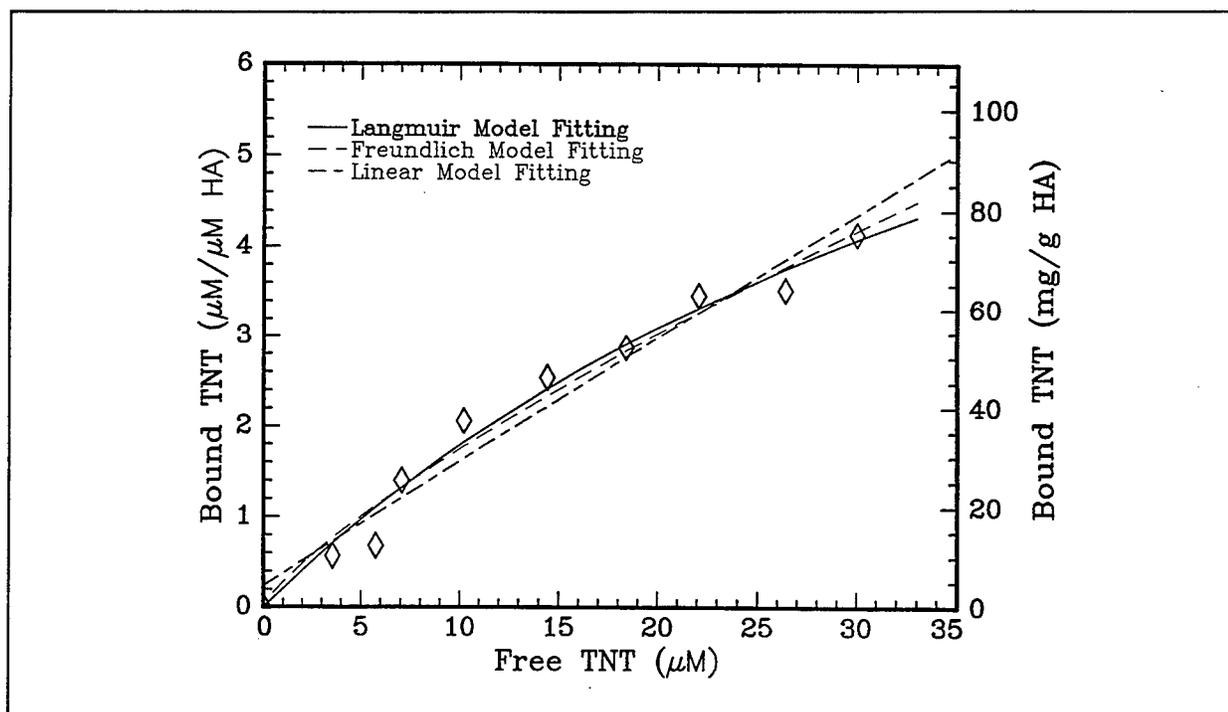


Figure 4. Comparison of model fitting (TNT and HA (2.5  $\mu\text{M}$ ) complexes were in 20-mM sodium phosphate pH 7.1 equilibrium for 50 hr. Curve was fit by the Langmuir, Freundlich, and linear model)

### Ionic strength and pH effects

Ionic strength and pH are expected to be important factors affecting ligand binding to HA. To study ionic strength effects, TNT at varying concentrations was interacted with 2.5  $\mu\text{M}$  HA at pH 7.1 at two different ionic strengths,

4 mM and 20 mM. In Figure 5, the concentration of bound TNT in micromolar/micromolar HA ( $Y_{left}$ ) and milligrams/gram HA ( $Y_{right}$ ), is plotted versus free TNT in micromoles. The two curves were best fit by the Langmuir model (Figure 4, Table 1).

The coefficients of the Langmuir fit for the above data are presented in Table 1 lines 3 and 4). The Langmuir constant,  $k$ , for the two curves are very close, but the association capacity parameters  $C_{L(B)max}$  are  $6.388 \mu\text{M}/\mu\text{M}$  HA in 4 mM and  $11.367 \mu\text{M}/\mu\text{M}$  HA in 20 mM. The results indicate that the higher ionic strength, 20 mM, allowed higher TNT binding to HA at all TNT concentrations, and the difference in maximum binding level was nearly a factor of two.

The effect of pH was measured over more than a two pH unit difference, corresponding to ranges in many soils. Figure 6 illustrated the interaction of TNT at varying concentrations with  $2.5 \mu\text{M}$  HA at 10 mM ionic strength at pH 4.6 and 6.8. Curves were best fit by the Langmuir model (Table 1, lines 1 and 2). The association capacity parameter  $C_{L(B)max}$  is  $17.057 \mu\text{M}/\mu\text{M}$  HA at pH 4.6 and  $29 \mu\text{M}/\mu\text{M}$  HA at pH 6.8. Inspection of Figure 6 and Table 1 shows that at pH 6.8, the binding level was always higher than at pH 4.6.

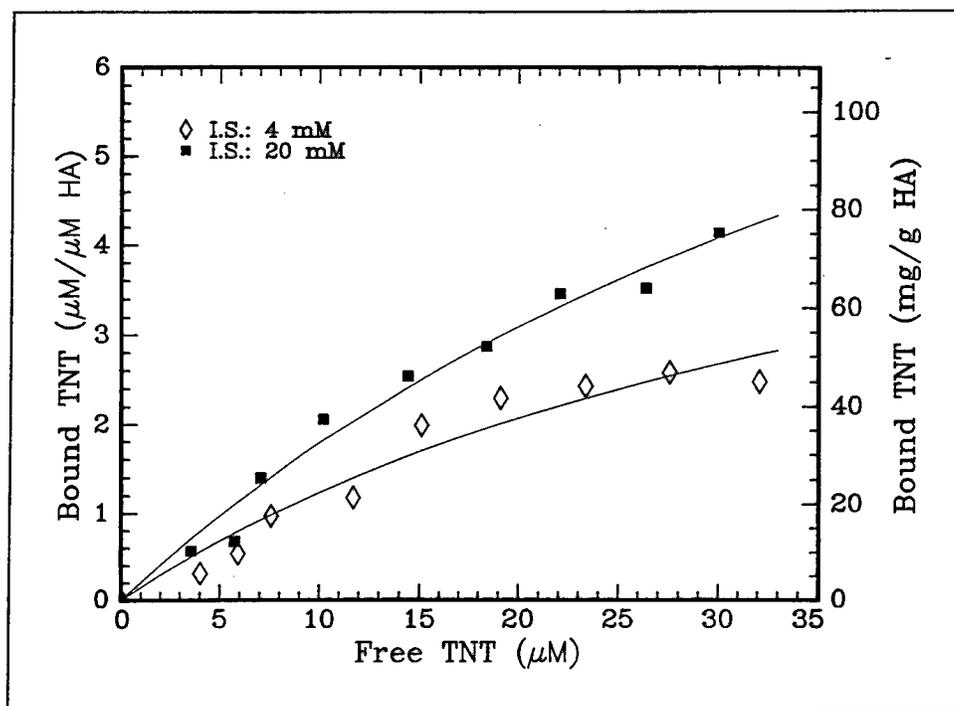


Figure 5. Effect of ionic strength on the binding of TNT to HA (TNT and HA ( $2.5 \mu\text{M}$ ) complexes were in sodium phosphate buffer pH 7.1 at differed ionic strengths, 4.0 and 20 mM. The total equilibrium time (incubation plus dialysis equilibrium) was 50 hr. The curves were fit by the Langmuir model)

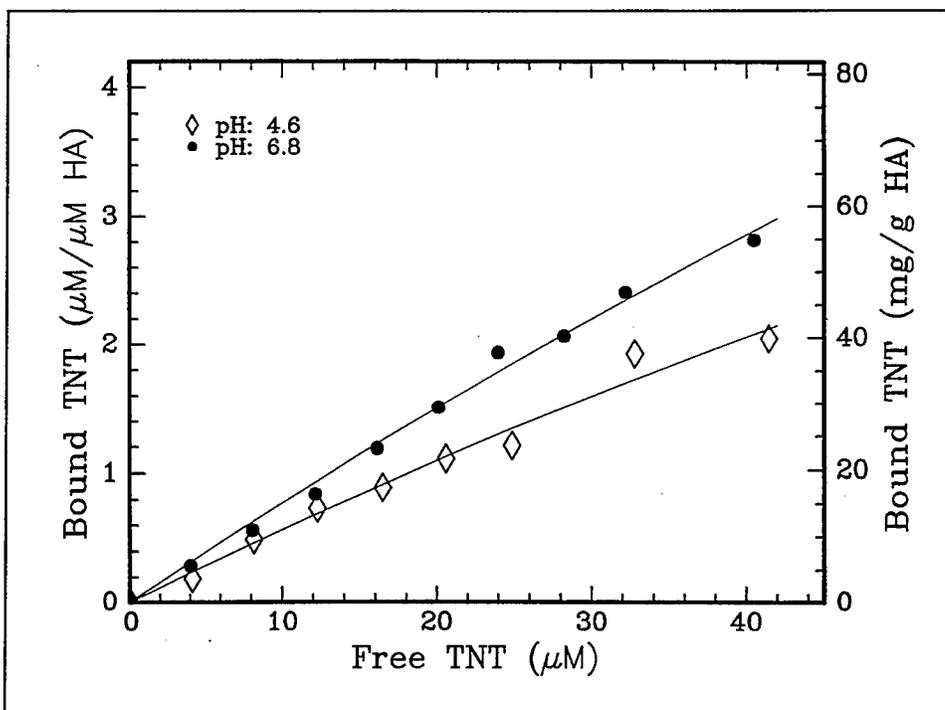


Figure 6. pH effect on the binding of TNT to HA (TNT and HA (2.5  $\mu\text{M}$ ) complexes were in 10 mM potassium phosphate at pH 4.6 and 6.8. The total equilibrium time was 48.3 hr. The curves were fit by the Langmuir model)

These results were supported by another set of experiments (data not shown) where pHs 4.6 and 7.3 were compared.

The pH dependent binding of 2,6DAmNT to HA was reversed from that of TNT. Figure 7 shows that more binding occurred at pH 4.6 than at the higher pH of 6.8, while the other conditions, HA concentrations at 5.0  $\mu\text{M}$  and ionic strength at 10 mM, were identical. Table 2 presents the linear model association coefficient,  $k_1$ ,  $0.0258 (\mu\text{M HA})^{-1}$  for pH 4.6 and  $0.0199 (\mu\text{M HA})^{-1}$  for pH 6.8. The same conclusion was reached from another set of data (not shown) where higher binding took place at pH 3.9 than at pH 7.1 at 5.12  $\mu\text{M}$  HA concentration and 10 mM ionic strength. The fact that Figure 7 was best fit by the linear model means that these ligands bind proportional to free ligand over a wide range (0-60  $\mu\text{M}$ ) without apparent saturation of HA.

### HA concentration effects

The effect of HA concentration on binding of nitroaromatics was investigated at 2.56 and 5.16  $\mu\text{M}$  HA for ligand, 2,6DAmNT in 10 mM sodium phosphate buffer at pH 7.1 (Figure 8). A linear increase was observed in the normalized molar ratio of binding versus free 2,6DAmNT concentration at

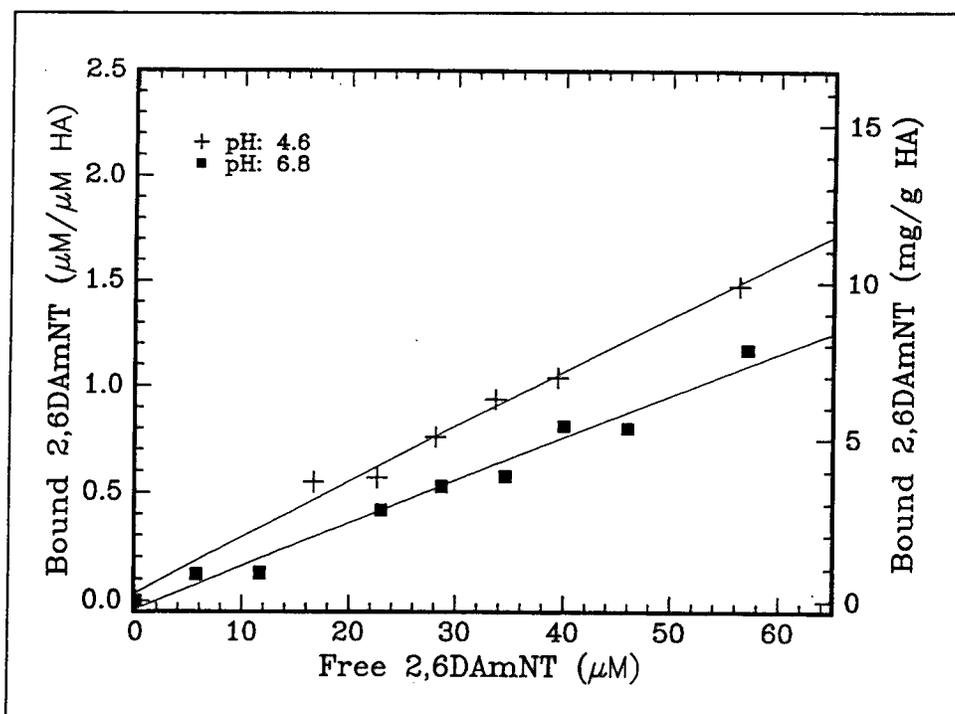


Figure 7. pH effect on the binding of 2,6DAmNT to HA (2,6DAmNT and HA (5.0 μM) complexes were in 10-mM potassium phosphate at pH 4.6 and 6.8. The total equilibrium time was 49.0 hr. The curves were fit by the linear model)

Ligand	HA Concentration, μM	pH	$k_f$ , 1/μM HA	Model	R <sup>2</sup>
2,6DAmNT	5.00	4.6	0.0258	Linear	1.00
2,6DAmNT	5.00	6.8	0.0199	Linear	0.99

Note: R<sup>2</sup>: regression coefficient;  $k_f$ : association coefficient.

both HA concentrations. Linear model fitting indicates a higher binding level at the lower HA concentration. The association coefficient,  $k_f$ , of the linear model is  $0.0266 (\mu\text{M HA})^{-1}$  in  $5.12 \mu\text{M HA}$  and  $0.0465 (\mu\text{M HA})^{-1}$  in  $2.56 \mu\text{M HA}$ . A similar HA dependence was observed for TNT binding and is consistent with previous publications (Carter and Suffet 1982; Landrum et al. 1984).

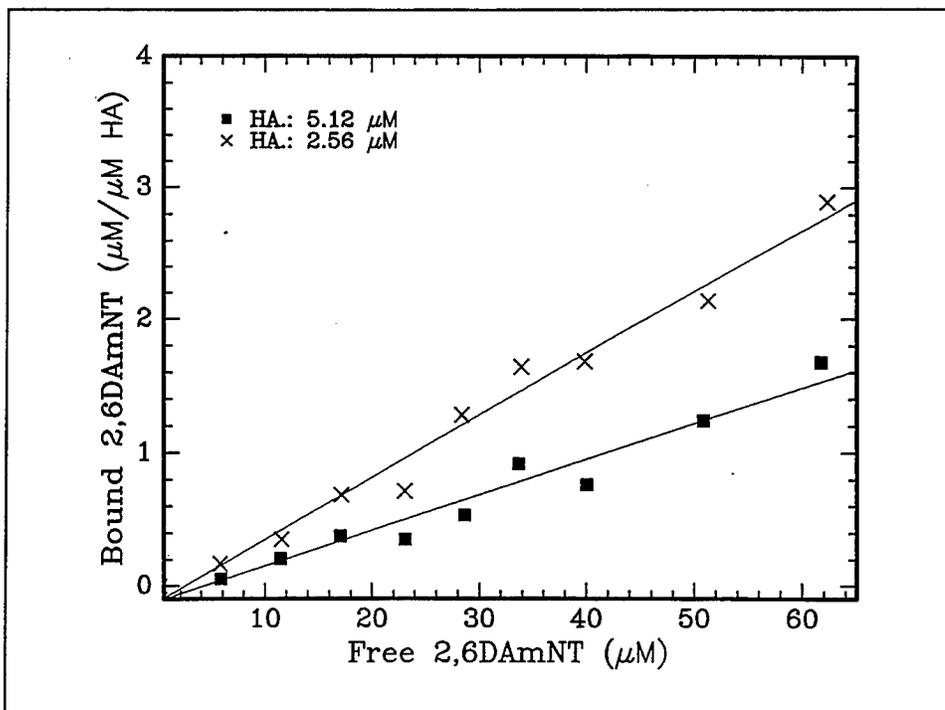


Figure 8. Effect of HA concentration (2.56 and 5.12  $\mu\text{M}$ ) on binding of 2,6DAmNT (Samples were incubated in 10 mM sodium phosphate pH 7.1, and total equilibrium time was 48 hr. Curves were fit by the linear model)

### Ligand comparison

A direct comparison of HA normalized ligand binding levels between TNT, 2AmDNT, and 2,6DAmNT was carried out under identical experimental conditions. The 2.5  $\mu\text{M}$  HA interacted with ligands in 10 mM potassium phosphate at pH 6.8. Three curves were fit by the linear model (Figure 9), which shows that the TNT binding levels are close to that of 2AmDNT, although the TNT binding levels level off while 2AmDNT continued to increase. By contrast, the binding levels of 2,6DAmNT are higher than the other two ligands and continue to increase over the entire ligand experimental range. The  $R^2$  value, which evaluates the fitness of the linear model, shows that 2,6DAmNT had the best fit ( $R^2 = 1.0$ ), compared with the other two ligands. The linear model was chosen rather than Langmuir or Freundlich models to fit curves for TNT and 2AmDNT to provide a convenient comparison of binding levels between the three ligands. The association coefficient,  $k_1$ , was  $0.0838 (\mu\text{M HA})^{-1}$  for 2,6DAmNT,  $0.065 (\mu\text{M HA})^{-1}$  for 2AmDNT, and  $0.0524 (\mu\text{M HA})^{-1}$  for TNT. The order of the ligand binding levels, 2,6DAmNT > 2AmDNT > TNT, is consistent with the conclusion from a previous study (Bryniok et al. 1993).

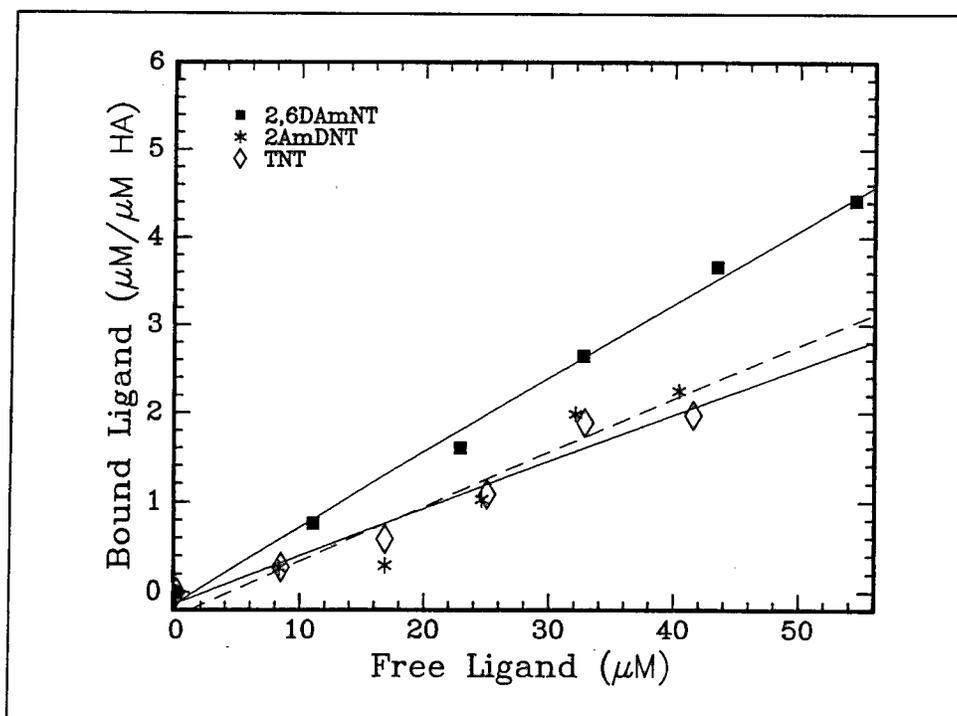


Figure 9. Comparison of ligand effect on binding of HA (Complexes of HA (2.50  $\mu\text{M}$ ) with TNT, 2AmDNT, and 2,6DAmNT were in 10 mM potassium phosphate pH 6.8; equilibrium time was 46 hr. Three curves were fit by linear model)

## Discussion

New observations are presented on the binding between hydrophobic pollutants, TNT, 2,6DAmNT, and 2AmDNT and hydrophilic HA, a polymeric component of soil. TNT, 2AmDNT, and 2,6DAmNT bind to HA at different levels that are influenced by many factors, including HA concentration, ligand concentration, pH, ionic strength, ligand structure, temperature, and incubation time. A common observation for the three nitroaromatic compounds binding to HA was the slow binding kinetics. This is consistent with that observed previously for TNT binding to whole soil (Pennington and Patrick 1990).

### Role of polyelectrolyte HA

HA exhibits flexible, linear, polyelectrolyte behavior under experimental conditions of low HA concentration (<3.5 g/L), pH > 3.5, and moderate ionic strength (<50 mM) (Schnitzer 1986; Ghosh and Schnitzer 1980; Hayes and Himes 1986; Murphy et al. 1994). Thus, the charge possessed by HA plays an important role in its behavior. The presence of significant levels of -COOH functional groups and phenolic structures in the structure of HA are likely

involved in some of the binding reactions. Thus, the charge, configuration, and aggregation of HA will be varied by its ionic and pH environment and its own concentration. At high concentrations, HA tends to be coiled and aggregated, hence leading to a decrease in the available exposed binding sites that can explain the binding increase with decreasing HA level.

At high pH, the HA molecules may shift to a more open configuration in solution due to the charge repulsion between ionized functional groups (Hayes and Himes 1986; Murphy et al. 1994). The most prominent structural feature, phenolic -COOH groups, would possess increased charges as the pH increased from 4.6 to 6.8. The higher charge density in HA would cause a decrease in aggregation to become an increasingly linear and therefore more planar polyelectrolyte. The extended polymer may create more binding sites to associate with the nitroaromatic ligands. This is consistent with the TNT data showing higher binding to HA at higher pH (6.8) compared with the lower pH (4.6).

The net charge on the HA polymer will decrease as the ionic strength is increased, resulting in coiling and aggregation. The HA thus becomes less hydrophilic while its charge is neutralized with increasing ionic strength. It seems reasonable that the less hydrophilic configuration of HA would bind the hydrophobic ligand TNT (Carter and Suffet 1982) more effectively. The effect of ionic strength ranging over a factor of five resulted in an increase in binding of TNT by close to a factor of two. This the binding level is related to one factor being more dominant than another (hydrophilicity versus coiling/aggregation).

### **Role of nitroaromatic structure**

The reduction of nitrogroups on TNT to amines had important effects upon binding. The diamine ligand, 2,6DAmNT, had a marginally higher HA binding level compared with 2AmDNT and TNT. The measurements are consistent with TNT being uncharged while 2AmDNT and 2,6DAmNT possess partial (+) charged amines. Potential binding sites for these (+) charges are located at the numerous carboxylic acid derivatized phenolic rings found in HA. Uncharged ring sites in HA, being less numerous, provide potential sites for the binding of uncharged TNT. This interpretation is consistent with experimental findings and reference (Bryniok et al. 1993). Based on the numbers and types of sites, the observations that 2,6DAmNT ligand could continue the rate of binding to higher concentrations than TNT can be explained. The binding level slightly decreases while the pH increases for 2,6DAmNT. This may be due to a slight loss of charge level on the 2,6DAmNT ligand as the pH is increased from 4.6 to 6.8, or from 3.9 to 7.1, based on the measured  $pK_a$  value 3.37 (Glover, Hoffsommer, and Kubose 1977).

## Model fitting comparison

It is interesting to note that the functional relationship between the HA bound ligand concentration and the free ligand concentration was found to be different for TNT and 2,6DAmNT. A simple linear relationship was observed to best fit all the 2,6DAmNT data (Table 2). The results were more complicated in the case of TNT, where a nonlinear relationship was observed. However, the TNT data were best fit by the Langmuir model. Table 1 shows that for all four sets of TNT data, the  $\chi^2$  values, which represent the goodness of fit, keep the same relative order. That is,  $\chi^2$  has the smallest (best fit) value for the Langmuir model; intermediate value for the Freundlich model; and the largest value for the linear model. Figure 5 demonstrates the model fitting comparison visually. It is clear that the Langmuir model is the best fit. This fact and these data indicate that TNT binding to HA would reach a maximum level as the binding sites were all filled. Table 1 tabulates the maximum binding levels for TNT; under the conditions of 4 to 20 mM ionic strength and pH 4.6 to 6.8, the binding limit varies from about 6 to 30  $\mu\text{M}$  TNT/ $\mu\text{M}$  HA. The different model fitting for TNT and 2,6DAmNT infer that the binding mechanism of the above two ligands for HA are different.

## Acknowledgment

The authors acknowledge Dr. J. C. Pennington for grant support (DAAL03-91-C-0034); Dr. D. Robertson, Dr. J. Carlson, and Ms. R. Bagalawis for analytical support; and Dr. D. Gunnison, Dr. T. Jenkins, and Dr. U. Dharmawardana for helpful discussions.

## References

- Beckett, R. (1987). *Aquatic humic substances*. I. H. Suffet and P. MacCarthy, ed., ACS 65-80.
- Bryniok, D., Daun, G., Knackmuss, H. J., and Lenke, H. (1993). *Spektrum der Wissenschaft*, October 106-8.
- Cantor, C. R., and Schimmel, P. R. (1980). *Biophysical chemistry, Part III*. W. H. Freeman and Company, New York, 1334-38.
- Carter, C. W., and Suffett, I. H. (1982). *Environ. Sci. Technol.* 16, 735-40.
- Cornel, P. K., Summer, R. S., and Roberts, P. V. J. (1986). *Colloid Interface Sci.* 110, 149-64.
- Ghosh, K., and Schnitzer, M. (1980). *Soil Sci.* 129, 266-76.

- Glover, D. J., Hoffsommer, J. C., and Kubose, D. A. (1977). *Analytica Chimica Acta* 88, 381-84.
- Hayes, M. H. B., and Himes, F. L. (1986). *Interactions of soil minerals with natural organics and microbes*. P. M. Huang and M. Schnitzer, ed., SSSA Special Publication Number 17, Soil Science of America, Masison, WI, 103-58.
- Jenkins, T. F., Walsh, M. E., Schumacher, P. W., and Miyares, P. H. (1989). *J. Assoc. Off. Anal. Chem.* 72, 890-9.
- Kaplan, D. L. (1992). *Current Opinion in Biotechnology* 3, 253-60.
- \_\_\_\_\_. (1996). *Organic energetic compounds*. P. Marinkas, ed., Nova Science Publishers, Inc., New York, 373-416.
- Landrum, P. F., Nihart, S. R., Eadle, B. J., and Gardner, W. S. (1984). *Environ. Sci. Technol.* 18, 187-92.
- Murphy, E. M., Zachara, J. M., Smith, S. C., Phillips, J. L., and Wietsma, T. W. (1994). *Environ. Sci. Technol.* 28, 1291-99.
- Pennington, J. C., and Patrick, W. H., Jr. (1990). *J. Environmental Quality* 19, 559-67.
- Schnitzer, M. (1986). *Interactions of soil minerals with natural organics and microbes*. P. M. Huang and M. Schnitzer ed., SSSA Special Publication Number 17, Soil Science of America, Masison, WI, 77-101.
- Tsutsuki, K., and Kuwatsuka, S. (1984). *Soil Sci. Plant Nutr.* 30, 151-62.
- Wolfram, S. (1991). *Mathematica*. Wolfram Research, Inc., New York.
- Walker, J. E., and Kaplan, D. L. (1992). *Biodegradation* 3, 369-85.
- Weber, W. J., Jr. (1972). *Physicochemical processes for water quality control*. Wiley-Interscience, New York.
- Weder, H. G., Schildknecht, J., and Kesselring, P. (1971). *Amer. Lab., No. 10*, 15-21.

# 3 Hydrolytic Release of Bound Residues From Composted TNT-Contaminated Soil<sup>1</sup>

---

## Introduction

The U.S. Army is obligated to restore land previously contaminated with the explosive TNT (2,4,6-trinitrotoluene). One remediation method that is being tried is composting. In this method, the soil to be treated is mixed with a readily decomposable source of organic carbon, which is usually also a mixture of readily available waste products, such as cow manure, alfalfa, sawdust, and vegetable wastes (Kaplan and Kaplan 1982a; Pennington et al. 1995). Previous work on TNT/soil composts indicates that TNT is rapidly converted to solvent-extractable reduction products and to residues that are bound in a nonsolvent-extractable form (Pennington et al. 1995; Kaplan and Kaplan 1982b; Caton et al. 1994). Similar observations have been made with respect to TNT-amended soils (Pennington 1988; Harvey et al. 1990) and activated sludge (Carpenter et al. 1978). Similarly, tissues of plants grown hydroponically in TNT solutions metabolized TNT reductively. Reduction residues were bound in a nonsolvent-extractable form (Harvey et al. 1990; Palazzo and Leggett 1986a,b). A considerable quantity of two reduction products, 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT), was released by acid hydrolysis of the harvested plants after first extracting unbound metabolites with benzene (Palazzo and Leggett 1986a,b). This appears to be the only instance in which bound metabolites have been identified and quantified using high performance liquid chromatography (HPLC) or gas liquid chromatography (GLC). In all the other studies, the analysis was done radiochemically, indicating only that the bound species contains the radiolabeled benzene ring from the TNT.

---

<sup>1</sup> Published as the following journal article: Thorne, P. G., and Leggett, D. C. (1997). "Hydrolytic release of bound residues from composted soil contaminated with 2,4,6-trinitrotoluene," *Environmental Toxicology and Chemistry* 16, 1132-1134.

In experiments at Oak Ridge, base hydrolysis after solvent extraction recovered about 60 percent of the applied label from TNT/soil composts even after 90 days (Caton et al. 1994). Although the hydrolysates were not analyzed for known TNT reduction products, the analogy between living plants and compost systems containing mostly plant matter and wastes suggests that standard HPLC analysis of these hydrolysates should be the first step taken in elucidation and determination of bound TNT residues in soil compost. A combination of radiochemical and chemical analyses done together would provide even more information.

It is generally believed that TNT must be reduced before it can be bound covalently and that solvent extraction can remove essentially all residues that are not covalently bound if enough time is allowed for the slow kinetics of desorption (Jenkins and Grant 1987). The reduction pathways for TNT appear to be essentially the same in a wide diversity of species and environmental systems including mammals, higher and lower plants, bacteria, soil, activated sludge, and compost. TNT metabolism and disposition in the environment have been thoroughly discussed in several reviews (McCormick, Feeherry, and Levinson 1976; Walsh 1990; Gorontzy et al. 1994). The first stable reduction products are 4-ADNT and 2-ADNT, of which the 4-ADNT generally predominates. The occurrence of hydroxylamine intermediates and their condensation to form azoxy dimers has also been reported (Kaplan and Kaplan 1982b; McCormick, Feeherry, and Levinson 1976). The mono-aminoDNTs are ubiquitous in fully aerobic environments and are chemically analogous to several dinitroaniline herbicides. The analogy is more obvious if reference is made to 4-ADNT as 3,5-dinitro-4-methyl aniline. The nitroaniline herbicides as a class also undergo reductive metabolism and binding behavior in soils (Kearney et al. 1976). However, no studies were located in which parent compounds or their metabolites were recovered and determined after hydrolysis or other forms of cleavage.

The chemistry of chloroaniline binding in soil has been studied far more extensively, but the extent to which they are analogous to nitroanilines is in doubt. The substitution of chloro- for nitro-groups may change their chemistry significantly. Nevertheless, parent chloroanilines have frequently been recovered in soil or organic matter hydrolysates following solvent extractions to remove unbound species (Bartha and Pramer 1970; Bartha 1971; Hsu and Bartha 1974, 1976; You and Bartha 1982). Similarly, a nitrophenyl ether herbicide applied to soils exhibited the same pattern of reduction, followed by binding that was reversible on hydrolysis (Yamada 1983). Residues were recoverable many years after application.

Bound chloroaniline was extracted with supercritical methanol 16 years after application of a chlorophenyl urea herbicide to soil (Scheunert, Mansour, and Andreux 1992). Supercritical carbon dioxide with methanol modifier was used to extract explosives residues from compost (Martinez, Ho, and Griest 1995). Preliminary results indicated that recoveries of reduction products may have been higher than with solvent extraction alone. Of interest here is

whether the release of analytes was due to methanolysis of the complex or to some other mechanism.

The fact that residues could be recovered by hydrolysis does not prove that a single mode of binding is occurring. Many different kinds of linkages are known to be present in soil organic matter and plants that are hydrolyzable, e.g., lignin and quinone adducts, amides, glucose, and acyl conjugates.

The objectives of this work were to differentiate between bound and unbound explosives and their transformation products in composted soil; to investigate the time course of metabolite evolution and binding; and to evaluate various hydrolysis methods.

## Materials and Methods

Compost samples from Umatilla Army Depot Activity, Hermiston, OR, were obtained from Black and Veatch Waste Sciences, Tacoma, WA. These samples were from aerated and unaerated windrow composting pilot studies. The compost-amendment mixture was composed of 30 vol. percent cow manure, 25.4 vol. percent sawdust, 25.4 vol. percent alfalfa, 14.3 vol. percent chopped potato waste, and 4.9 vol. percent chicken manure. The final compost consisted of 70 vol. percent amendment and 30 vol. percent of explosives-contaminated sediment excavated from a dried-out explosives waste lagoon. Samples of composted amendment that contained 10 vol. percent of uncontaminated sediments were also available as controls. Samples of contaminated compost were taken on Days 1, 5, 10, 20, and 40. Control composts were sampled on Day 55.

Solvents used for extractions and analysis were HPLC-grade from Alltech. Concentrated  $H_2SO_4$ ,  $Na_2HPO_4$ , and NaOH were reagent-grade from Baker. Sep-Pak Poropak<sub>RDX</sub> solid-phase extraction cartridges were from Waters. Standards for HPLC analysis and spikes were made from Standard Analytical Reference Materials (SARMs) obtained from the U.S. Army Environmental Center, except for 2,4-diamino-6-nitrotoluene (2,4-diANT) and 2,6-diamino-4-nitrotoluene (2,6-diANT), which were supplied by Dr. Ronald Spanggord, Stanford Research Institute (Menlo Park, CA).

HPLC analysis was performed using either a Spectra-Physics system (8875 autosampler, 8800 pump, 8490 detector, Hewlett-Packard 3396 integrator) or a Waters system (717 autosampler, 616 pump, 600S controller, 996 photodiode array detector, Millennium workstation). A Phenomenex Ultracarb 5 ODS (20) (4.6 mm by 250 mm, 5  $\mu$ m) reverse phase column with an Alltech C-18 guard cartridge was used for the analytical separations. The aqueous/methanol (volume percent/volume percent) gradient elution time steps were as follows: start at 85/15, ramp to 65/35 at 8 min, ramp to 42/58 at 10 min and hold for 13 min, ramp to 0/100 at 28 min and hold for 7 min, ramp down to 85/15 at 40 min and hold for 10 min before the next injection. The flow rate

was 0.8 ml/min. Quantification was performed at 254 nm, while peak identities and purities were assessed by comparing sample and standard peak spectra and retention times. Confirmation of analyte identities was performed using a Supelco CN (4.6 mm by 250 mm, 5  $\mu$ m) cyanopropyl column that separates the analytes in a different order from the ODS column.

Compost samples had been air-dried overnight then ground in a Wiley mill to pass a 2-mm screen prior to receipt. Subsamples weighing 2.00 g were added to 40-ml glass vials and sealed with Teflon-lined caps. A 10-ml aliquot of acetonitrile was added and the sample vortexed for 1 min. The vials were then placed in a cooled, sonic bath and sonicated overnight. Following sonication, 10 ml of aqueous calcium chloride solution (5 g/L) was added and the sample vortexed. The vial was centrifuged at 1,500 rpm for 5 min. The aqueous  $\text{CaCl}_2$ /acetonitrile extract was decanted, filtered, and saved for HPLC analysis. The vials were then refilled with fresh  $\text{CaCl}_2$ /acetonitrile solution, vortexed, centrifuged, and decanted repeatedly until no more unbound explosives residues or free amino transformation products were detectable by HPLC. The solvent-extracted residues were then air-dried overnight.

Two hydrolysis procedures were evaluated. The first was an acid hydrolysis. Triplicate subsamples from aerated and unaerated compost time-series were analyzed. The air-dried residues from the acetonitrile extractions were transferred to 22-ml vials. A 10-ml aliquot of 50-percent aqueous  $\text{H}_2\text{SO}_4$  was added, the samples vortexed for 1 min, then placed in a sonic bath for 6 hr. The temperature in the bath was allowed to increase to 30  $^\circ\text{C}$ . The vials were then centrifuged at 1,500 rpm for 5 min. A 5-ml aliquot of the acid digest was removed and neutralized by adding 100 ml of aqueous, 1.2 M  $\text{Na}_2\text{HPO}_4$  (pH 8.4). The resulting solution had a pH of 6.5. The neutralized solution was pulled through a 6-ml Sep-Pak cartridge at 10ml/min using vacuum. The cartridge was washed with an additional 20 ml of reagent grade water to remove salts then evacuated for 5 min to remove residual water. Then a 5-ml aliquot of acetonitrile was added and allowed to drip through the cartridge at 5 ml/min. A 5-ml aliquot of reagent grade water was then washed through the cartridge into the acetonitrile extract. The mixture was diluted to 10.0 ml using reagent grade water and analyzed by HPLC.

The second hydrolysis procedure combined a base and acid hydrolysis and included spike-recovery assessments. Triplicate subsamples from the Day 15 aerated composts were analyzed. Residues from the exhaustive acetonitrile extractions were air-dried as above. After being transferred to 22-ml vials, 10 ml of 0.5 M NaOH was added. The samples were mixed for 3 min and sonicated overnight at 30  $^\circ\text{C}$ . The vials were centrifuged, and a 5-ml aliquot of the basic extract was removed and diluted to 10 ml with concentrated  $\text{H}_2\text{SO}_4$ . The remaining 5 ml of basic extract was left in the vial with the residue, and 5 ml of concentrated  $\text{H}_2\text{SO}_4$  was added. Both acidified extracts were sonicated for 6 hr at 30  $^\circ\text{C}$ . After sonication, the vials were centrifuged and 5-ml aliquots removed and neutralized as above.

## Results and Discussion

The exhaustive, acetonitrile extractions of the time-series showed that the concentration of unbound TNT is rapidly reduced by the composting processes, both aerated and unaerated (Table 1). These results agree with previous analyses of these composts (Griest et al. 1993). The rapid increase of unbound (free), solvent-extractable aminoDNTs and slower increase of free diaminoNTs were followed by subsequent increases in these compounds in the acid hydrolyzed, bound fractions (Figures 1 and 2). After 40 days of composting, very little of the amino and diamino reduction products were released by acid hydrolysis. The acid hydrolysis degraded the matrix substantially, but no more detectable TNT was released even though TNT is stable under these acidic conditions. This result confirms that solvent extraction removes all unbound residues from sorption sites deep within the matrix. Traces of azoxy compounds were found in a few of the samples. No hydroxylaminos were detected.

The second, base-acid hydrolysis experiment was performed using Day 15 aerated compost. The amounts of amino and diamino compounds released by the base hydrolysis were roughly equal to those released by the acid-only hydrolysis (Table 2). However, the subsequent acid hydrolysis of the base-hydrolyzed residues released considerably more of these reduction products. A graphical comparison of the two treatments emphasizes the dramatic increase (Figure 3). The routine acetonitrile, sonic extraction of Day 15 compost released 50 mg/kg of TNT and reduction products (2 percent of the original 2,826 mg/kg TNT and aminos). The two-step hydrolysis released an additional 480 mg/kg of reduction products, which accounts for an additional 17 percent of initial TNT.

The disproportionately low recoveries of 2-ADNT and 2,6-diANT is a puzzle. There could be differential microbial production of these isomers in the compost system; however, that would not account for the low spike recoveries. A recent investigation (Haderlein, Weissmahr, and Schwarzenbach 1996) showed that adsorption to clays may be quite different for the different isomers of the aminoDNTs and diANTs. Problems with recoveries of aminoDNTs from acid-preserved well-water samples (Jenkins et al. 1995) indicate that the mechanisms of loss are complex.

## Conclusions

Previous researchers using radio-labeled composts (Pennington et al. 1995; Caton et al. 1994) found that the majority of TNT reduction products were bound in nonsolvent extractable forms. Results from the time-series analysis confirm these findings and have important implications for the routine operation of compost-remediation systems. The transformation of TNT to solvent-nonextractable reduction products appears to go through two stages of covalent binding to the compost matrix. In the first stage, a significant percentage

<b>Table 1</b> <b>Umatilla Windrow Composts-Time Series (Overnight, sonic acetonitrile extracts (ACN) and 6-hr sonic H<sub>2</sub>SO<sub>4</sub> digests (Acid))</b>					
<b>Concentration, mg/kg</b>					
<b>Aerated</b>					
	<b>TNT</b>	<b>4-ADNT</b>	<b>2-ADNT</b>	<b>2,6-diANT</b>	<b>2,4-diANT</b>
Day 1 ACN extract	2,360	291	170	nd <sup>1</sup>	nd
Acid hydrolysate	nd	23.6	4.6	nd	15.3
Day 5 ACN extract	1,060	210	220	nd	nd
Acid hydrolysate	nd	31.2	4.7	1.6	34.4
Day 10 ACN extract	28.6	149	20.3	5.3	54
Acid hydrolysate	nd	63.1	5.1	4.5	75.1
Day 15 ACN extract	14.7	24.5	2.2	nd	8.3
Acid hydrolysate	nd	46.4	3.7	4.2	66.2
Day 20 ACN extract	11.6	9.2	1.4	nd	4.6
Acid hydrolysate	nd	21.2	2	2.4	53.1
Day 40 ACN extract	3.3	13.3	2.3	nd	nd
Acid hydrolysate	nd	31.4	3.5	2.6	34.4
<b>Unaerated</b>					
Day 1 ACN extract	2,000	379	215	nd	nd
Acid hydrolysate	nd	20.3	3.9	nd	12.7
Day 5 ACN extract	113	1,090	450	4.6	20.9
Acid hydrolysate	nd	33.7	4.4	4	57.3
Day 10 ACN extract	40	154	7.5	14.5	172
Acid hydrolysate	nd	40.3	2.7	5.49	111
Day 15 ACN extract	22.6	10.7	1.9	nd	10.7
Acid hydrolysate	nd	25.4	1.7	4.9	93.6
Day 20 ACN extract	9.8	3.2	0.8	nd	nd
Acid hydrolysate	nd	5.8	0.4	2.4	53.8
Day 40 ACN extract	3.8	3.7	1	nd	nd
Acid hydrolysate	nd	6.3	0.9	1.4	20.2

<sup>1</sup>nd = <0.1 mg/kg.

of the bonds are hydrolyzable by acid alone or a combination of base and acid. As composting continues, a second stage occurs in which the bonds are either altered to form different functional groups that are not hydrolyzable or additional bonds form as the bound transformation products are further reduced. The termination of composting as soon as solvent-extractable TNT concentrations drop below a specified action level may stop the binding processes at the first, hydrolyzable stage. This may result in the potential for long-term releases of TNT metabolites that might not occur if composting is continued into the second stage of nonhydrolyzable binding.

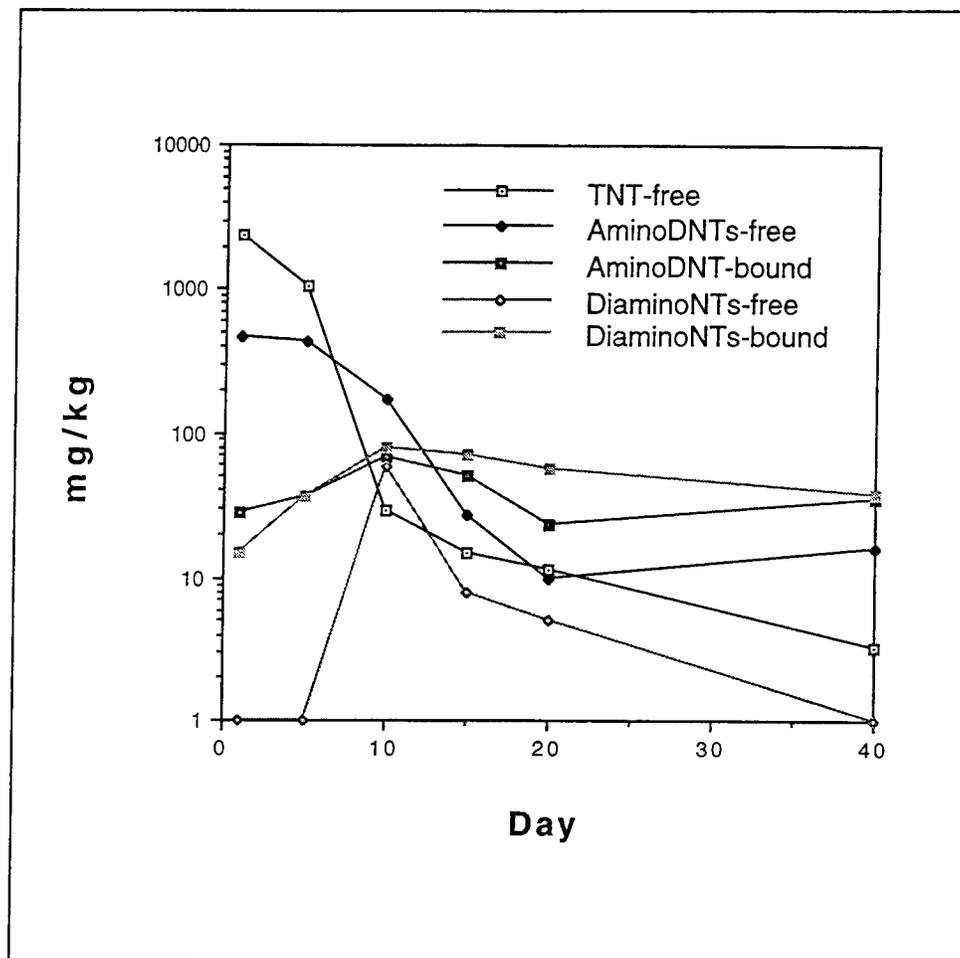


Figure 1. Concentration of free and bound TNT and metabolites in aerated compost

Optimization of base/acid hydrolysis methods and assessments of analyte recoveries are continuing. Investigations of a full-scale composting operation and residues from pilot-scale anaerobic digester are underway.

### Acknowledgment

This research was funded by the U.S. Army's Strategic Environmental Research and Development Program, Dr. Judith C. Pennington, U.S. Army Engineer Waterways Experiment Station, Project Monitor. The cooperation of Mr. Harry Craig, District 10 EPA, Portland, OR; Ms. Elona Tuomi, Remediation Technologies, Seattle, WA; Ms. Ginger Ferguson, Black and Veatch Waste Sciences, Tacoma, WA; and Dr. William Lowe and Roy F. Weston, West Chester, PA, is greatly appreciated.

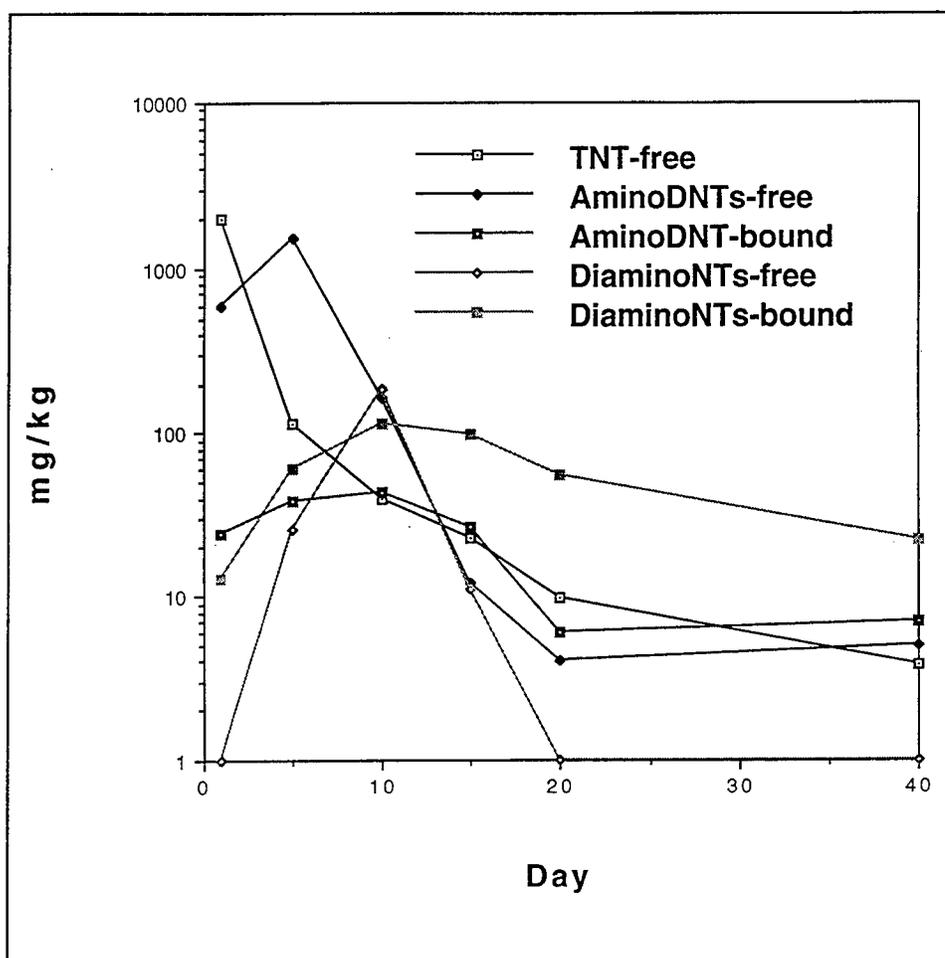


Figure 2. Concentration of free and bound TNT and metabolites in unaerated compost

Table 2 Day 15 Aerated Compost (Overnight, sonic acetonitrile extracts (ACN) and 6-hr sonic H <sub>2</sub> SO <sub>4</sub> digests of NaOH extracts (Base) and insoluble residues (Acid))					
Concentration, mg/kg					
	TNT	4-ADNT	2-ADNT	2,6-diANT	2,4-diANT
Day 15 ACN Extract	14.7	29.9	1.4	0.5	17.7
Base hydrolyste	nd <sup>1</sup>	19.8	nd	2	55.2
Acid Hydrolyste	nd	81	7.8	7.8	276

<sup>1</sup>nd = <0.1 mg/kg.

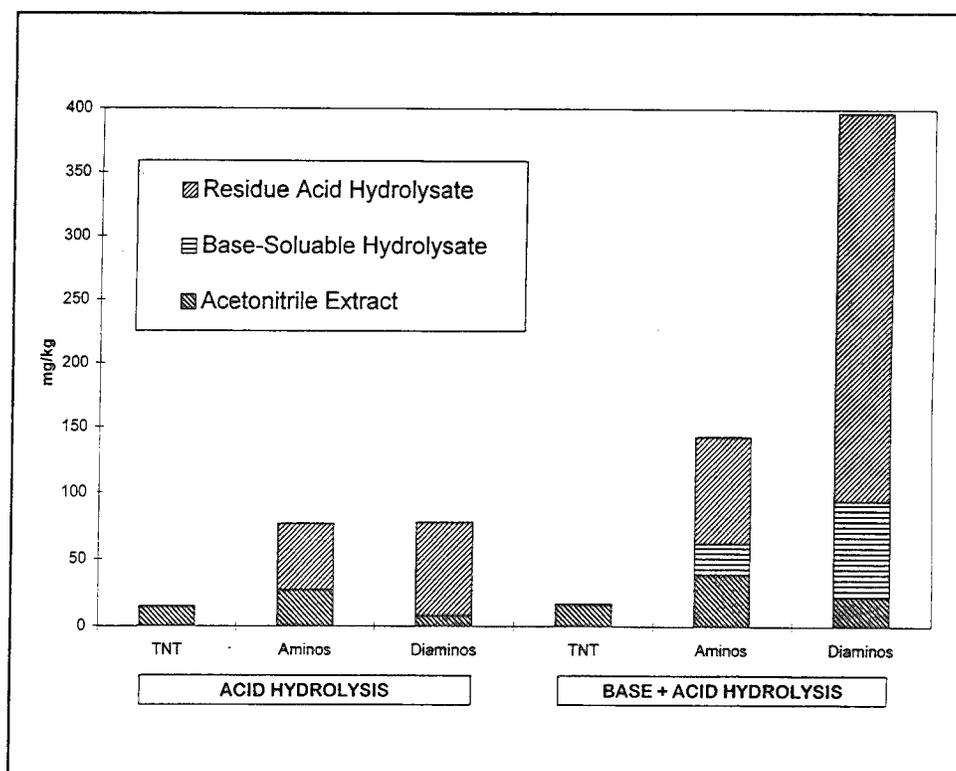


Figure 3. Comparison of recoveries of amino- and diamino compounds using acetonitrile extractions, acid digestions, and base-acid digestions

## References

- Bartha, R. (1971). "Fate of herbicide-derived chloroanilines in soil," *J. Agric. Food Chem.* 19, 385-7.
- Bartha, R., and Pramer, D. (1970). "Metabolism of acylanilide herbicides," *Adv. Appl. Microbiol.* 13, 317-41.
- Carpenter, D. F., McCormick, N. G., Cornell, J. H., and Kaplan, A. M. (1978). "Microbial transformation of  $^{14}\text{C}$ -labeled 2,4,6-trinitrotoluene in a activated-sludge system," *Appl. Environ. Microbiol.* 35, 949-54.
- Caton, J. E., Ho, C. H., Williams, R. T. and Griest, W. H. (1994). "Characterization of insoluble fractions of TNT transformed by composting," *J. Environ. Sci. Health. A.* 29, 659-70.
- Gorontzy, T., Drzyzga, O., Kahl, M. W., Bruns-Nage, D., Breitung, J., von Lowe, E., and Blotevogel, K. H. (1994). "Microbial degradation of explosives and related compounds," *Crit. Revs. Microbiol.* 20, 265-84.

- Griest, W. H., Stewart, A. J., Canton, J. E., Ho, C. H., Ironside, K. S., Caldwell, W. M., and Tan, E. (1993). "Chemical and toxicological testing of composed explosives-contaminated soil," *Environmental Toxicology and Chemistry* 12, 1105-16.
- Haderlein, S. B., Weissmahr, K. W., and Schwarzenbach, R. P. (1996). "Specific adsorption of nitroaromatic explosives and pesticides to clay minerals," *Environmental Science and Technology* 30, 612-22.
- Harvey, S. D., Fellows, R. J., Cataldo, D. A., and Bean, R. M. (1990). "Analysis of 2,4,6-trinitrotoluene and its transformation products in soils and plant tissues by high-performance liquid chromatography," *J. Chromatogr.* 518, 361-74.
- Hsu, T. S., and Bartha, R. (1974). "Interaction of pesticide-derived chloroaniline residues with soil organic matter," *Soil Sci.* 116, 444-52.
- \_\_\_\_\_. (1976). "Hydrolyzable and nonhydrolyzable 3,4-dichloroaniline-humus complexes and their respective rates of biodegradation," *J. Agric. Food Chem.* 24, 118-22.
- Jenkins, T. F., and Grant, C. L. (1987). "Comparison of extraction techniques for munitions residues in soil," *Anal. Chem.* 59, 1326-31.
- Jenkins, T. F., Thorne, P. G., McCormick, E. F., and Myers, K. F. (1995). "Preservation of water samples containing nitroaromatics and nitramines," Special Report 95-16, U.S. Army Cold Regions Research and Engineering Laboratory, Hanover, NH.
- Kaplan, D. L., and Kaplan, A. M. (1982a). "Thermophilic biotransformations of 2,4,6-trinitrotoluene under simulated composting conditions," *Appl. Environ. Microbiol.* 44, 757-60.
- \_\_\_\_\_. (1982b). "Composting industrial wastes- biochemical consideration," *Biocycle* 23, 42-44.
- Kearney, P. C., Plimmer, J. R., Wheeler, W. B., and Kontson, A. (1976). "Persistence and metabolism of dinitroaniline herbicides in soils," *Pest. Biochem. Physiol.* 6, 229-38.
- Martinez, G., Ho, C. H., Griest, W. H. (1995). "Supercritical fluid extraction of explosives and metabolites from composted soil," *Analytical Letters* 28, 1499-511.
- McCormick, N. G., Feeherry, F. E., and Levinson, H. S. (1976). "Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds," *Appl. Environ. Microbiol.* 31, 949-58.

- Palazzo, A. J., and Leggett, D. C. (1986a). "Effect and disposition of TNT in a terrestrial plant," *J. Environ. Qual.* 15, 49-52.
- \_\_\_\_\_. (1986b). "Effect and disposition of TNT in a terrestrial plant and validation of analytical methods," CRREL Report 86-15, U.S. Army Cold Regions Research and Engineering Laboratory, Hanover, NH.
- Pennington, J. C. (1988). "Soil sorption and plant uptake of 2,4,6-trinitrotoluene," Ph.D. diss., Louisiana State University, Baton Rouge, LA.
- Pennington, J. C., Hayes, C. A., Myers, K. F., Ochman, M., Gunnison, D., Felt, D. R., McCormick, E. F. (1995). "Fate of 2,4,6-trinitrotoluene in a simulated compost system," *Chemosphere* 30, 429-38.
- Scheunert, I., Mansour, M., and Andreux, F. (1992). "Binding of organic pollutants to soil organic matter," *Intl. J. Environ. Anal. Chem.* 46, 189-99.
- Walsh, M. E. (1990). "Environmental transformation products of nitroaromatics and nitramines- literature review and recommendations for analytical method development," CRREL Special Report 90-2, U.S. Army Cold Regions Research and Engineering Laboratory, Hanover, NH.
- Yamada, T. (1983). "Bound residues of 4-aminophenyl 2,4,6-trichlorophenyl ether and 4-aminophenyl 2,4-dichlorophenyl ether in paddy soil treated with CNP (Chlornitrofen)," *J. Pest. Sci.* 8, 33-39.
- You, I. S., and Bartha, R. (1982). "Evaluation of the Bleidner technique for analysis of soil-bound 3,4-dichloroaniline residues," *J. Agric. Food Chem.* 30, 1143-47.

# Appendix to Chapter 3

---

## Initial Analytical Development

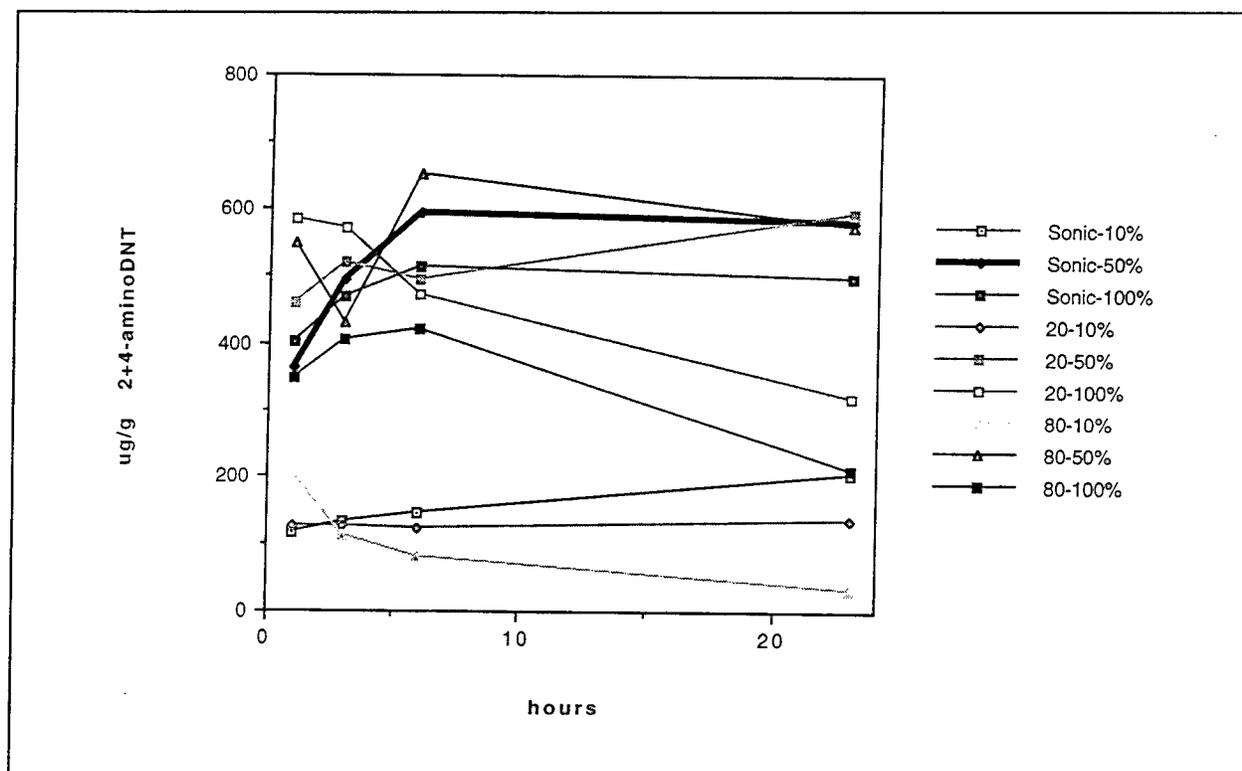
Initial extractions and digestions were performed using the U.S. Army Engineer Waterways Experiment Station composts. The first acid used for digestions was 6 N HCl. The release of additional quantities of aminoDNTs was noticed. The switch to concentrated H<sub>2</sub>SO<sub>4</sub> was pragmatic: it is a stronger acid and does not produce obnoxious fumes. Optimization of digestion conditions was determined by a single experiment with single samples. Results are plotted in Appendix Figure 1. Ten-percent acid was clearly insufficient. One hundred-percent acid caused analyte losses. High temperatures produced large interfering peaks in the chromatograms. Fifty-percent H<sub>2</sub>SO<sub>4</sub> in a sonic bath for 6 hr appeared to be both sufficient and conservative.

## Residues of RDX and HMX

Analyses for HMX, RDX, monitroso-RDX, trinitroso-RDX, and azoxy-nitrotoluenes were performed for all compost experiments. Digester sludges were not analyzed for these compounds due to large interferences using Method 8330 analysis. These data were not included in the journal article, which was restricted to TNT and its reduction products. Tables containing the additional data are included in this appendix (Appendix Tables 1-3)). RDX and HMX concentrations decreased at a slower rate than TNT concentrations. HMX concentrations were reduced more in the unaerated windrows than in the aerated windrows or static piles. Some HMX and RDX appeared in the acid digests because the washing step after the acetonitrile extraction had not yet been optimized.

## Anaerobic Digester Sludge From Bangor

These sludges were received from Elena Tuomi (ReTec). They were wet and not ground. This experiment was done before the gradient separation was developed, so there are no data on the diaminoNTs.



Appendix Figure 1. Acid digests of ACN extracted compost

Appendix Figure 2 is a flowchart for this experiment. Anaerobic digester residue (4.5 g) was split two ways. Sample heterogeneity was large. Two treatments were applied: (a) 10 ml of ACN overnight sonic, 30 °C and (b) 10 ml of aqueous, 50 mM  $\text{KH}_2\text{PO}_4$  (pH 5.5), 35 °C with 0.5 g of cellulase. Vials were spun, then the extracts filtered and saved. Extracts were either diluted with MQ or MeOH or diluted with 50-percent  $\text{H}_2\text{SO}_4$ , then MeOH and analyzed by HPLC Method 8330. ACN-extracted residue was washed with 20 ml of ACN and the last few drops of wash examined—no more analytes were detected. The cellulase residue was washed with 50 ml of MQ. The rinsed ACN-extracted residue was split in half. Half was cellulase digested—no more analytes were released. The other half was digested with 50-percent  $\text{H}_2\text{SO}_4$  for 18 hr at 37 °C. 4ADNT and a shoulder of 2ADNT were recovered. The cellulase residue was washed with MQ to remove the enzyme, then ACN extracted. TNT and 4 and 2ADNT were recovered. This residue was then washed with 20 ml of ACN, dried, and digested with 50-percent  $\text{H}_2\text{SO}_4$ . 4ADNT was recovered; but band broadening obscured the possible detection of 2ADNT. The sums of analytes from the two treatment paths were not equal. The acid digests of the soluble fractions (ACN, aqueous buffer and cellulase digest) did not release additional analytes.



Appendix Table 1 (Concluded)

CWR8-unaerated		Concentration, mg/kg												
		TNT	4-ADNT	2-ADNT	2,6-diANT	2,4-diANT	TNB	2,4DNT	HMX	RDX	mononitroso-RDX	trinitroso-RDX		
Day 1 ACN extract %rsd	2,000 0	379 8	215 9	nd	nd	8 2	3.3 7	221 4	1,060 4	nd	nd			
Day 1 ACID hydrolysate %rsd	nd	20.3 12	3.9 13	nd	12.8 49	nd	nd	nd	2.5 5	nd	nd			
Day 5 ACN extract %rsd	113 21	1,090 12	450 16	4.6 29	20.9 16	nd	nd	237 7	1,070 6	31.1 12	nd			
Day 5 ACID hydrolysate %rsd	nd	33.7 11	4.4 9	4 16	57.2 15	nd	nd	nd	nd	nd	nd			
Day 10 ACN extract %rsd	40 17	154 4	7.5 5	14.5 5	172 10	nd	nd	230 5	940 6	23 2	nd			
Day 10 ACID hydrolysate %rsd	nd	40.3 6	2.7 0	5.4 21	111 27	nd	nd	nd	nd	nd	nd			
Day 15 ACN extract %rsd	22.6 36	10.6 2	1.9 14		10.7 7	nd	nd	142 4	114 8	15.2 9	10.8 6			
Day 15 ACID hydrolysate %rsd	nd	25.4 2	1.7 1	4.9 0	93.6 11	nd	nd	nd	nd	nd	nd			
Day 20 ACN extract %rsd	3.8 10	3.2 16	0.8 6	nd	nd	nd	nd	nd	nd	nd	nd			
Day 20 ACID hydrolysate %rsd	nd	5.8 1	0.4 0	2.4 0	53.8 9	nd	nd	nd	nd	nd	nd			
Day 40 ACN extract %rsd	3.8 2	3.7 14	1 19	nd	nd	nd	nd	nd	4.9 9	7.5 7	nd			
Day 40 ACID hydrolysate %rsd	nd	6.3 1	1 0	1.4 22	20.2 3	nd	nd	nd	nd	nd	nd			

Appendix Table 2 Day 15 Aerated Compost (Overnight, sonic acetonitrile extracts (ACN) and 6-hr sonic H <sub>2</sub> SO <sub>4</sub> digests of NaOH extracts (Base) and insoluble residues (Acid))							
Concentration, mg/kg							
	TNT	4-ADNT	2-ADNT	2,6-diANT	2,4-diANT	HMX	RDX
Day 15 ACN extract	14.7	29.9	1.4	0.5	17.7	173	116
Base hydrolysate	nd <sup>1</sup>	19.8	nd	2	55.2	nd	nd
Acid hydrolysate	nd	81	7.8	7.8	276	nd	nd

<sup>1</sup>nd = <0.1 mg/kg.

## Resampled Composts From Umatilla Static Piles and Windrows

These samples were received from Weston. They were 40-day composts that had been bagged and left onsite for several months. Overnight, sonic ACN extractions and TCLP (pH 5.0 aqueous, 18 hr, 20 °C) leachings were performed on single samples. ACN-extracted residues were acid digested with 50-percent H<sub>2</sub>SO<sub>4</sub> for 6 hr in the sonic bath. Results are listed in Table 3. The results for the ACN extracts were similar to the results reported by Greist for these Composts. Acid digestions of the TCLP leachates and ACN extracts did not release additional analytes. The acid digests from these new samples were run without neutralization.

## Neutralization of Acid Digests

Before a large number of samples from a complete time-series was run, a neutralization method was developed that would not only reduce the risk of damaging the HPLC column, but also provide some sample cleanup, since resolution deteriorated rapidly when analyzing digests directly. It was also found that the diaminoNTs were protonated in strong acid and were not retained by the C-18 column. The best way (and safest!) to neutralize a strong acid is by dilution with a moderately strong, basic buffer. This was easily accomplished for 5 ml of acid digest by using 100 ml of 1.2 M Na<sub>2</sub>HPO<sub>4</sub>. The neutralized solution could be analyzed directly; however, the detection limit would be raised to approximately 100 µg/g due to the dilution—and the interfering substances would still be present. A SepPak Poropak<sub>RDX</sub> solid-phase extraction cartridge was used to reconcentrate the digest back to the original 5 ml, while not retaining many of the interfering compounds. Recoveries from spiked Milli-Q of all analytes except the nitroso-RDXs were close to 100 percent. The nitrosos were recovered at about 30 percent. The relative standard deviations of the results for the time-series were good. Appendix Figure 3-5 are representative chromatograms of the standards, acetonitrile extracts, and acid digests.

Appendix Table 3

Umatilla Resampled Windrows and Static piles (tank reactors) (Overnight, sonic acetonitrile extracts (ACN), 6-hr sonic H2SO4 dligests and 18-hr aqueous TCLP leachates)

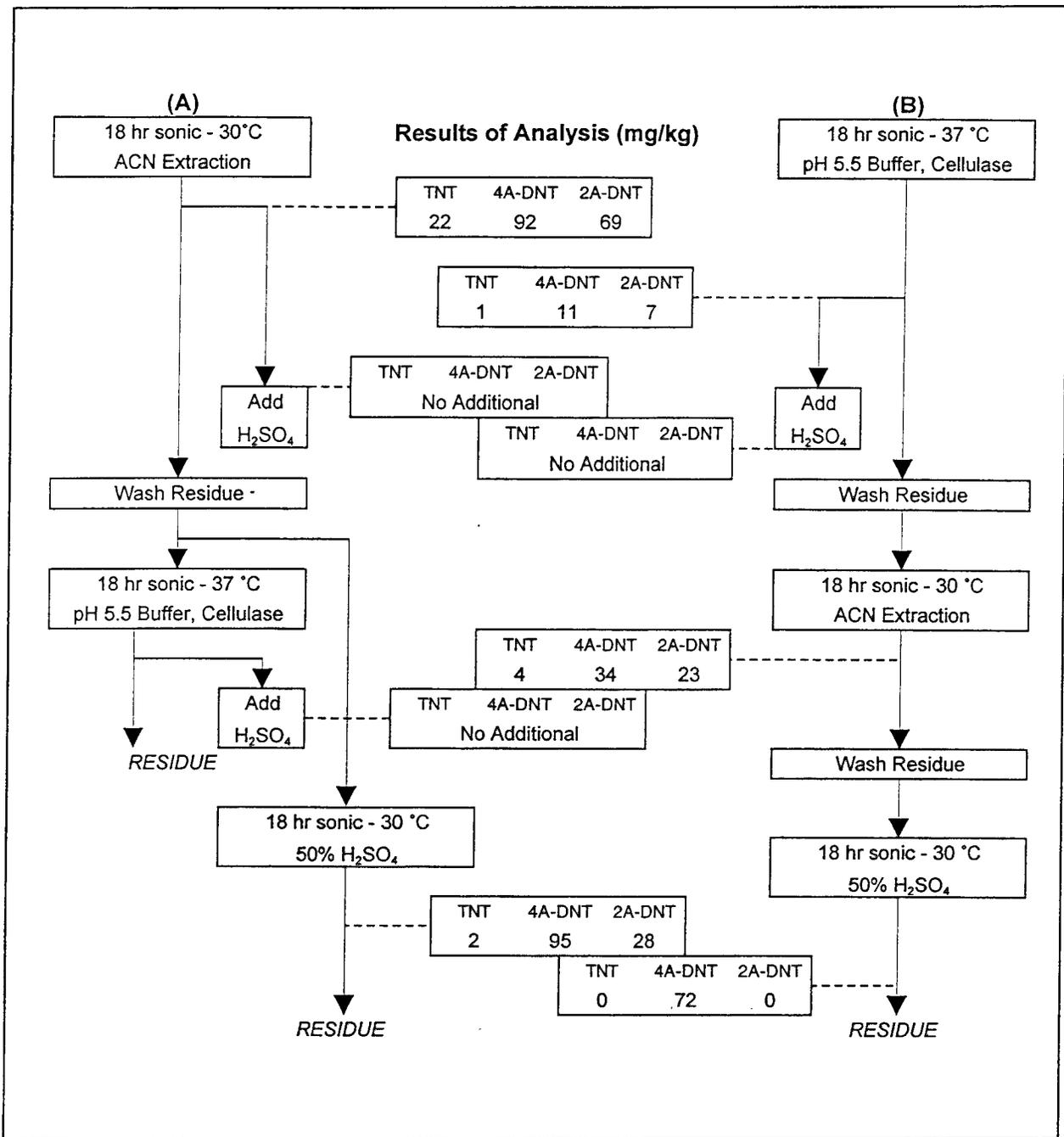
		Concentration, mg/kg											mononitroso-	
		TNT	4-ADNT	2-ADNT	2,6-diANT	2,4-diANT	TNB	2,4DNT	2-2Azoxy	4-4Azoxy	HMX	RDX	RDX	
CWR7-Aerated Windrow														
ACN extract	5.1	3.4	1.6	nd <sup>1</sup>	nd	0.9	nd	1.4	6.4	16.5	4.3	nd	nd	
ACID hydrolysate	nd	6.9	1.2	nd	5.5	0.5	0.2	nd	nd	nd	nd	nd	nd	
TCLP leachate	nd	1.7	nd	nd	nd	nd	nd	nd	nd	13.6	2.2	nd	nd	
CWR8-Unaerated Windrow														
ACN extract	1.3	1	0.5	nd	nd	0.9	nd	nd	nd	1.8	1.8	nd	nd	
ACID hydrolysate	nd	4	0.8	nd	nd	0.5	nd	nd	nd	2.7	nd	nd	nd	
TCLP leachate	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.5	1.6	nd	nd	
SEED C-Aerated Static Pile														
ACN extract	6.9	29.2	1.5	nd	nd	nd	nd	8.4	200	514	nd	nd	nd	
ACID hydrolysate	nd	47.1	5.5	0.3	36.5	nd	1	nd	7	49.4	6.5	nd	nd	
TCLP leachate	nd	12.2	1.5	nd	nd	nd	nd	nd	59.9	404	nd	nd	nd	
CONTROL C-Aerated Static Pile														
ACN extract	63.1	31.2	6.9	nd	nd	nd	nd	nd	200	545	nd	nd	nd	
ACID hydrolysate	nd	43.2	5.7	0.3	47.5	nd	1.1	nd	9.9	64.6	9.4	nd	nd	
TCLP leachate	1.1	16.6	3.2	nd	nd	nd	nd	nd	64.7	366	nd	nd	nd	
SEED D-Aerated Static Pile														
ACN extract	3	30.7	6.7	nd	nd	nd	nd	3.7	183	175	nd	nd	nd	
ACID hydrolysate	nd	33.3	6	0.5	34.8	nd	1.1	nd	9.3	49.4	0.9	nd	nd	
TCLP leachate	nd	8.3	1.3	nd	nd	nd	nd	nd	51.1	139	nd	nd	nd	
CONTROL D Static Pile														
ACN extract	6.3	22.2	5.5	nd	nd	nd	nd	nd	147	256	nd	nd	nd	
ACID hydrolysate	nd	33.1	6.1	0.5	27.4	nd	1	nd	5.8	31.5	6.3	nd	nd	
TCLP leachate	nd	8.4	1.6	nd	nd	nd	nd	nd	44	213	nd	nd	nd	

<sup>1</sup>nd = <0.1 mg/kg.

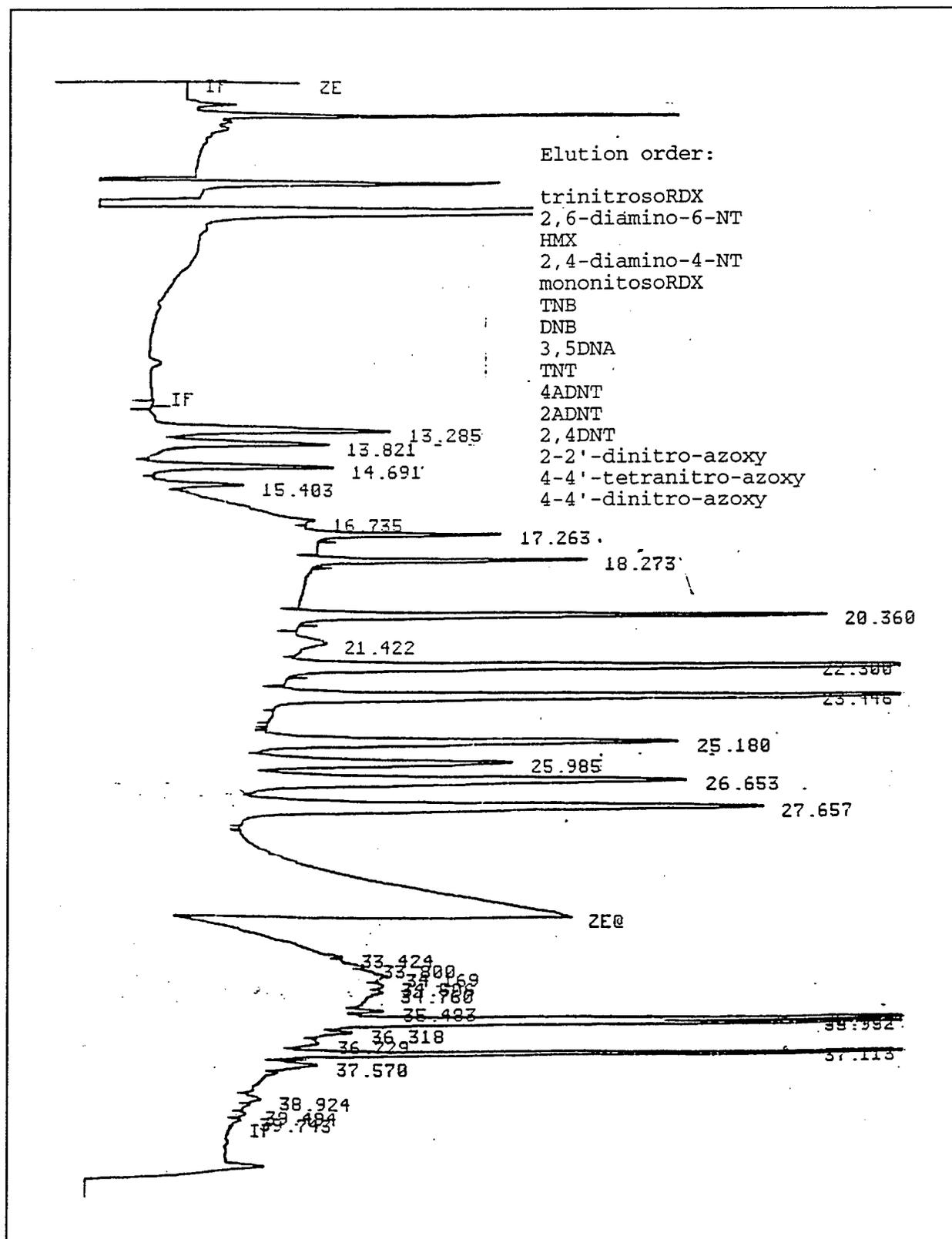
The samples for the time-series were received from Black and Veatch but had been prepared by Weston. Their initial tests determined that air-drying, milling, and riffing could produce subsamples that had analyte concentrations with RSDs of around 4 percent using 250-g samples. For the time-series samples, they took 500- to 1,000-g samples from 14 locations in each windrow, composited this material, then proceeded with the processing.

## Future Experiments

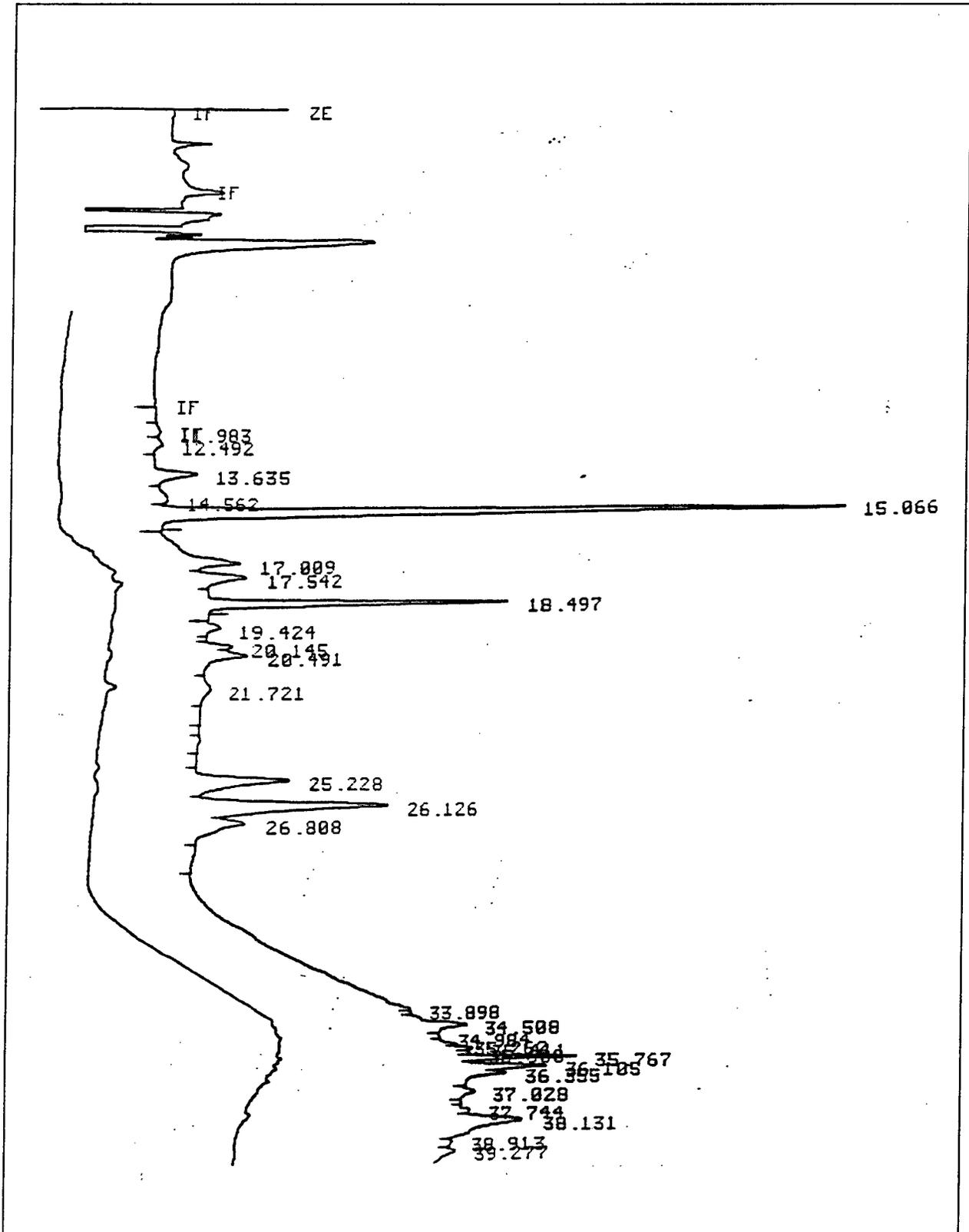
- a.* Do some spike-recovery tests with the diAnts and ADNTS using the Control composts.
- b.* Work out a neutralization/Sep-Pak recovery of the base-soluble fraction. Were some of the reduction products hydrolyzed by the base, before the acid digestion.
- c.* Configure a continuous acid digestion-neutralization-Sep-Pak recovery system using low-pressure chromatography hardware.
- d.* Perform the improved base/acid hydrolysis on a time-series from the optimized Umatilla operation. How much can be recovered from the "finished" compost?
- e.* Perform the hydrolysis on more anaerobic digester sludge. There is a time-series from the Bangor digesters.



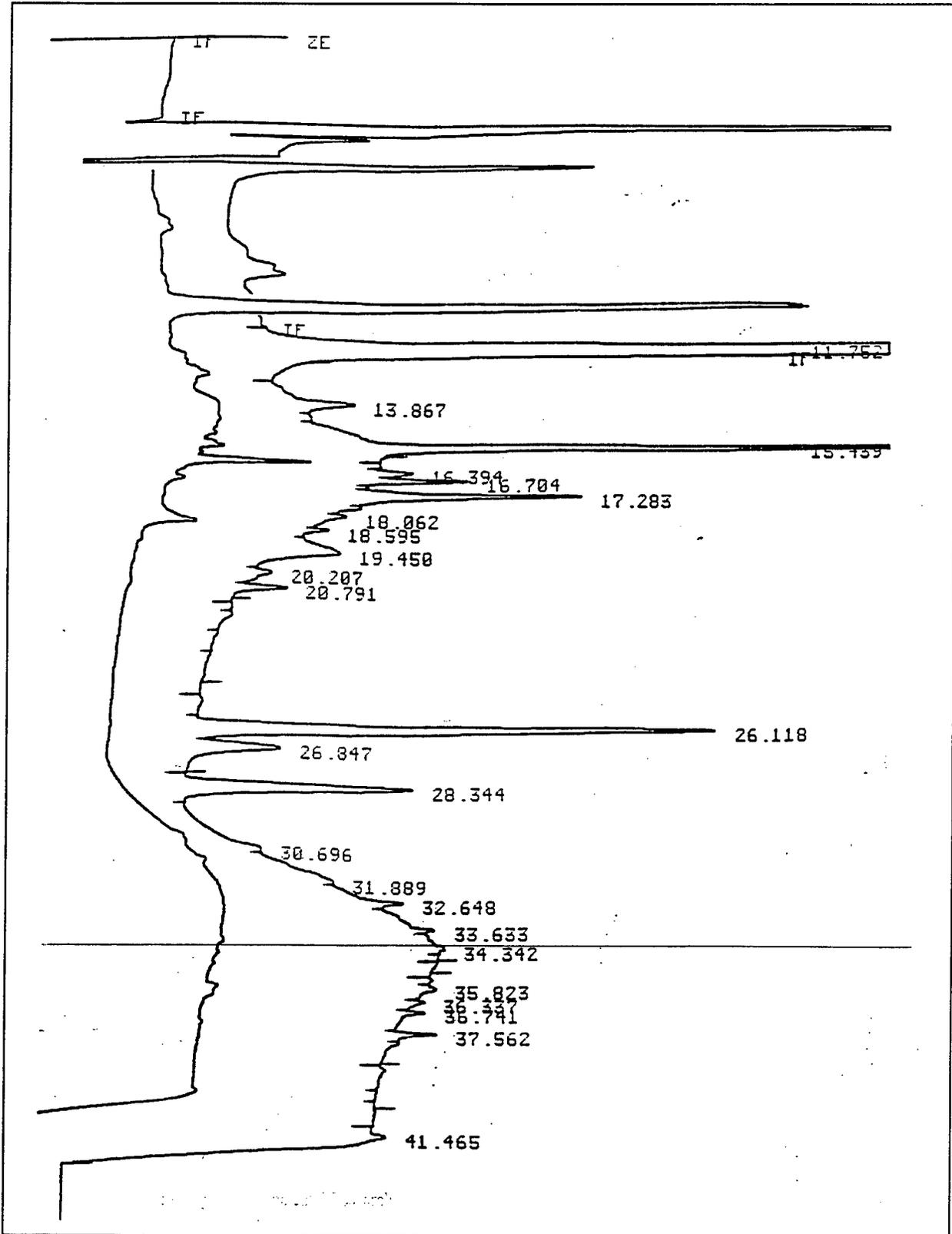
Appendix Figure 2. Analysis of anaerobic digester sludge



Appendix Figure 3. Sixteen analyte standard (1,000 µg/L), gradient elution



Appendix Figure 4. Aerated Day 40 compost and Day 55 control-acetonitrile extract



Appendix Figure 5. Aerated Day 40 compost and Day 55 control AcLa digest

# 4 Microbial Degradation of Conjugated Fractions<sup>1</sup>

---

## Objectives

The objectives of this chapter are (a) to assess the ability of enzymes active in modifying substituents on aromatic rings to modify TNT and/or mediate reactions between TNT and major soil organic constituents, and (b) to evaluate the ability of soil microorganisms to degrade radiolabeled TNT bound to various organic components in the soil.

## Methods and Materials

### Enzyme assays

Polyphenol oxidase (tyrosinase or catecholase), laccase, and peroxidase were purchased from Sigma Chemical Company, St. Louis, MO. Some general properties of each commercial enzyme, together with the test assays conducted to ensure the effectiveness of each enzyme, are given below.

**Polyphenol oxidase.** This enzyme was assayed with the procedure specified by Worthington (1993), which indicates that the polyphenol oxidase is bifunctional, possessing both phenol hydroxylase and polyphenoloxidase activities that convert a monophenol via *o*-diphenol to the *o*-quinone. Optimal pH is in the range of 6-7, below the point where abiotic conversion of TNT to azoxydinitrotoluenes may occur. The enzyme is able to oxidize a variety of parasubstituted catechols (Duckworth and Coleman 1970), but is inhibited by compounds that complex with copper. Benzoic acid will competitively inhibit activity against catechol, while cyanide will competitively inhibit interactions with oxygen (Duckworth and Coleman 1970).

---

<sup>1</sup> Authors of this chapter are D. R. Felt, C. A. Hayes, and B. E. Porter, AScI Corporation, McClean, VA, and D. Gunnison and H. Fredrickson, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

The assay for polyphenol oxidase is based on the oxidation of tyrosine to dihydroxyphenylalanine. The latter compound is subsequently oxidized to *o*-quinone, yielding an increased absorbance at 280 nm. According to the Worthington Manual, the rate of increase is in proportion to the enzyme concentration and is linear for 5 to 10 min following initiation of the assay. A unit of activity is defined as an increased in absorbance of 0.001 per minute under the defined conditions at 25 °C.

The enzyme was assayed as follows. An aqueous solution of cold (5 °C) enzyme was dissolved in distilled water to a concentration of 1.2 mg/ml. The active control substrate solution was prepared by dissolving 0.001M of L-tyrosine in distilled water. The assay solution was formulated by mixing 1.0 ml of 0.5M phosphate buffer at pH 6.5 with 1.0 ml of the active control substrate solution and 0.9 ml of distilled water. To provide dissolved oxygen for the reaction, the solution was aerated for 5 min in the assay cuvette using a fine capillary tube. The reaction was initiated by adding 0.1 ml of the enzyme solution and following spectrophotometrically at 280 nm. Following verification of activity, the activity of the same enzyme solution was tested against TNT that had been recrystallized in methanol and dissolved in distilled water to a concentration of 0.001M. The TNT solution was then substituted for the active control substrate and assayed in the same manner. Following this, the enzyme assay was repeated, but with the addition of 10 mg of commercial lignin or humic acid per liter together with 0.001M of the crystallized TNT preparation.

**Laccase.** This assay was conducted with a procedure supplied by Sigma Chemical Company (1995), based on the procedure of Ride (1980), also known as essay EC 1.10.3.2. In this assay, the active control substrate, syringaldazine, is enzymatically combined with oxygen at 30 °C and pH 6.5 by the fungal enzyme laccase to yield oxidized syringaldazine and water. The reaction is measured spectrophotometrically at 530 nm.

The enzyme was assayed as follows. A 100 mM potassium phosphate buffer in water was prepared and adjusted to pH 6.5 (buffer solution). The syringaldehydrazine active control solution was prepared by dissolving sufficient syringaldazine in solution methanol to yield a 0.216 mM solution in a final volume of 3.0 ml. The laccase enzyme solution was prepared by dissolving 2.5 mg of the enzyme in 10 ml of cold distilled water. This solution was maintained on crushed ice until used. A total of 2.20 ml of phosphate buffer solution was gently mixed with 0.50 ml of the enzyme preparation in a cuvette contained in a thermostatted spectrophotometer and permitted to equilibrate to 30 °C. At this time, 0.30 ml of the syringaldazine solution was also added to the two cuvettes, and the contents of the cuvettes were mixed by inversion. The control used for this reaction was 0.5 ml of distilled water in place of the laccase enzyme solution. The reaction was allowed to run for 10 min at 530 nm, with the absorbance of the reaction being read every 30 sec.

To assay the activity of this preparation against TNT, a solution of 10.0 mg of TNT was dissolved in 100 ml of distilled water, and this solution was substituted for the syringaldazine active control solution.

**Peroxidase.** This assay was conducted with the horseradish peroxidase procedure given in the Worthington Manual (Method EC 1.11.1.7). This is based on peroxidase catalyzing an oxidation by hydrogen peroxide and a variety of substrates, including ascorbate, ferrocyanide, cytochrome C, and various dyes in the leuco form. The enzyme is highly specific and will show activity with hydrogen peroxide and methanol or ethanol.

The assay is conducted with 4-aminoantipyrine as the hydrogen donor and is monitored as change in absorbance at 510 nm. One unit of activity is defined as the decomposition of 1  $\mu$ M of hydrogen peroxide per minute at 25 °C and pH 7.0. A sufficient amount of the enzyme preparation was dissolved in distilled water to yield a solution containing 1 mg/ml. The active control solution was formulated by preparing a solution of 0.0025M 4-aminoantipyrine with 0.17 mg of phenol in distilled water. The buffer solution used was a 0.2M potassium phosphate solution in distilled water. The hydrogen peroxide substrate is used at a level of 0.0017M dissolved in 0.2M potassium phosphate buffer, pH 7.0. For TNT assays, 1 mg of TNT was dissolved in 1 L of the phosphate buffer.

### Enrichment cultures

**Culture medium.** Enrichment cultures were conducted using the mineral medium of Konovaltshikoff-Mazoyer and Senez (1956) as described in Aaronson (1970) amended with 2.5 ml of the A9 medium of Abriel et al. (1989) per liter of final medium. The mineral medium contained, per liter of distilled water,  $\text{NH}_4\text{Cl}$ , 0.5 g;  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.5 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g; and  $\text{NaCl}$ , 4.0 g. A9 salts solution contained, per liter of distilled water,  $\text{HBO}_3$ , 300 mg;  $\text{ZnCl}_2$ , 50 mg;  $\text{MnCl}_4 \cdot \text{H}_2\text{O}$ , 30 mg;  $\text{CoCl}_2$ , 200 mg;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mg; and  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 30 mg.

Substrates for enrichment cultures were obtained from the extraction and fractionation of humic substances developed by K. F. Myers (Pennington et al. 1995). In summary, this procedure consisted of extraction with acetonitrile (ACN), followed by 0.5N NaOH to produce soluble and insoluble fractions. The soluble fraction is acidified to pH 1 with HCl. The material remaining in the acidified fraction is fulvic acid, while that precipitated by acidification is humic acid and humin. This precipitate is treated with NaOH and centrifuged to produce a soluble humic acid and an insoluble humin fraction. The material not solubilized in the original acetonitrile 0.5N NaOH treatment contains cellulose and also some humin. Upon treatment of this material with methyl isobutyl ketone (MIBK), the cellulose remains insoluble, while the humin is dissolved and can be reprecipitated by acidification with HCl, as described above. This procedure was run on soil from the Weldon Springs Army Ammunition Plant to obtain the fractions indicated.

Enrichment cultures required the use of sterile solutions, particularly as the enrichment process progressed. Cellulose was sterilized by autoclaving 0.5 g of the material in the enrichment flask at 121 °C for 15 min. Fractions dissolved in residual MIBK (cellulose and humin removed, then neutralized) and ACN were already sterile from treatment with these solvents. To remove these solvents, 5.0 ml of each of the fractions was placed into separate sterile 50-ml Erlenmeyer flasks with cotton plugs. The solvents were allowed to evaporate through the cotton plugs for several days under the hood. The flasks were each covered with foil to prevent photodegradation. The aqueous humic acid and fulvic acid fractions were sterilized by passage through a 0.45- $\mu$ m microporous filter, and the resulting filtrates were neutralized aseptically with 1N HCl or N NaOH. One-half gram of the insoluble humin was stirred in 10 ml of fresh MIBK in a 50-ml beaker to form a slurry, and 5 ml of this was pipetted into a sterile 50-ml flask, and evaporated as for the MIBK and ACN fractions. All flasks were prepared in triplicate. When all preparations were dry, 9.5 ml of the mineral medium was added to each flask. All pH values were double checked and readjusted to 7.0 if needed. To determine if humic acid dissolved in MIBK could support microbial growth, a residual MIBK fraction (cellulose and humin removed, then neutralized) was also used as an enrichment substrate.

**Inoculation, propagation, and isolation of microorganisms.** One flask of each of the substrate fractions was inoculated with 0.5 g of a Vicksburg, MS, garden soil, covered with aluminum foil, and incubated for 1 week at room temperature on a shaker at 75 rpm. A 0.5-ml aliquot of each culture was aseptically transferred to a sterile flask containing the same medium and substrate. These flasks were covered with foil and replaced onto the shaker for another week of incubation. This process was repeated a third time before the cultures were checked for microbial growth by streaking onto nutrient agar. Enrichment cultures exhibiting positive growth at this point were streaked onto the same medium that had been solidified with 1.5-percent agar. Individual microbial isolates obtained by this process were inoculated into sterile medium with the substrate on which they were isolated.

**Mineralization and other fates of radiolabeled carbon in the fractions.** Fractions prepared from Umatilla Army Depot Activity (UMDA) soil that had been amended with radiolabeled TNT were assayed with radiorespirometry. Two hundred fifty milligrams of each radiolabeled fraction was sterilized and placed aseptically into 250-ml Bellco Biometer flasks, each containing 25 ml of the sterile mineral salts-A9 salts solution used for the enrichment cultures. The cellulose fraction was not sterilized, since the particles are too large for filtration sterilization. Sterilization by autoclaving was undesirable for all fractions because TNT is broken down by moist heat. Therefore, the control fractions were used to exhibit any mineralizing activity resulting from the presence of suitable microorganisms in the unsterilized material. Thus, any activity present in the cellulose fraction receiving the active culture above and beyond the value present in the controls would represent mineralization activity from the active culture.

These flasks were prepared in sets of seven, with the seventh flask containing the nonradiolabeled version of the fraction (nonradioactive control). Four of the flasks containing the radiolabeled fraction and the nonradioactive control each 5.0 ml of 1-week-old culture of the microorganism isolated for the fraction, as above. The remaining two flasks containing the radiolabeled fraction received 5.0 ml of the same 1-week-old culture, but this inoculum was sterilized separately by autoclaving prior to introduction into the flasks. One millimeter of the mixture in the center well of each flask was removed and placed into a 20-ml scintillation vial prior to sealing the flasks. At this time, 2.0 ml of 1.0N KOH was added to the sidearm of each unit to trap evolved  $^{14}\text{CO}_2$ . Flasks were incubated for 1 month at room temperature on a shaker at 75 rpm. The KOH in each sidearm was replaced at intervals of 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, and 30 days following inoculation. Upon completion of incubation, the contents of the main well of each flask were acidified with 3-4 drops of  $\text{H}_3\text{PO}_4$  and then resealed with 2.0 ml of fresh KOH in the sidearms to capture any  $^{14}\text{CO}_2$  evolving as carbonate. This mixture was incubated for 24 hr at room temperature and 75 rpm prior to removal of the KOH from the sidearm. An additional 1.0 ml of sample was removed from the main well of each flask to determine total radioactivity remaining in the flask. The contents of the flask were also separated via HPLC or thin-layer chromatography and compared with known standards to identify degradation products.

## Results and Discussion

### Enzyme assays

Of the enzymes assayed, laccase, peroxidase, and polyphenol oxidase, only polyphenol oxidase exhibited activity against TNT). The activity rose sharply against time for the first concentration tested (1.0 mg/L) (Figure 1). To determine if activity would increase with increasing substrate concentration, the assay was run a second time at a TNT level of 100 mg/L. Here the enzyme responded poorly, suggesting that the active sites may have become overloaded or otherwise removed from the reaction. While these results indicate that polyphenol oxidase (L-tyrosinase) could act on low levels of TNT, additional assays were run to determine the TNT concentration at which inhibition begins. The study was rerun at concentrations, 1, 10, 25, and 50 mg of TNT/L. The TNT used for this portion of the work was recrystallized from methanol. Controls containing the same levels of TNT as the active preparation, but lacking the enzyme preparation, were run for each of the TNT concentrations to eliminate any possible interference resulting from photodegradation of TNT. Although photodegradation was detected, the rate and extent did not account for the activity found with the enzyme and crude TNT (1 mg of TNT/L). The second study using recrystallized TNT gave no evidence of enzymatic activity at any concentration. A rerun of this test produced the same results. The polyphenol oxidase was reacting with some component in the crude TNT preparation that was removed by the recrystallization process.

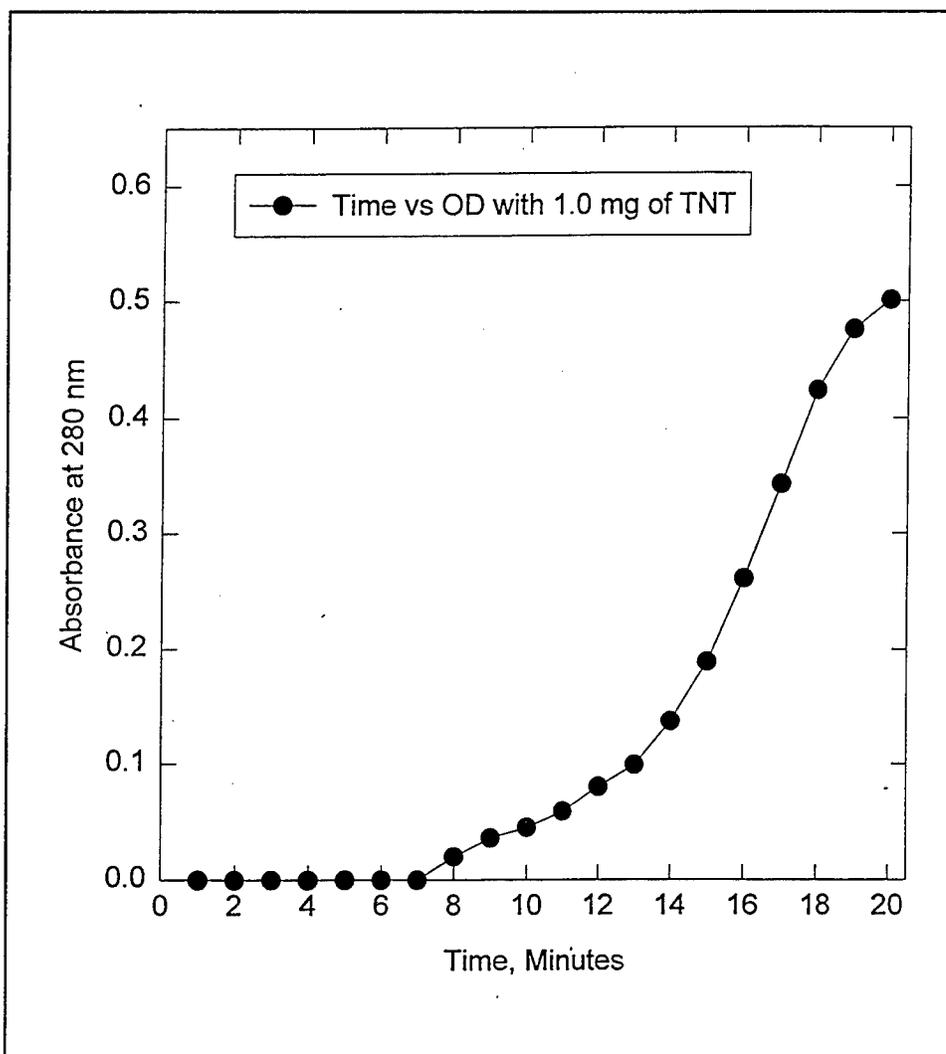


Figure 1. Change in absorbance of TNT over time with 1.0 mg of TNT incubated with polyphenol oxidase

### Enrichment cultures

This process was run in parallel with the extraction of radiolabeled fractions from the composted Umatilla soil. Three consecutive transfers of the enrichment culture were made, and apparently pure cultures of active microorganisms were obtained in the enrichment cultures containing cellulose, the humin and fulvic acid fractions, the acetonitrile extract, and the humic acid fraction, but not the residual MIBK fraction (from which the cellulose and humin had been removed). The active cultures are presently being identified. The residual MIBK fraction supported a poor, barely observable growth of microorganisms that failed to grow upon reculturing. Therefore, the residual MIBK fraction was dropped from further studies.

The results of radiorespirometry studies of the pure cultures for the cellulose, acetonitrile, humic acid, humin, and fulvic acid fractions are shown in Figure 2. The only fraction showing significant mineralization was the cellulose. The humic acid fraction also demonstrated some significant differences, but these are difficult to see because of the substantial widths of the error bars. Additional testing is being conducted on the cellulose and humic acid fractions to verify the observed differences, to provide more replicates to try and narrow the error bars in the humic acid fraction, and to produce enough material for detailed chemical analysis of the radiolabeled fractions. Of the remaining fractions, only the fulvic acid fraction demonstrated substantial differences between the active and sterile control treatments; however, the active and sterile control curves were close together and followed about the same pattern, suggesting that some other process besides biodegradation was occurring.

## Conclusions

The enzymes laccase and horseradish peroxidase were ineffective in transforming TNT, indicating that they are unlikely to have a major impact in forming conjugates between TNT molecules and between TNT and the soil fractions. Polyphenol oxidase was active in modifying dissolved TNT from a nonpurified batch at a concentration of 1 mg/L. However, the same level of enzyme failed to respond positively to increased levels of TNT in the range of 100 mg/L. An attempt to determine the limit at which inhibition occurred using a recrystallized TNT preparation demonstrated no activity at the 1, 10, 25, and 50 mg of TNT/L levels. This work was suspended in order to seek alternative means for measuring TNT modification and to conduct an analysis on the crude TNT preparation to determine the specific substrate(s) that may have been targeted by the polyphenol oxidase.

Microbial isolates were obtained from enrichment cultures grown of cellulose, humin and fulvic acids, acetonitrile extract, and humic acid, but not the residual MIBK fraction. When tested against extracts of U-[<sup>14</sup>C]-TNT containing compost via radiorespirometry, several of the fractions released <sup>14</sup>CO<sub>2</sub>. However, only the cellulose and humic acid fractions displayed a substantial difference between the active and sterile control treatments. The fulvic acid fraction also showed a substantial difference between treatments; however, in this case the two treatments paralleled each other very closely, indicating the abiotic processes are occurring.

## References

- Aaronson, S. H. (1970). *Experimental microbial ecology*. John Wiley and Sons, New York.
- Duckworth, H. W., and Coleman, J. E., (1970). *Biol. Chem.* 245, 1613-17.

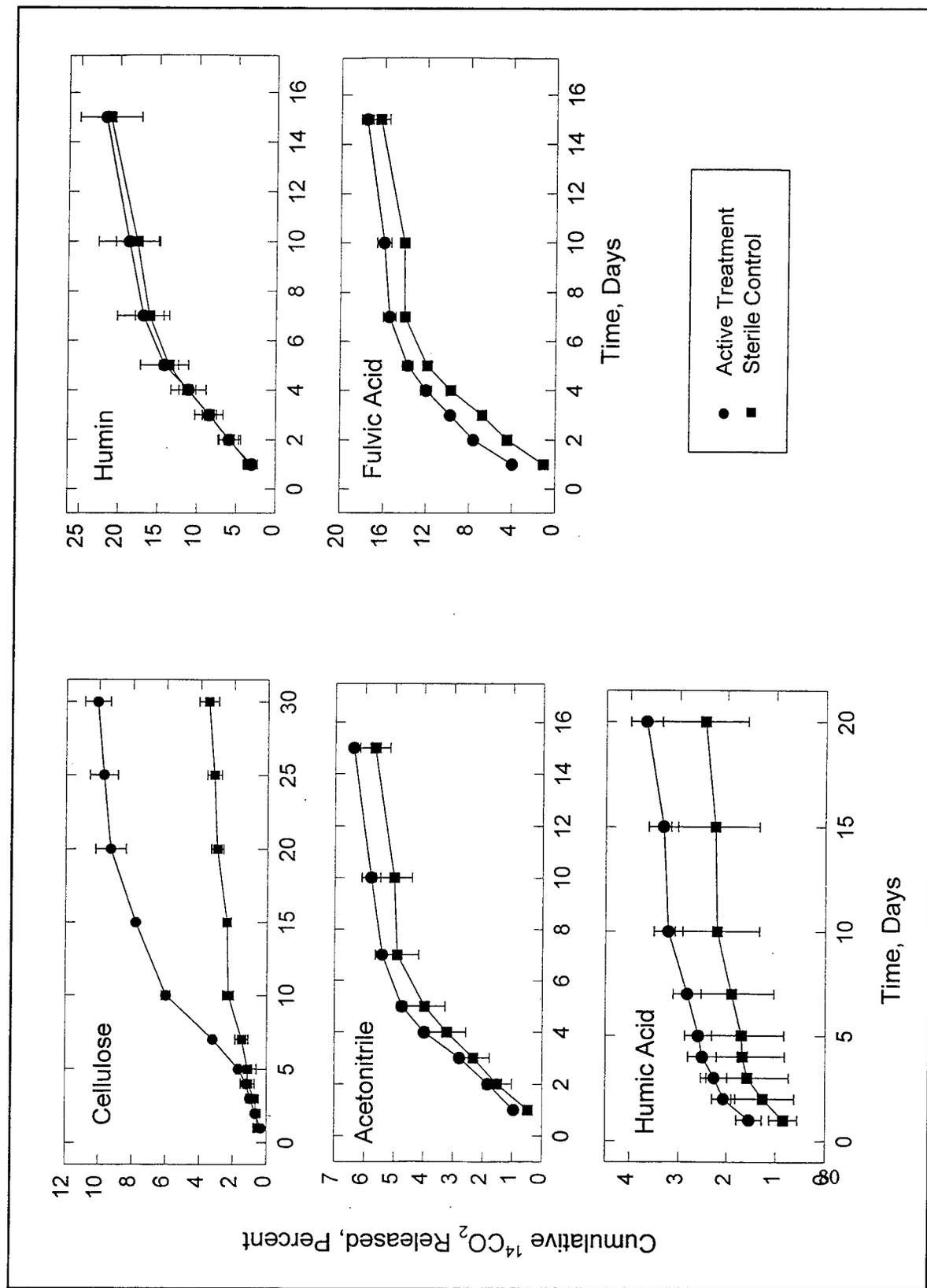


Figure 2. TNT mineralization in U- $^{14}\text{C}$ -TNT labeled fractions

Pennington, J. C., Hayes, A., Myers, K. F., Ochman, M., Gunnison, D., Felt, D. R., and McCormick, E. F. (1995). "Fate of 2,4,6-trinitrotoluene in a simulated compost system," *Chemosphere* 30, 429-38.

Ride, J. P. (1980). *Physiological Plant Pathology* 16, 187-96.

Worthington. (1993). *Worthington enzyme manual*. Worthington Biochemical Corporation, Freehold, NJ.

# 5 Analysis of Coupling Affinities Between TNT Degradation Products and Humic Acid Based on Surface Plasmon Resonance<sup>1</sup>

---

## Introduction

Recent research suggests that amino transformation products of TNT are attached to humus in surface soils (Lenke et al. 1993). Humic acid, a principal component of soil humus, is a structurally complex and heterogeneous material. Hypothetical structures have been proposed, but chemically specific characterization is difficult. However, many of the functional groups available for interactions with contaminants are known. The possibility of interactions between these functional groups and the amino transformation products of TNT warrant investigation.

Surface plasmon resonance (SPR) is an optical phenomenon that can be coupled to a biospecific interaction analysis system (BIA) to study the binding affinity between molecules. Molecular interactions occur at the surface of a sensor chip and are measured by an optical system. The parts of the system include a liquid handling system that supplies reagents, buffers and sample, an optical system for detection of coupled material by changes in refractive index, and a sensor chip in which the immobilization reactions occur. The sensor chip consists of three layers and four surface flow cells. The layers are a glass base, a thin gold film for enhancing the SPR, and a hydrogel of carboxylated dextran that provides the matrix for immobilization of one of the reactants of interest. The second reactant is introduced in the aqueous phase that flows through the hydrogel. The four surface flow cells allow four independent immobilization tests to occur simultaneously on the same chip.

---

<sup>1</sup> The author of this chapter is Christoph H. Allersmeier, U.S. Army Natick Research, Development and Engineering Center.

To study interactions between TNT transformation products and humic acid, the objective was to immobilize the smaller molecule, e.g., 4-amino-2,6-dinitrotoluene or 2,6-diamino-4-nitrotoluene, to the hydrogel and flow the larger molecule, the humic acid, through the matrix.

## Methods

The software controlling the system (BIACore) operations includes a methods definition language (MDL) to write sequences directing the test process. The programming language is derived from Qbasic and gives control over all applications. Each test requires a method, written as a text file in a sequence of special orders and exact syntax. Therefore, preliminary runs must be conducted to verify the method and eliminate syntax errors.

An appropriate buffer system for the test system must be developed. Suggestions are presented in the instrument manual (Pharmacia Biosensor AB 1991). The filter system must be degassed prior to uses. All solutions must be filtered through a 0.22-mm filter to remove particles and microorganisms. Once a new sensor chip is docked into the device and connected to the liquid handling system, a normalization procedure for the surface plasmon response signals is conducted using a glycerol solution.

A solution of mixed system specific chemicals are passed over the chip surface to activate it. These chemicals, NHS and EDC, are consumable products delivered with the equipment. A solution containing the analyte to be bound is passed through the activated matrix. Ethanolamine is then pumped through the flow cells to deactivate any unreacted bonds.

The progress of the experiments is shown on personal computer screen as a sensogram. A typical sensogram for a successfully immobilized experiment is illustrated in Figure 1. The numbered segments of the graph refer to the phases of the immobilization process as follows:

- 1 Baseline of the eluent buffer
- 2 NHS/EDC activation of the dextran chip surface
- 3 Baseline after activation
- 4 Ligand adsorption (immobilization) on the dextran layer
- 5 Level of immobilized ligand before deactivation
- 6 Deactivation of unreacted bonds with ethanolamine
- 7 Level of immobilized ligand after deactivation

Once immobilization has been achieved, the reactant can be introduced. Since coupling reactions depend upon the concentration of interacting components and the pH of the solutions, a series of tests must be conducted to optimize these variables. All tests of a series can be carried out in one flow cell of the sensor chip. Therefore, the method program contains a loop command to repeat the test under modified conditions as often as desired with a "stripping"

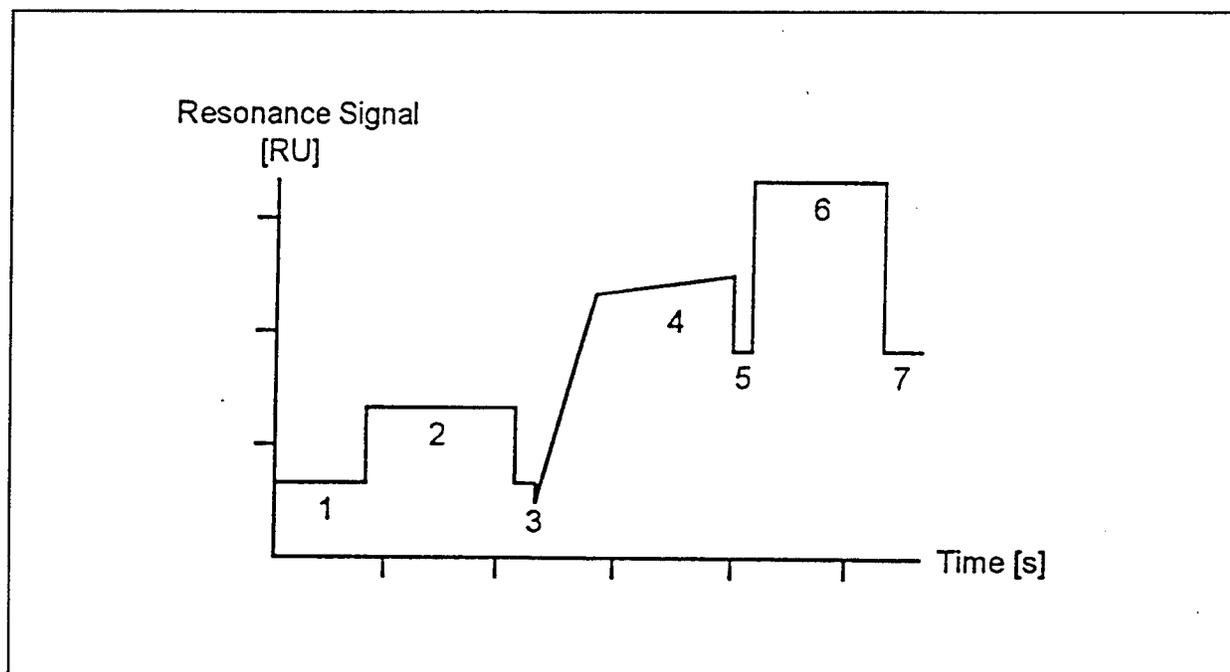


Figure 1. A typical sensorgram generated during successful immobilization of a substrate

step for regeneration at the end of every run. Stripping consists of rinsing analyte from the flow cell to recreate initial conditions.

When interaction has occurred, the sensorgram shows two characteristic sections (Figure 2). When analyte reaches the detection unit, the association phase is evident (“analyte” in Figure 2). The curve increases with decreasing steepness to a maximum that can become extended when all analyte has been linked. As soon as the analyte has passed the detection unit and eluent buffer reaches the flow cell, the curve slopes down as dissociation occurs (“buffer” in Figure 2). The peak of the curve marks the time of equilibrium, where association and dissociation rates are balanced.

The BIACore manual recommended binding of the smaller component to the chip surface if the two components are significantly different in size. After activation of the sensor chip surface with NHS and EDC, ligands can become immobilized via the primary amino groups. Therefore, tests using 4-amino-2,6-dinitrotoluene (4A) and 2,6-diamino-4-nitrotoluene (2,4DANT) coupling to the dextran layer of the chip surface were attempted to test applicability of the technology. The basic control program for this test is included in Appendix A. Variables tested included pH, concentration, and flow rate of ligand fluid and pH, concentration, and chemistry of the buffer solution. The ranges of these variables were those recommended by the manual. A second set of experiments attempted to couple the humic acid to the dextran.

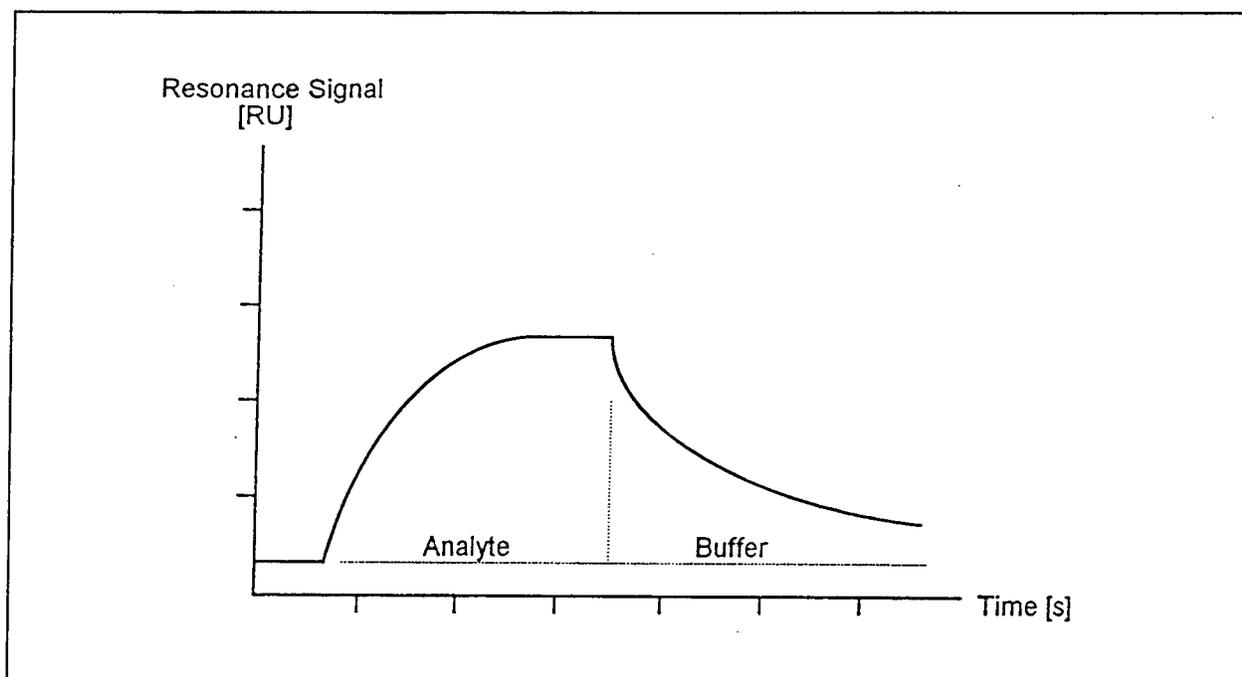


Figure 2. Sensorgram of an effective interaction showing the two typical phases

In order to confirm that the instrumentation and the program control were functioning properly, a set of negative experiments were conducted both with and without a stripping step. Sensorgrams were compared with those produced in explosives tests.

## Results

None of the experiments successfully immobilized 4A, 2,4DANT nor humic acid to the dextran base (see Appendix A for programs and sensorgrams). Typical sensorgrams for a negative outcome and a positive outcome (immobilization of the protein lysozyme that served as a positive control) are shown in Figures 3 and 4, respectively.

Results of negative interactive sensorgrams with and without stripping (Figures 5 and 6, respectively) indicated how the bulk refractive index changes by alterations in the liquids flowing through the system when no interactions occur. Similarities between these sensorgrams and the ones generated for 4A, 2,4DANT and humic acid (Figure 3 and Appendix A) were obvious. Therefore, the instrumentation and control program were confirmed to be functioning properly.

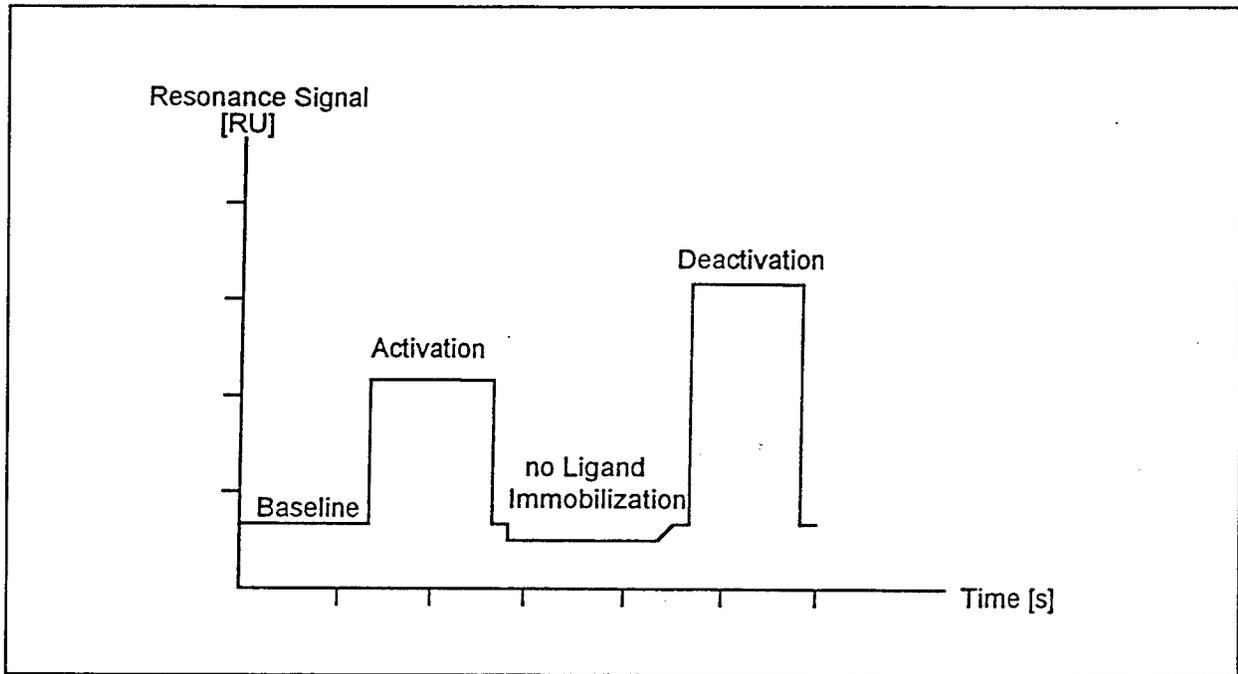


Figure 3. A typical sensorgram for a negative immobilization result

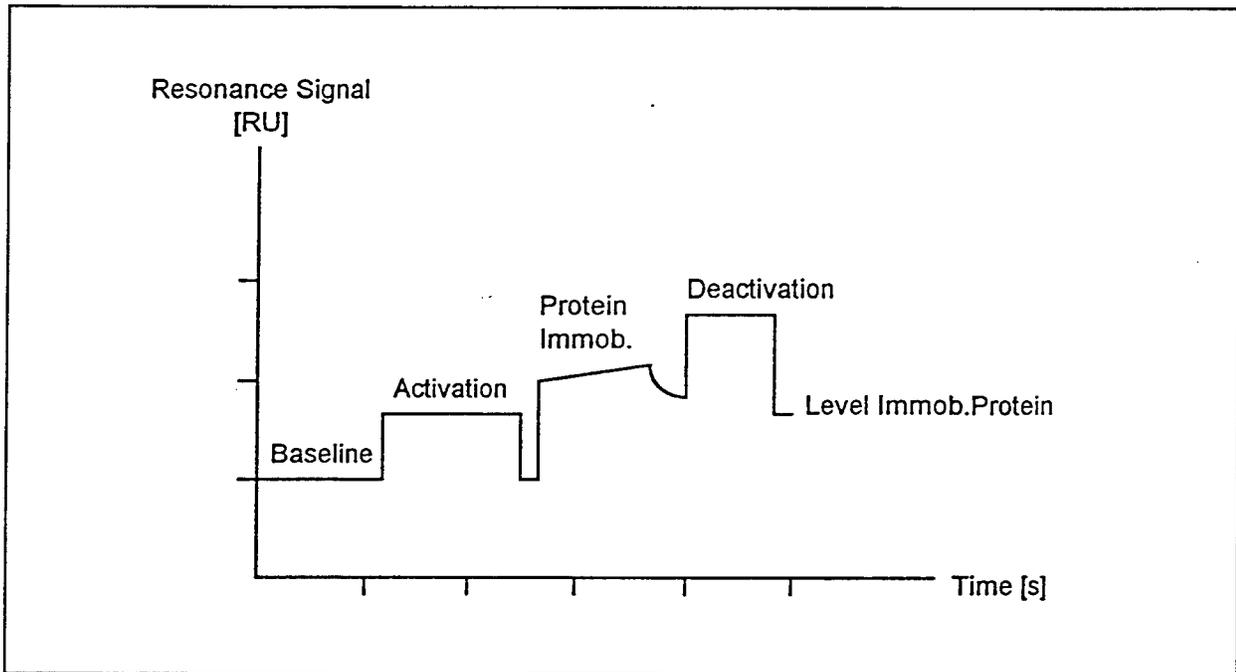


Figure 4. A typical sensorgram for a successful protein immobilization

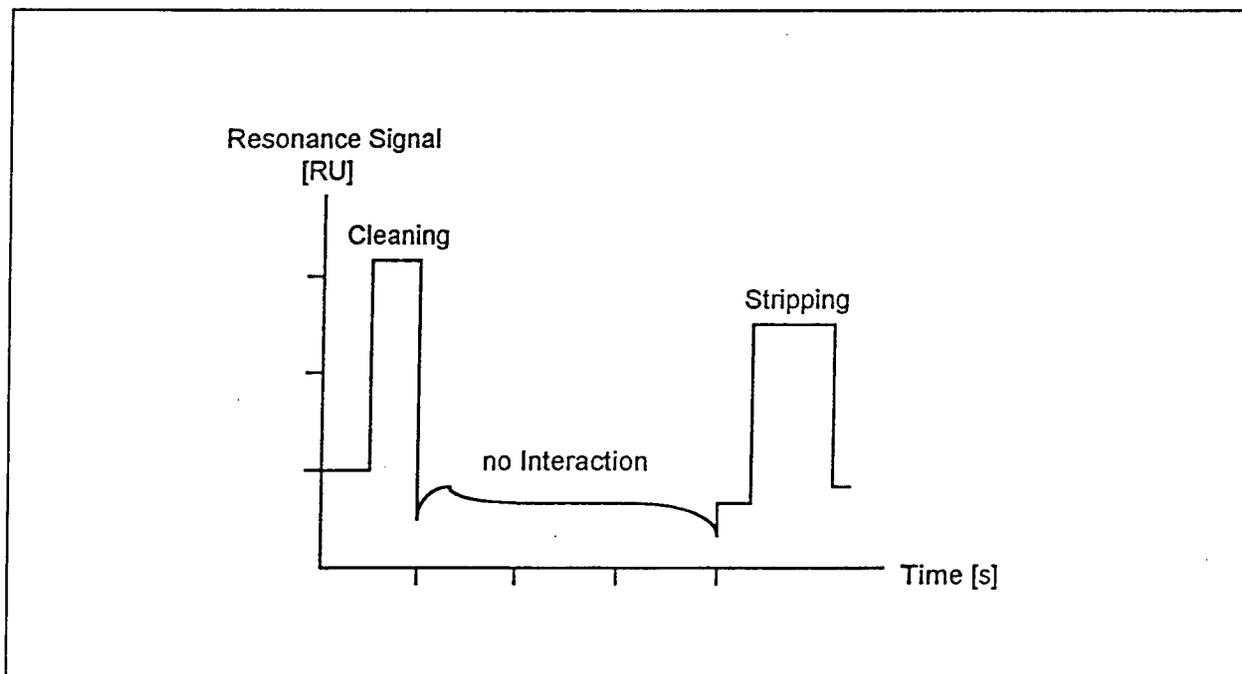


Figure 5. Sensorgram of a negative interaction test with stripping

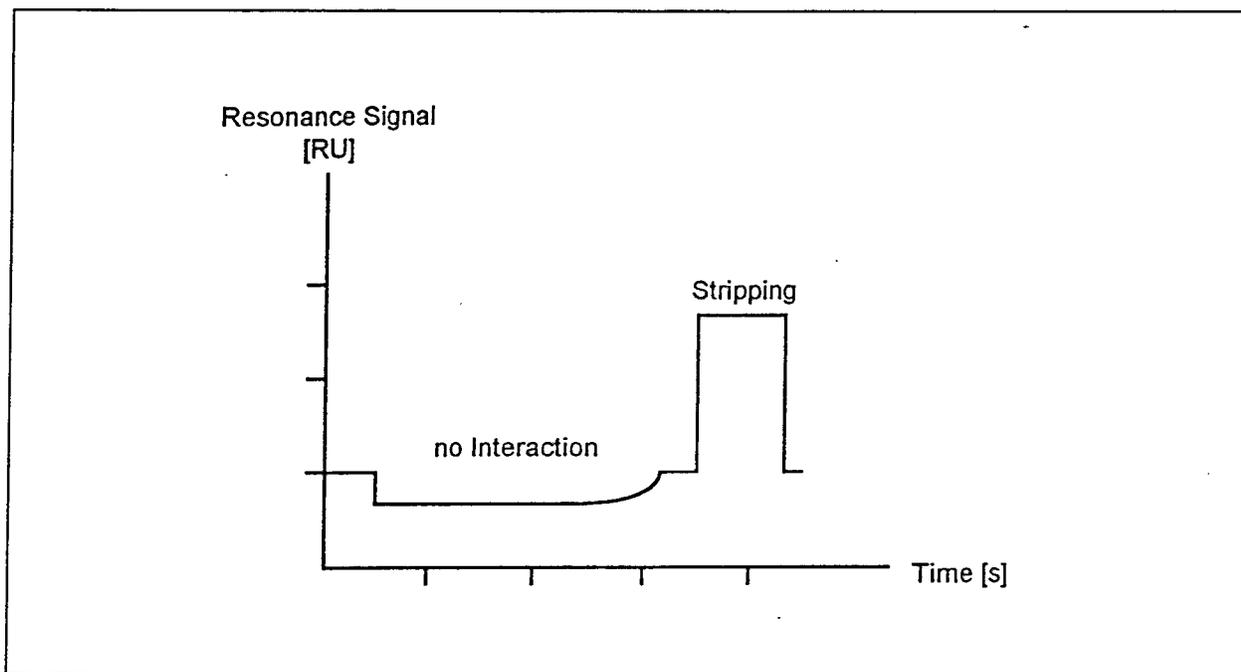


Figure 6. Sensorgram of a negative interaction without stripping

## Conclusions

Results indicated that use of the dextran matrix in its current configuration is inappropriate for immobilization of the compounds of interest. The instrument was designed for real-time biospecific interaction analysis. Prior applications of the instrument have involved the use of proteins and protein-like compounds that have a characteristic affinity for the dextran. The compounds used in this study lack that affinity. Therefore, the interactions between humic acid and the two aminated transformation products of TNT could not be studied via this techniques.

## References

- Lenke, H., Daun, G., Bryniok, D., Knackmus, H. J. (1993). "Biologische Sanierung von Rustungsaltslasten," *Spektrum der Wissenschaft*.
- Pharmacia Biosensor AB. (1991). BIACore System Manual.

# Appendix A

## BIOCore Programs and Data

---

Example System Check .....	A2
Basic Immobilization Program .....	A3
Immobilization Tests .....	A4
Basic Interaction Program .....	A73
Interaction Tests .....	A74
Summary of Immobilization and Interaction Tests .....	A80

Name: \_\_\_\_\_  
 Instrument ID: \_\_\_\_\_ Date: \_\_\_\_\_

**RESULTS**

**A. Noise**

Short term      Sd:      0.7 (**<1.0**)

**B. IFC test**

Area 1: 1202494      Area 3: 1180771  
 Area 2: 1197353      Area 4: 1125624  
 % diff      0.4      4.8  
                  (**<7%**)      (**<7%**)

**Flow parameter**

	5 ul loops	50ul loops
	1.0	1.0
	1.0	1.1
	( <b>0.9-1.1</b> )	( <b>0.9-1.1</b> )

Cliptest      0.003      Dispersion factor      0.988  
                  0.002      (**0.96-1.00**)  
                  0.003  
 Average:      0.003  
                  (**<0.05**)

**C. Refractometer test**

FC 15% Sucrose  
 1      20873  
 2      20951  
 3      20971  
 4      20978  
 Average:      20943      (**22000-23000**)  
 SD:      48      (**<200**)

**D. Mixing**

Mix 1:      10746  
 Mix 2:      10448  
 Mix 3:      10250  
 Average:      10481  
 SD:      249.67  
 CV %:      2.4      (**<2%**)

Mix dilution factor      0.946  
                  (**0.93-0.98**)

**EVALUATION**

The shaded figures in the text above are approximate acceptance limits.

If the results are outside acceptance limits, compare the sensorgrams in the system check result file with the reference sensorgrams in the System Manual or in the files in C:\BIA\GUIDE.

Check

- cycles 2-5 for noise and refractometer test
- cycles 1 and 7 for IFC test
- cycles 6 and 7 for mixing

If your sensorgrams are disturbed by frequent airspikes, degas the eluent, run the working tool INITIATE and re-run the system check.

Some common causes of deviations from acceptance limits are listed below. See system manual for troubleshooting and maintenance procedures.

- A. Noise.      Airspikes, mechanical vibrations, electric disturbance.
- B. IFC test      Low flow parameter can indicate leakage in flow system.  
                  Low dispersion factor can indicate bad loop filling (clogged IFC, leakage in autosampler/injection system).  
                  High cliptest can indicate defective valve functions or incomplete washing.
- C. Refractometer      Are the solutions correctly prepared?
- D. Mixing      Inadequate air segment in autosampler tube.  
                  Leakage in autosampler/injection system.

Immobilization Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    CAPTION      Immobilization of ..... (pH ....)
    FLOW         ...      !  $\mu$ l/min

    ! Mix NHS and EDC
    TRANSFER    R1F1 R1F4 ... ! Quantity
    TRANSFER    R1F2 R1F4 ... ! Quantity
    MIX         R1F4 ...      ! Quantity

    ! Activate sensorchip with EDC/NHS
    INJECT      R1F4 ...      ! Quantity

    ! Inject ligand onto chip surface
    INJECT      R2D1 ...      ! Quantity

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT      R1F6 ...      ! Quantity

END

MAIN
    RACK        1 thermo_b
    RACK        2 thermo_a
    FLOWCELL    ...
    APROG       immobilization
END
```

Program

```
DEFINE APROG IMMOB1
    !R1F1 EDC
    !R1F2 nhs
    !R1F4 EMPTY
```

```
FLOW          5
    !MIX NHS AND EDC
TRANSFER      R1F1 R1F4 70
TRANSFER      R1F2 R1F4 70
MIX           R1F4 115
```

```
    !ACTIVATE SENSORCHIP
INJECT        R1F4 50
```

```
    !INJECT 2,6-DA-4-NT ONTO CHIP SURFACE
INJECT        R2E1 50
```

```
    !DEACTIVATE REACTIVE GROUPS WITH ETHANOLAMINE
INJECT        R1F6 40
```

END

MAIN

```
RACK          1 THERMO_b
RACK          2 THERMO_a
FLOWCELL      1
APROG         IMMOB1
WASH          b
```

END

Notebook

Date: 07/25/95  
Operator:

Purpose: immobilize ligand flow cell 1  
Results: no immobilization

Comments:

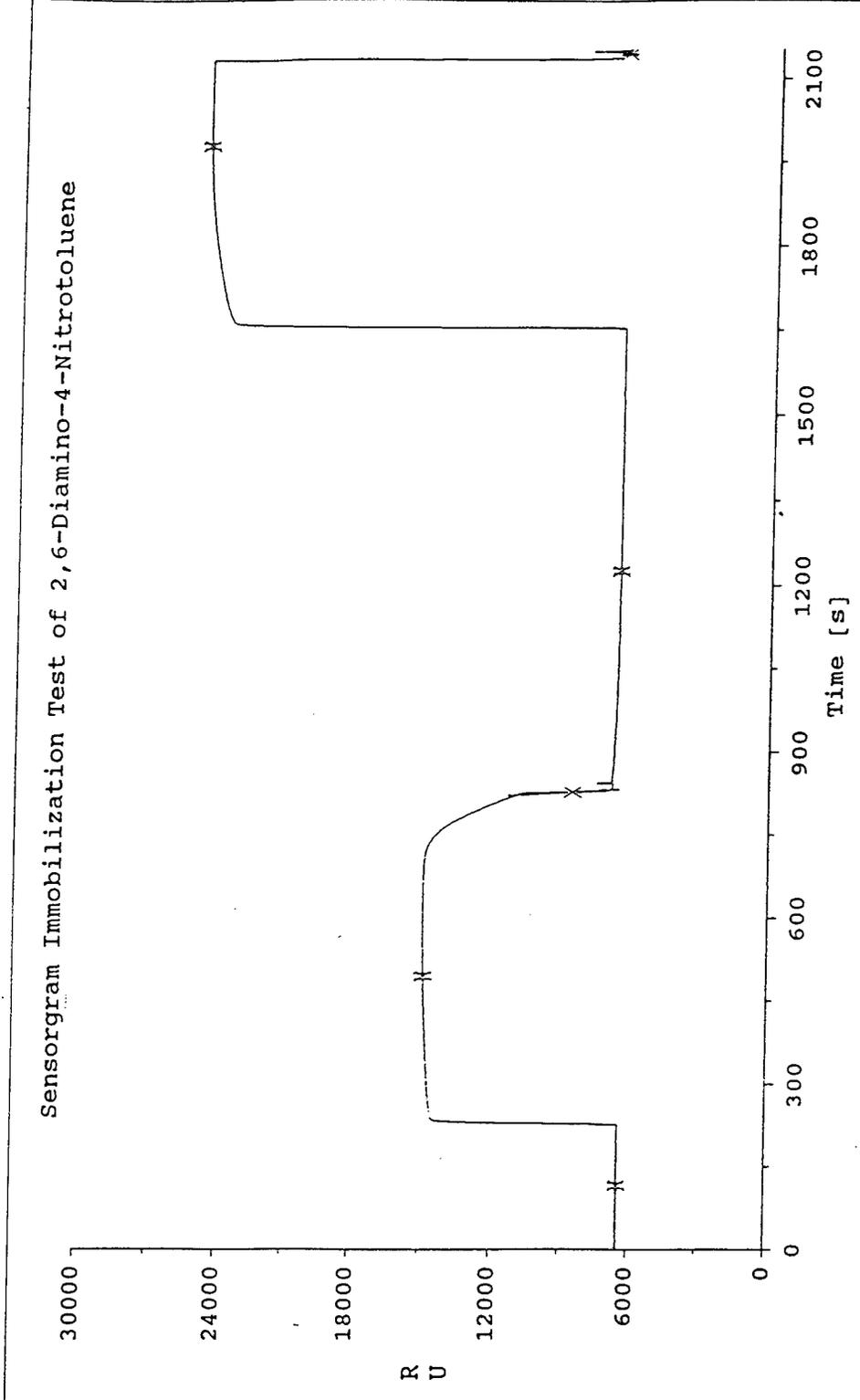
Flow cell: 1  
SensorChip: CM5 DANT  
EluentBuffer: sodium phosphate  
Regeneration:  
Flow: 5 ul/min

~~Immobilized~~ Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 50 ul  
Conc: 194 uM  
Buffer: sodium phosphate  
Immobilization File: 25jul-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Sensorgram Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	1	116.00	10.00	6450	0.48883	0.05292	0.48092	YES	0	Baseline
IMMOB1	1	494.00	10.00	14918	2.08163	0.62322	0.25971	NO	8468	EDC/NHS
IMMOB1	1	827.00	10.00	8990	1856.67268	-528.57301	644.59267	NO	2540	EndEDC
IMMOB1	1	1225.00	10.00	6632	0.82070	-0.21524	0.42879	NO	182	ImmobArea
IMMOB1	1	1977.00	10.00	24848	1.91268	0.55977	0.48494	NO	18398	Ethanolamine
IMMOB1	1	2144.00	3.00	6748	21.65683	-16.73047	1.93798	NO	298	EndSensorgr

Program

```
DEFINE APROG IMMOB1
  !R1F1 EDC
  !R1F2 nhs
  !R1F4 EMPTY

  FLOW      5
  !MIX NHS AND EDC
  TRANSFER  R1F1 R1F4 70
  TRANSFER  R1F2 R1F4 70
  MIX       R1F4 115

  !ACTIVATE SENSORCHIP
  INJECT    R1F4 50

  !INJECT 2,6-DA-4-NT ONTO CHIP SURFACE
  INJECT    R2E1 50

  !DEACTIVATE REACTIVE GROUPS WITH ETHANOLAMINE
  INJECT    R1F6 40

END

MAIN
  RACK      1 THERMO_b
  RACK      2 THERMO_a
  FLOWCELL  3
  APROG     IMMOB1
  WASH      b

END
```

Notebook

Date: 07/25/95

Operator:

Purpose: immobilize ligand flowcell 3

Results: no immobilization

Comments:

Flow cell: 3

SensorChip: CM5 DANT

EluentBuffer: sodium phosphate

Regeneration:

Flow: 5 ul/min

~~Immobilized~~ Ligand: 2,6-Diamino-4-Nitrotoluene

Volume: 50 ul

Conc: 194 uM

Buffer: sodium phosphate

Immobilization File: 25jul-3.blr

Comments:

Analyte 1:

Volume:

Conc:

Buffer:

Comments:

Analyte 2:

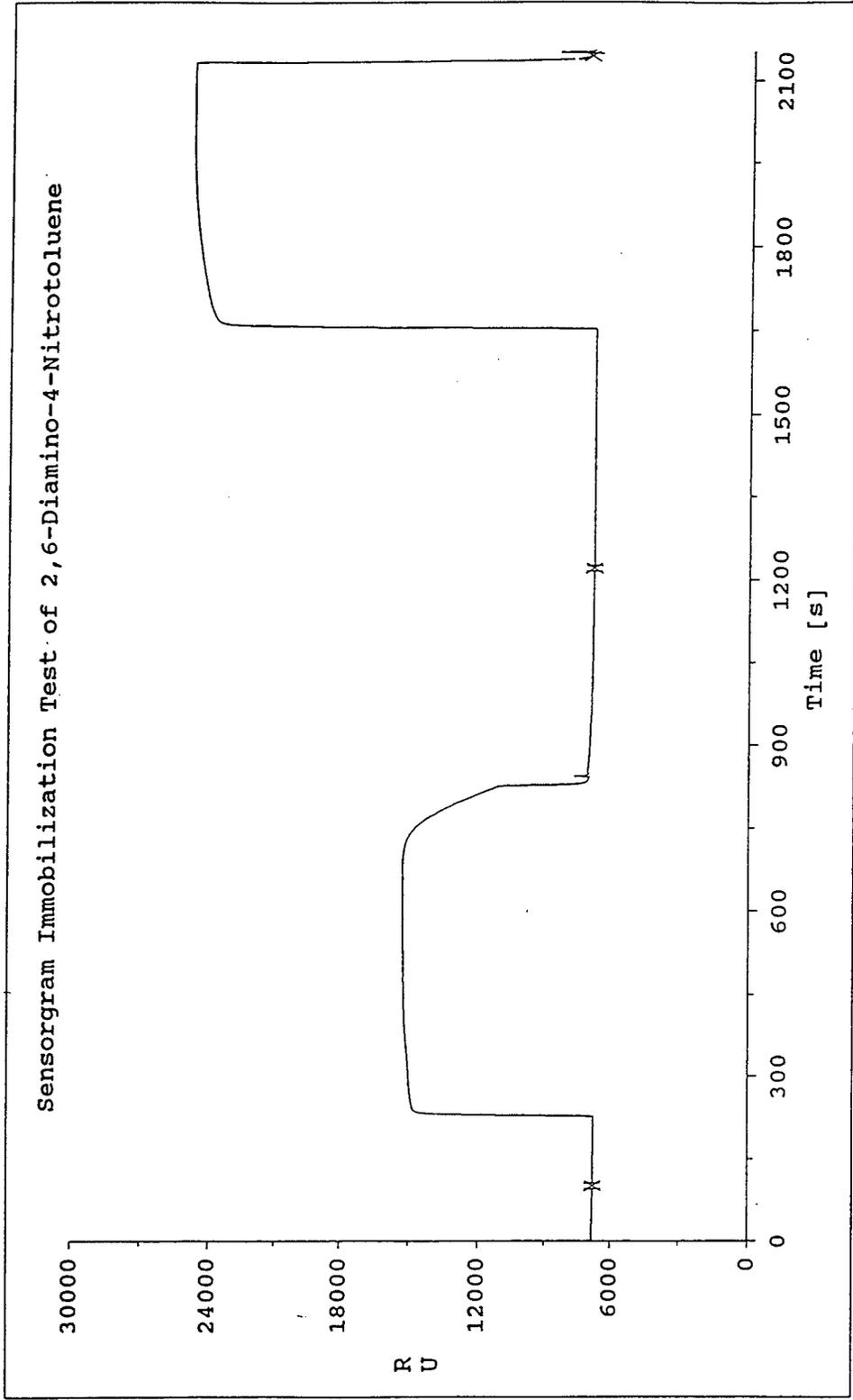
Volume:

Conc:

Buffer:

Comments:

Sensorgram Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	3	101.00	10.00	6791	1.18236	-0.34527	0.31027	YES	0	Baseline
IMMOB1	3	1221.00	10.00	6822	0.58581	-0.15020	0.32489	NO	31	ImmobilArea
IMMOB1	3	2145.00	10.00	7219	234.91591	-64.90898	99.11021	NO	428	EndSensogr

Program

```
DEFINE APROG IMMOB1
    !R1F1 EDC
    !R1F2 nhs
    !R1F4 EMPTY

    FLOW      5
    !MIX NHS AND EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    !ACTIVATE SENSORCHIP
    INJECT    R1F4 50

    !INJECT 2,6-DA-4-NT ONTO CHIP SURFACE

    INJECT    R2E1 50

    !DEACTIVATE REACTIVE GROUPS WITH ETHANOLAMINE
    INJECT    R1F6 40

END

MAIN
    RACK      1 THERMO_b
    RACK      2 THERMO_a
    FLOWCELL  4
    APROG     IMMOB1
    WASH      b

END
```

Notebook

Date: 07/28/95  
Operator:

Purpose: immobilize ligand flowcell 4  
Results: no immobilization  
Comments:

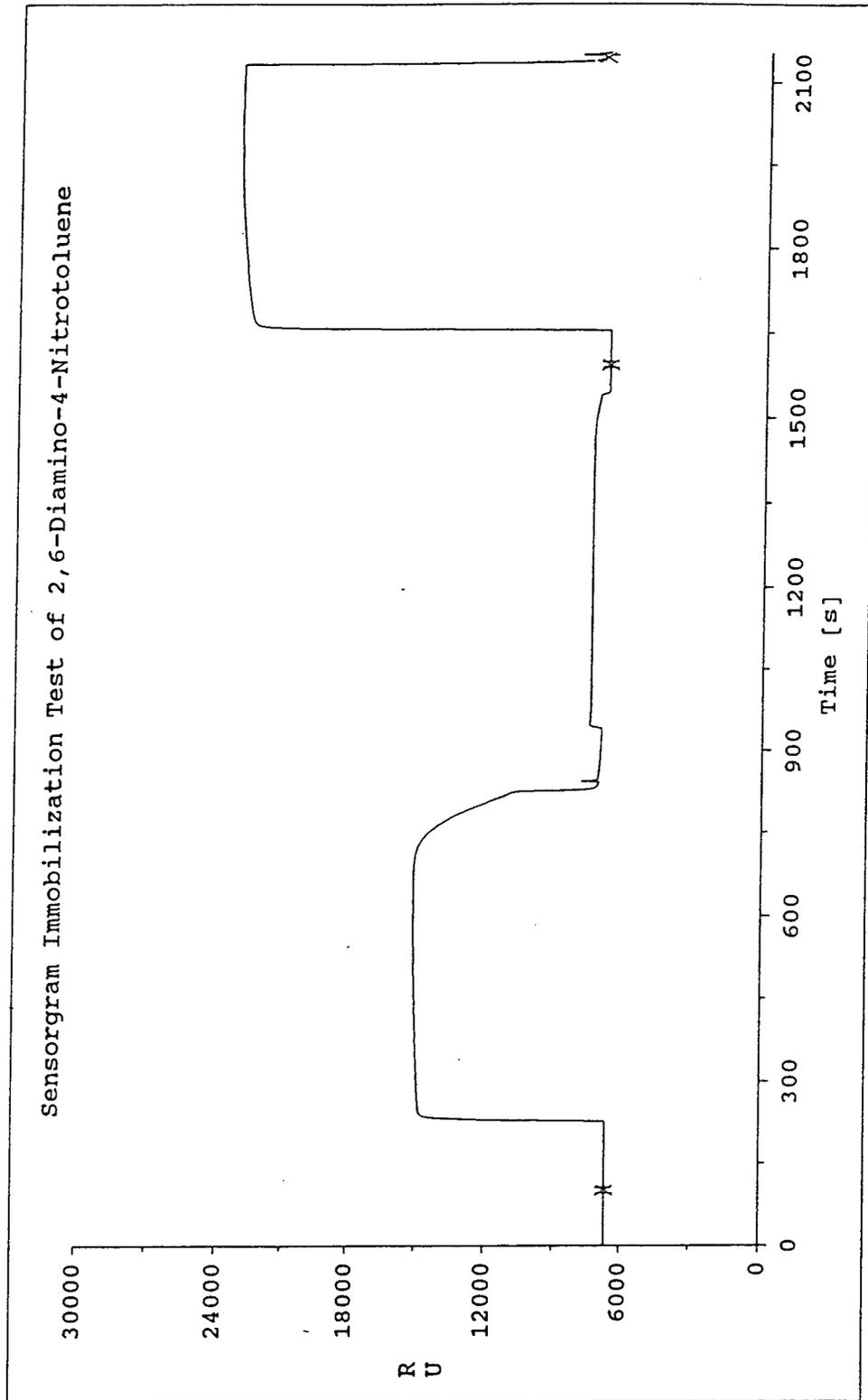
Flow cell: 4  
SensorChip: CM5 DANT  
EluentBuffer: sodium phosphate  
Regeneration:  
Flow: 5 ul/min

~~Immobilized~~ Ligand: 2,6-diamino-4-nitrotoluene  
Volume: 50 ul  
Conc: 424 uM  
Buffer: sodium phosphate  
Immobilization File: 28july95.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Sensorgram Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	4	101.00	10.00	6672	0.50085	0.02917	0.51800	YES	0	Baseline
IMMOB1	4	1596.00	10.00	6779	0.65832	-0.11536	0.56470	NO	107	ImmobilArea
IMMOB1	4	2147.00	10.00	7203	192.47449	-54.36298	71.00672	NO	531	EndSensogr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 25

    ! Inject 2,6-DA-4-NT onto chip surface
    INJECT    R2D1 25

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 25
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  1
    APROG     immobilization
END
```

Notebook

Date: 11/27/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments:

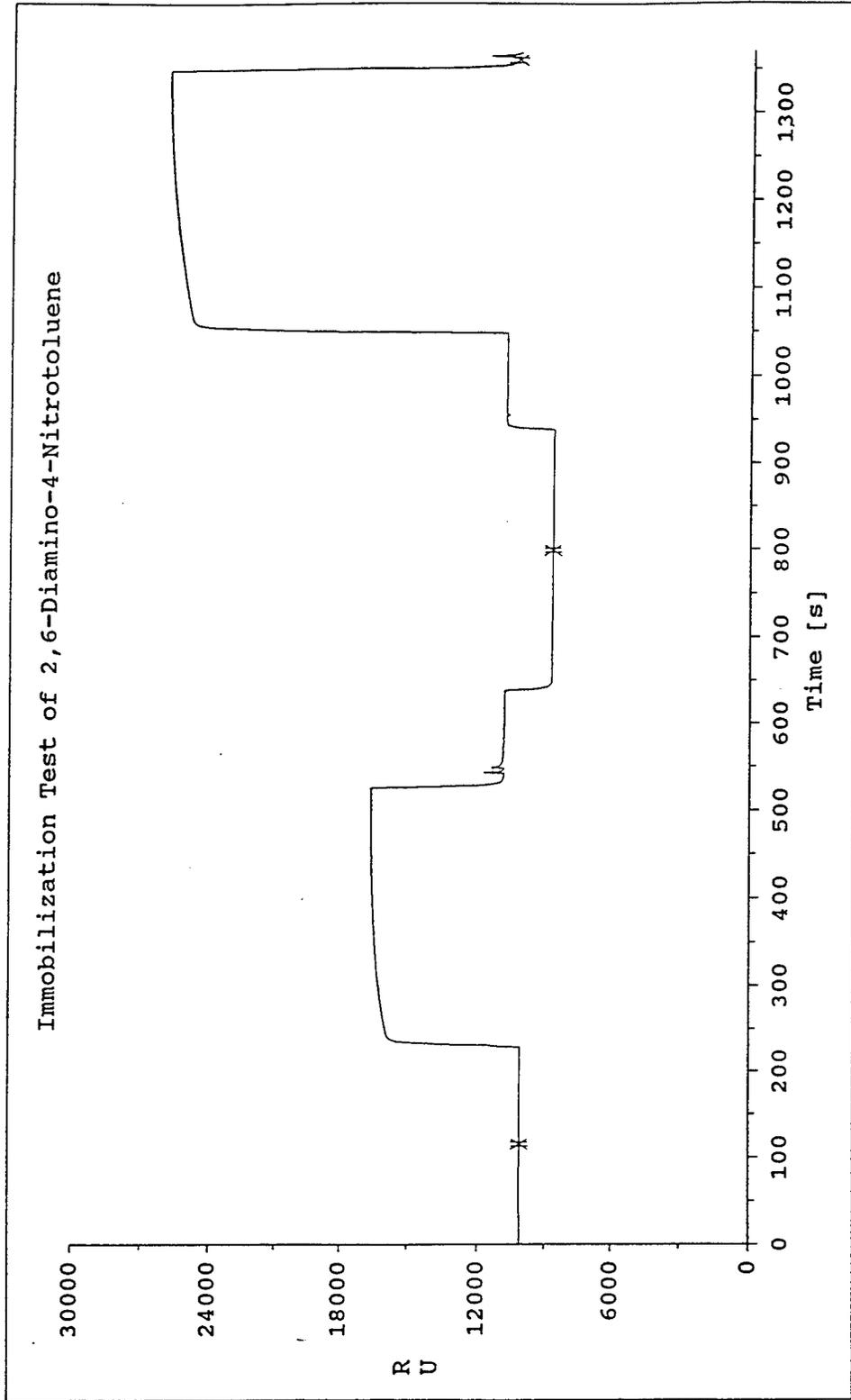
Flow cell: 1  
SensorChip: (B) CM5 DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 25  $\mu$ l, pH 6.57  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 27nov.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	1	115.50	5.00	10112	0.49288	-0.14506	0.45346	YES	0	Baseline
immobilization	1	799.00	5.00	8679	0.96026	-0.34126	0.81775	NO	-1434	ImmobbArea
immobilization	1	1360.50	5.00	10403	48.30988	-28.46151	10.86213	NO	290	End-Sensogr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 50

    ! Inject 2,6-DA-4-NT onto chip surface
    INJECT    R2D1 50

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 40

END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  4
    APROG     immobilization
    WASH      b

END
```

Notebook

Date: 11/15/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments: ? pH of 7.06 too high  
          ? DANT conc too low  
          ? DANT prep not useful

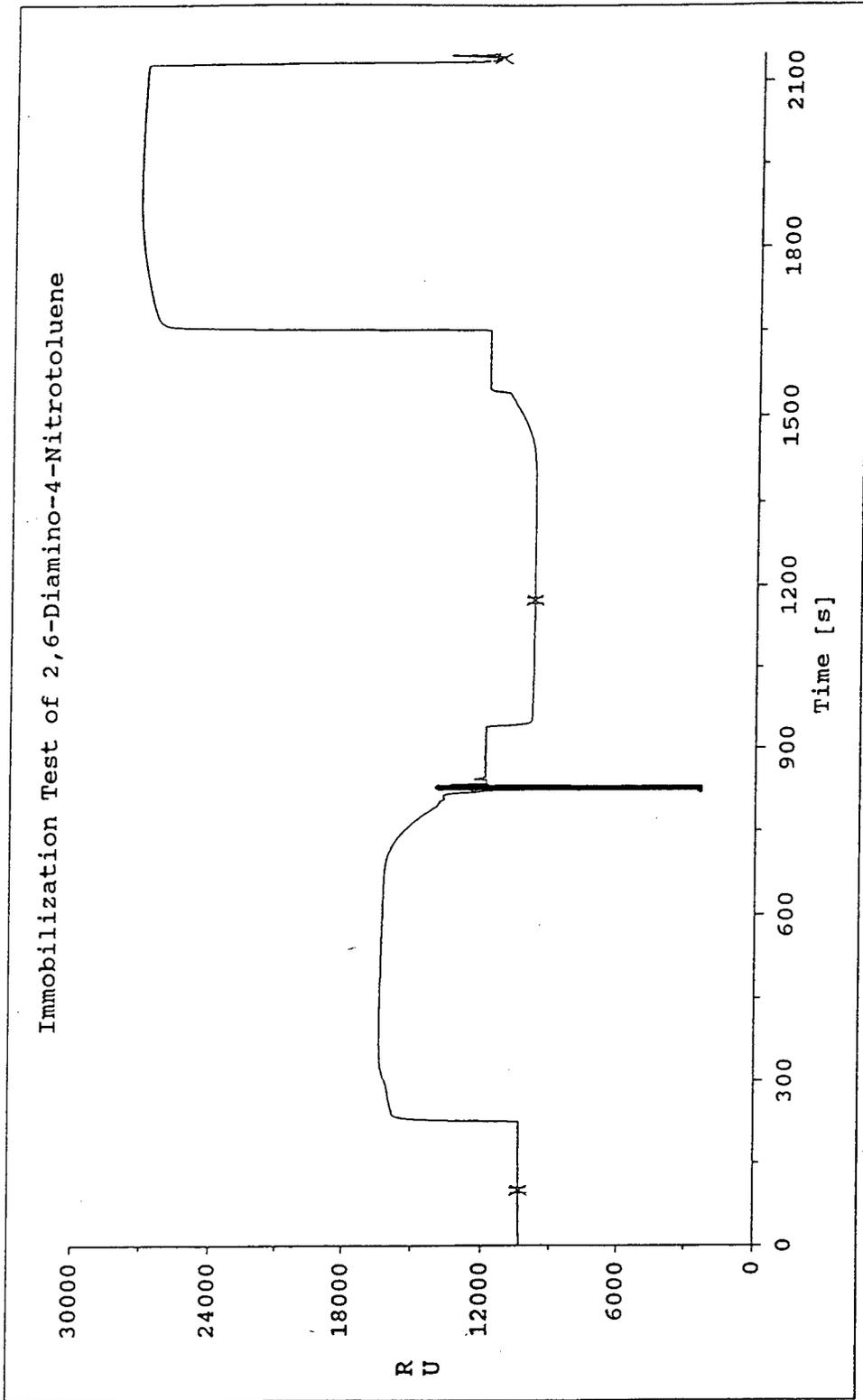
Flow cell: 4  
SensorChip: CM5 Lyso/DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 50  $\mu$ l, pH 8.00  
Conc: 194  $\mu$ M  
Buffer: Sodium phosphate, pH 7.06  
Immobilization File: 15nov.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	EC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	4	101.00	10.00	10394	0.36108	-0.00154	0.38057	YES	0	Baseline
immobilization	4	1173.00	10.00	9830	1.57165	-0.04890	1.64782	NO	-564	ImmobArea
immobilization	4	2141.00	10.00	11788	546.11671	37.96984	560.14350	NO	1394	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6
```

```
FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
TRANSFER  R1F1 R1F4 70
TRANSFER  R1F2 R1F4 70
MIX       R1F4 115
```

```
    ! Activate sensorchip
INJECT    R1F4 50
```

```
    ! Inject 2,6-DA-4-NT onto chip surface
INJECT    R2D1 50
```

```
    ! Deactivate excess reactive groups with ETHANOLAMINE
INJECT    R1F6 40
```

END

MAIN

```
RACK      1 thermo_b
RACK      2 thermo_a
FLOWCELL  1
APROG     immobilization
END
```

Notebook

Date: 11/16/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments:

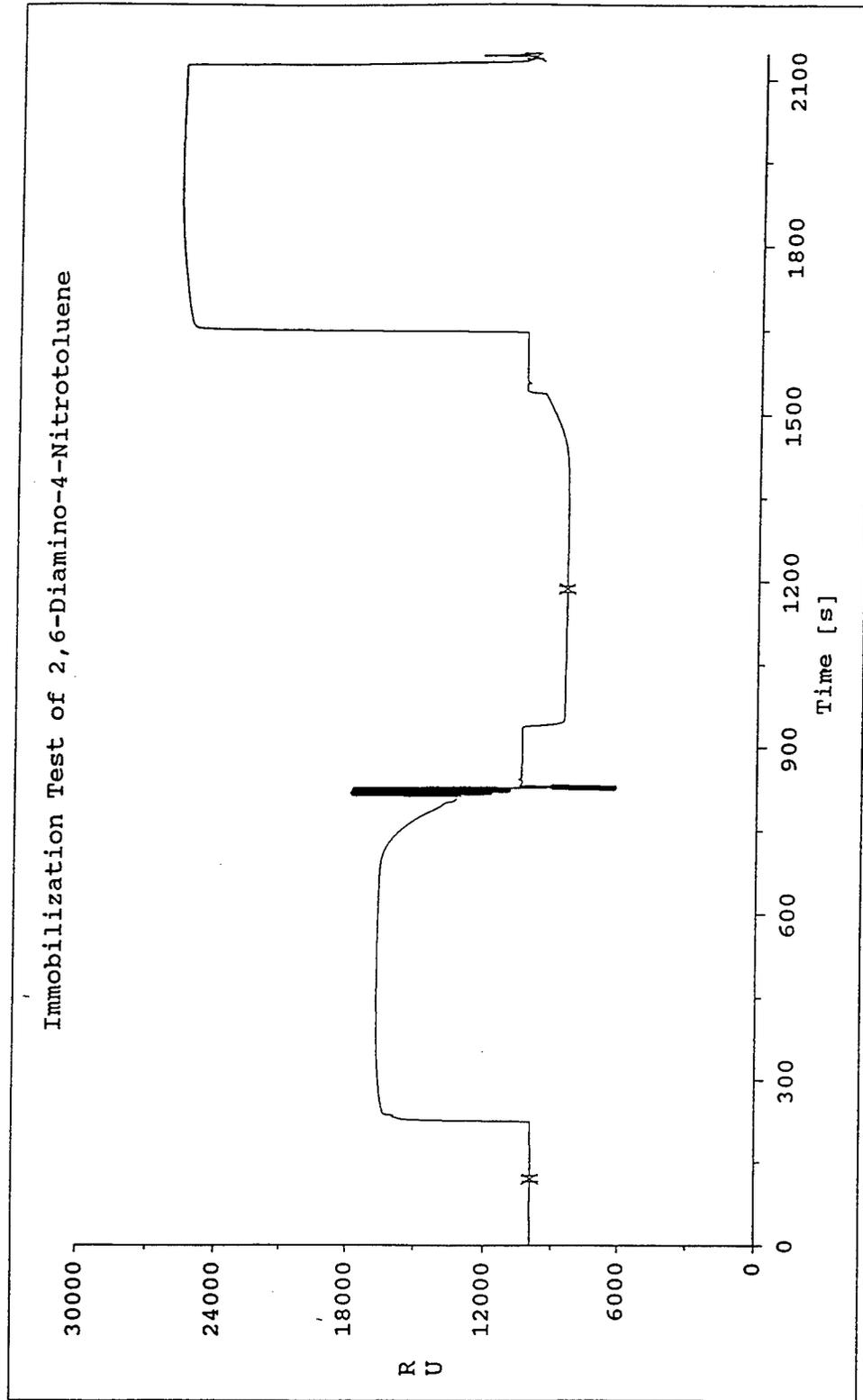
Flow cell: 1  
SensorChip: CM5 Lyso/DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 50  $\mu$ l  
Conc: 194  $\mu$ M  
Buffer: Sodium phosphate pH 7.00  
Immobilization File: 16nov-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
Immobilization	1	121.00	10.00	9923	0.51785	-0.01180	0.54430	YES	0	Baseline
Immobilization	1	1188.00	10.00	8523	0.83582	-0.18416	0.60142	NO	-1400	ImmobArea
Immobilization	1	2144.00	10.00	10660	715.36277	54.98059	729.14895	NO	738	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 50

    ! Inject 2,6-DA-4-NT onto chip surface
    INJECT    R2D1 50

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 40
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  1
    APROG     immobilization
END
```

Notebook

Date: 16/11/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments:

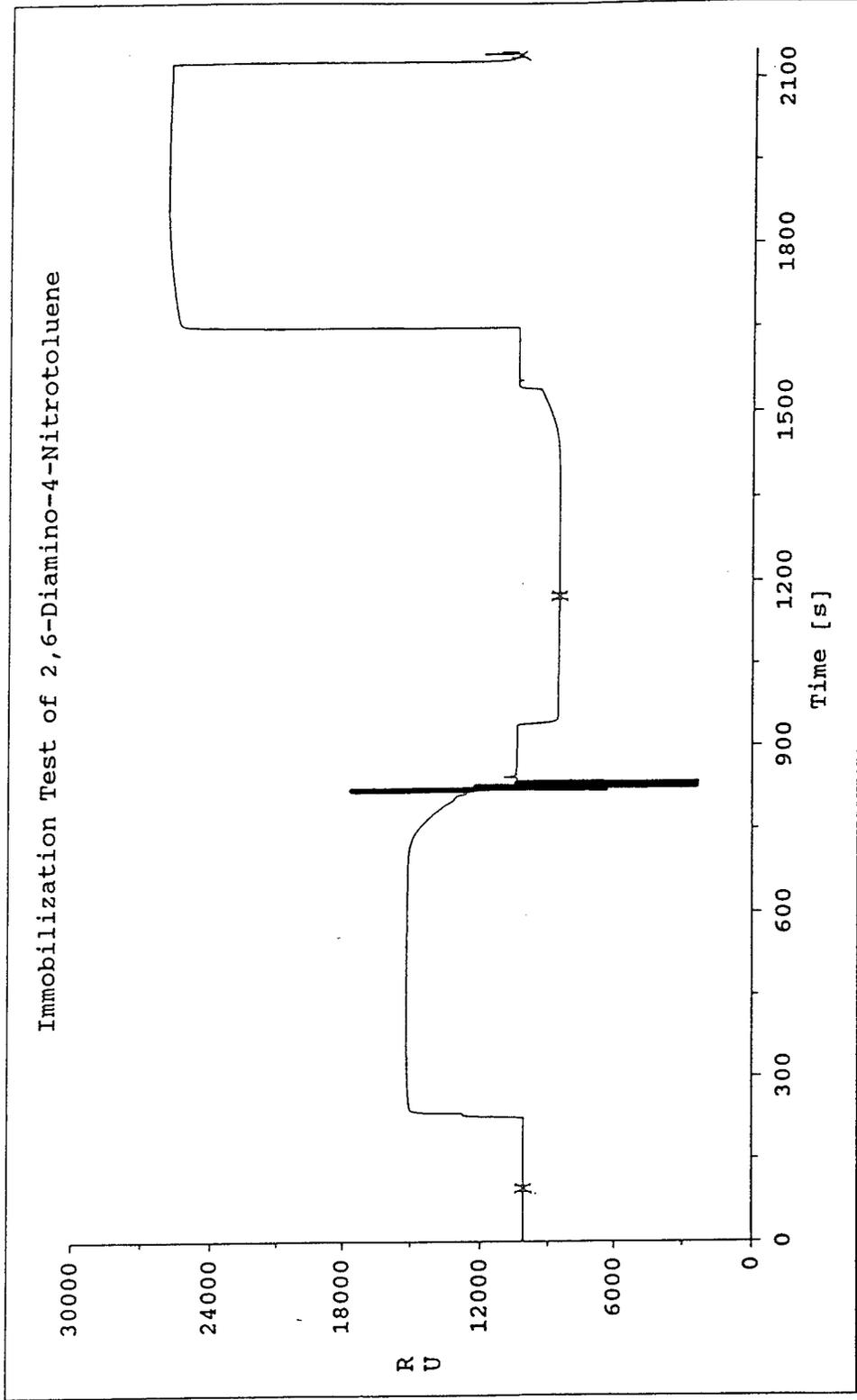
Flow cell: 1  
SensorChip: CM5 Lyso/DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 50  $\mu$ l  
Conc: 194  $\mu$ M  
Buffer: Sodium phosphate, pH 4.42  
Immobilization File: 16nov-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	1	96.00	10.00	10113	0.64157	0.01763	0.67346	YES	0	Baseline
immobilization	1	1173.00	10.00	8534	1.04032	-0.23264	0.73554	NO	-1579	ImmobArea
immobilization	1	2144.00	10.00	10748	508.03484	67.73969	480.30554	NO	634	EndSensorgr

Program

DEFINE APROG immobilization

! EDC in R1F1  
! NHS in R1F2  
! Empty vial in R1F4  
! ETHANOLAMINE in R1F6

FLOW 5 !  $\mu$ l/min  
! Mix NHS and EDC  
TRANSFER R1F1 R1F4 70  
TRANSFER R1F2 R1F4 70  
MIX R1F4 115

! Activate sensorchip  
INJECT R1F4 25

! Inject 2,6-DA-4-NT onto chip surface  
INJECT R2D1 25

! Deactivate excess reactive groups with ETHANOLAMINE  
INJECT R1F6 40

END

MAIN

RACK 1 thermo\_b  
RACK 2 thermo\_a  
FLOWCELL 1  
APROG immobilization  
END

Notebook

Date: 11/17/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments: No more air spikes after cut of  
activation time in half (injection  
of 25  $\mu$ l instead of 50  $\mu$ l);  
injection time of ligand also cut  
in half by reducing the amount

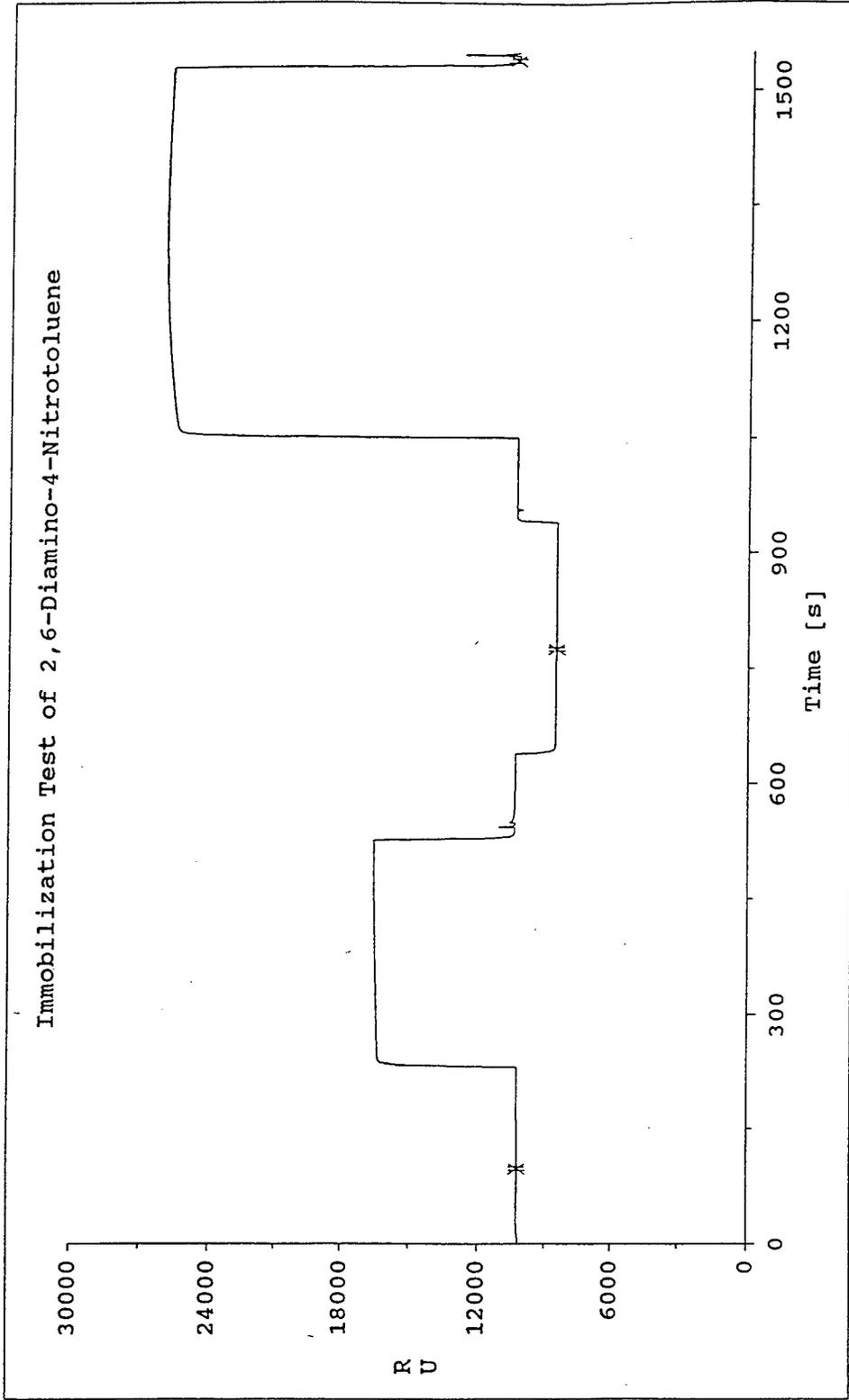
Flow cell: 1  
SensorChip: CM5 Lyso/DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 25  $\mu$ l  
Conc: 194  $\mu$ M  
Buffer: Sodium phosphate, pH 4.4  
Immobilization File: 17nov.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	1	98.00	5.00	10249	0.39440	-0.10265	0.37502	YES	0	Baseline
immobilization	1	774.50	5.00	8536	0.67878	-0.05101	0.70992	NO	-1714	ImmobArea
immobilization	1	1539.00	5.00	10538	50.08553	-29.30792	12.75567	NO	288	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 25

    ! Inject 2,6-DA-4-NT onto chip surface
    INJECT    R2D1 25

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 25
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  2
    APROG     immobilization
END
```

Notebook

Date: 11/28/95  
Operator:

Purpose: Immobilization of 2,6-Diamino,4-Nitrotoluene  
Results: No immobilization  
Comments:

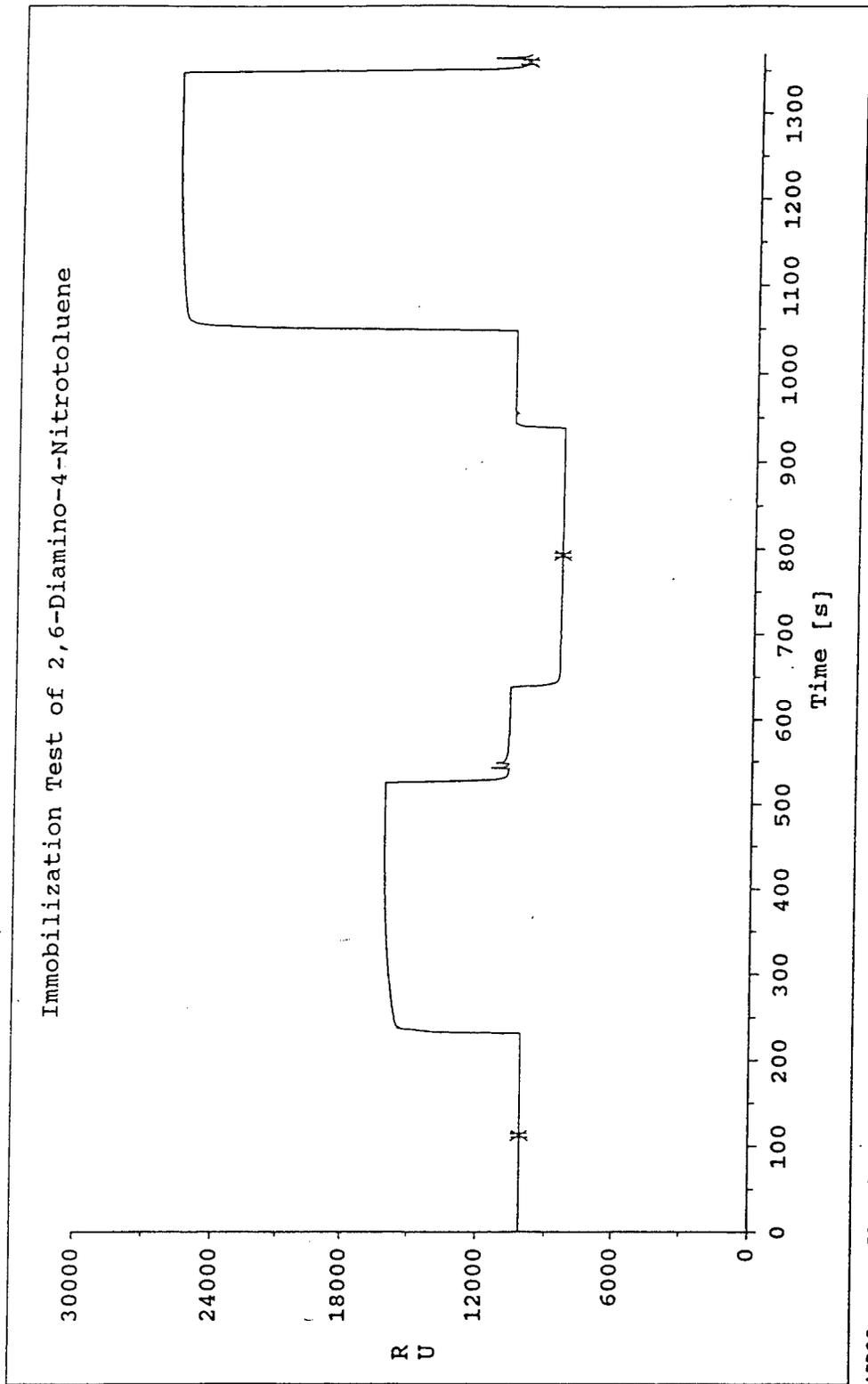
Flow cell: 2  
SensorChip: (B) CM5 DANT  
EluentBuffer: HEPES/P20  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 25  $\mu$ l, pH 4.55  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 28nov.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
Immobilization	2	112.50	5.00	10127	0.55966	-0.02298	0.58857	YES	0	Baseline
Immobilization	2	792.50	5.00	8520	0.73189	-0.30483	0.55790	NO	-1607	ImmobArea
Immobilization	2	1360.50	5.00	10394	38.14120	-22.69979	6.47474	NO	267	EndSensorgr

Program

DEFINE APROG immobilization

! EDC in R1F1  
! NHS in R1F2  
! Empty vial in R1F4  
! ETHANOLAMINE in R1F6

FLOW 2 !  $\mu$ l/min  
! Mix NHS and EDC  
TRANSFER R1F1 R1F4 70  
TRANSFER R1F2 R1F4 70  
MIX R1F4 115

! Activate sensorchip  
INJECT R1F4 25

! Inject 2,6-DA-4-NT onto chip surface  
INJECT R2D1 25

! Deactivate excess reactive groups with ETHANOLAMINE  
INJECT R1F6 30

END

MAIN

RACK 1 thermo\_b  
RACK 2 thermo\_a  
FLOWCELL 1  
APROG immobilization  
END

Notebook

Date: 12/01/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments:

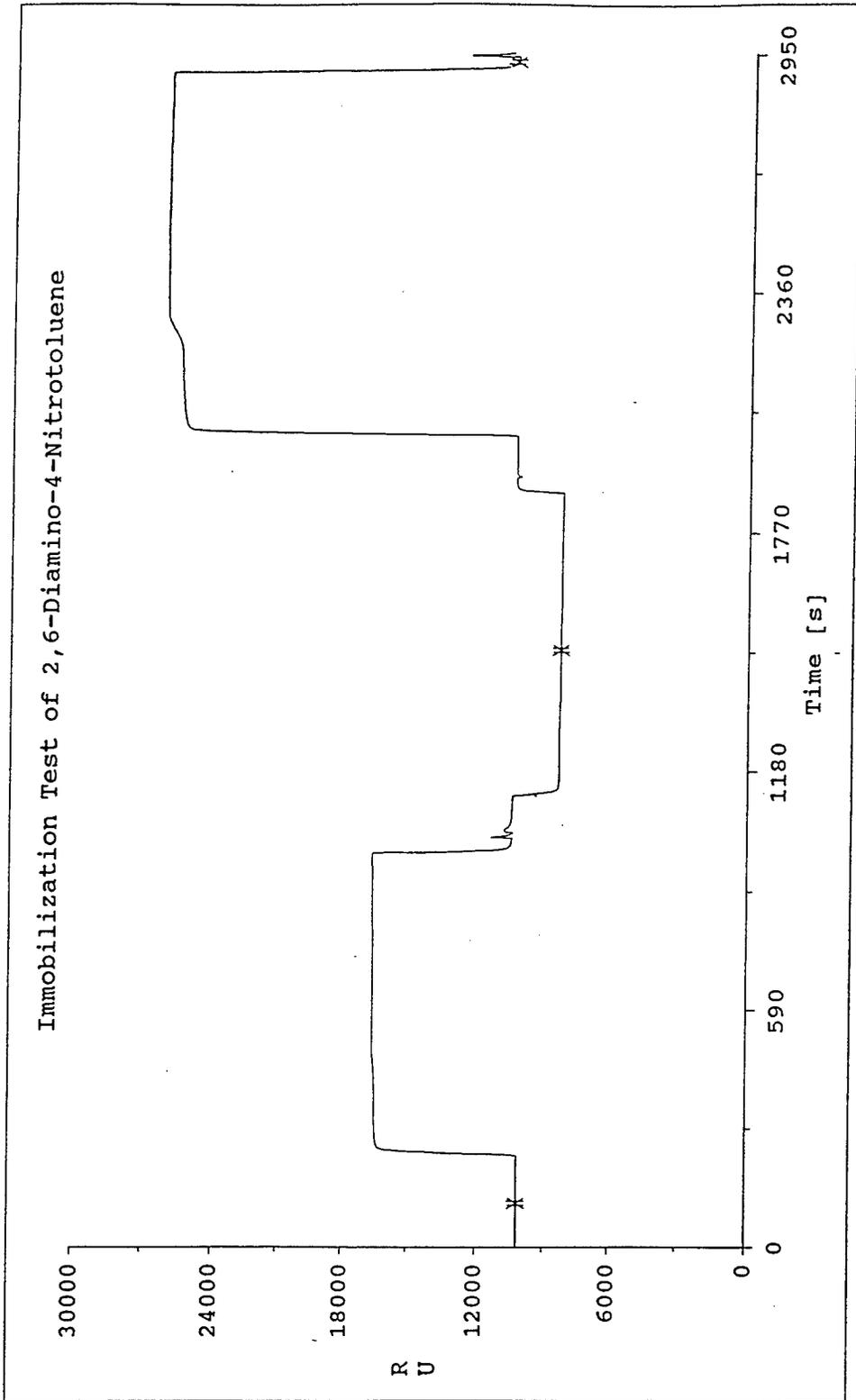
Flow cell: 1  
SensorChip: CM5-DANT/DNAT/Humic Acid  
EluentBuffer: HEPES/P20  
Regeneration:  
Flow: 2  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 25  $\mu$ l, pH 4.55  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 01dec-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	1	111.00	10.00	10183	0.41512	-0.01937	0.43230	YES	0	Baseline
immobilization	1	1481.00	10.00	8313	0.73004	-0.14725	0.57199	NO	-1870	ImmobaArea
immobilization	1	2934.00	10.00	10643	40.04016	-11.94065	6.22215	NO	459	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      1      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 25

    ! Inject 2,6-DA-4-NT onto chip surface
    INJECT    R2D1 25

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 30
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  2
    APROG     immobilization
END
```

Notebook

Date: 12/01/95  
Operator:

Purpose: Immobilization of 2,6-Diamino-4-Nitrotoluene  
Results: No immobilization  
Comments:

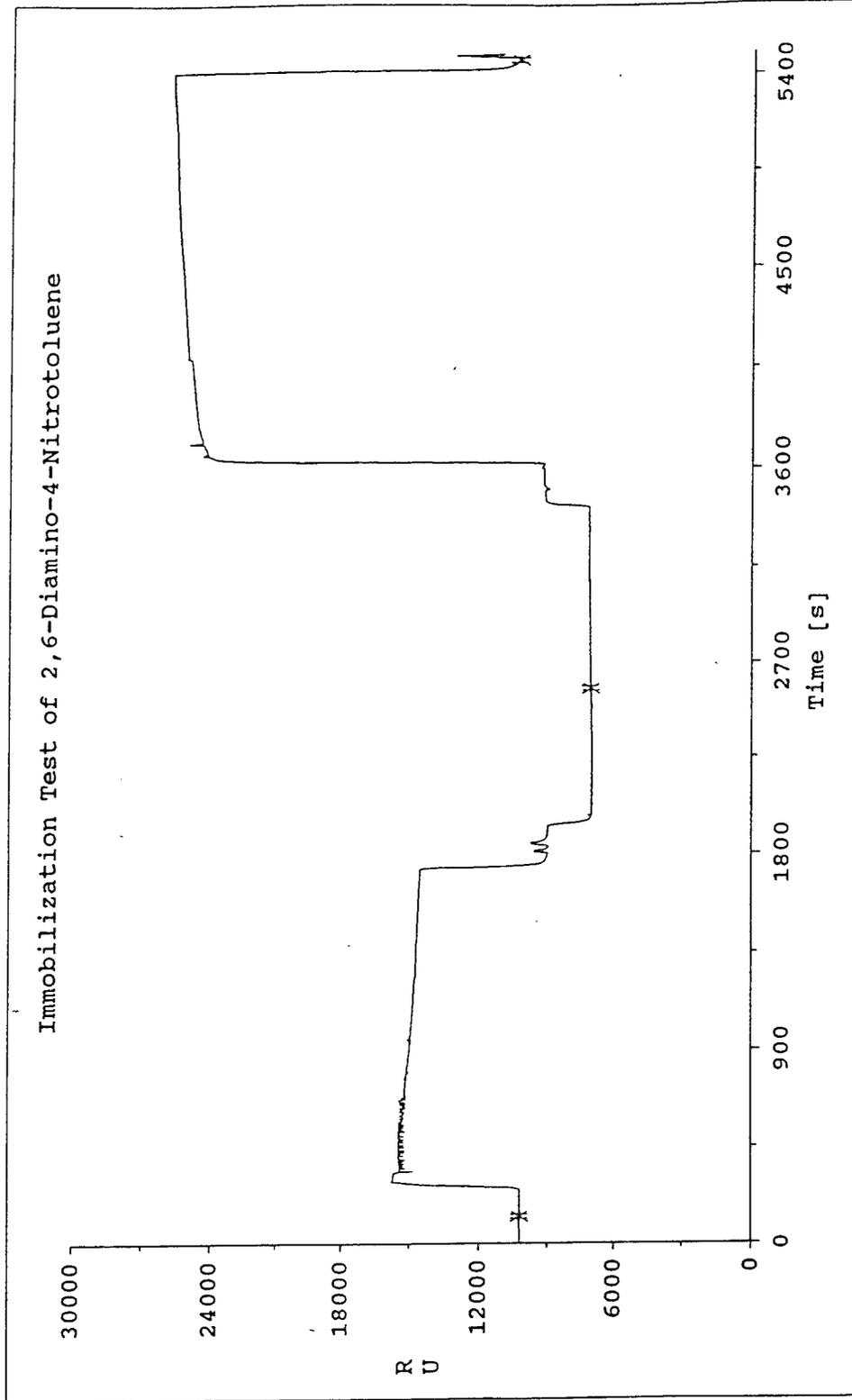
Flow cell: 2  
SensorChip: CM5-DANT/DNAT/Humic Acid  
EluentBuffer: HEPES/P20  
Regeneration:  
Flow: 1  $\mu$ l/min

Ligand: 2,6-Diamino-4-Nitrotoluene  
Volume: 25  $\mu$ l, pH 4.63  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 01dec-3.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 2,6-Diamino-4-Nitrotoluene



APROG	FC	Itime	Window	AbsResp	SD	Slope	LRSD	Baseline	RollResp	Id
Immobilization	2	128.00	20.00	10231	1.11598	-0.14973	0.53643	YES	0	Baseline
Immobilization	2	2576.00	20.00	7077	2.04945	0.19693	1.68459	NO	-3154	ImmobArea
Immobilization	2	5472.00	20.00	10388	46.04866	-6.90622	4.92820	NO	157	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 25

    ! Inject 4-A-2,6-DNT onto chip surface
    INJECT    R2D1 25

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 25
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  3
    APROG     immobilization
END
```

Notebook

Date: 11/29/95  
Operator:

Purpose: Immobilization of 4-Amino-2,6-Dinitrotoluene  
Results: No immobilization  
Comments:

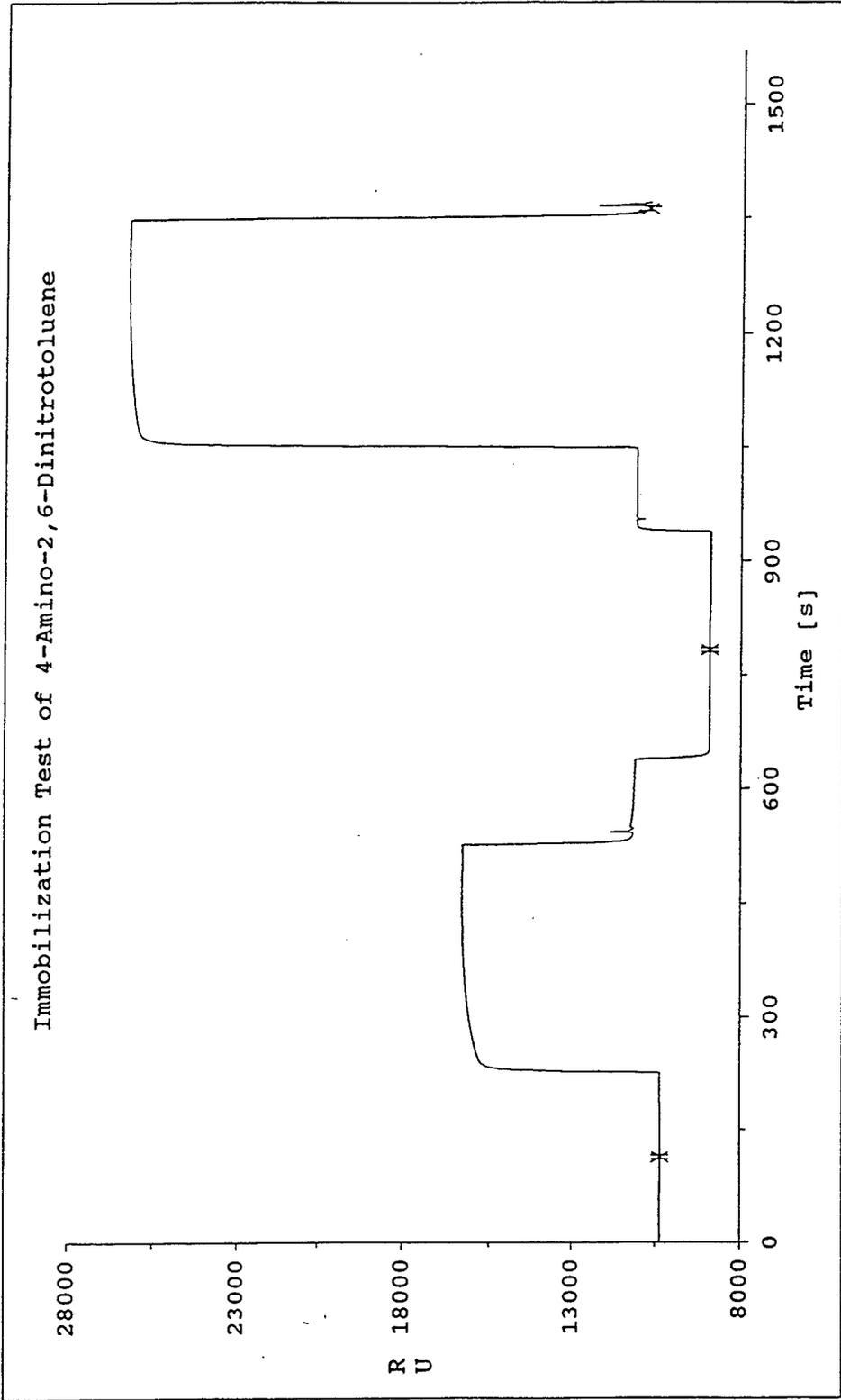
Flow cell: 3  
SensorChip: CM5-DANT/DNAT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 4-Amino-2,6-Dinitrotoluene  
Volume: 25  $\mu$ l, pH 6.25  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 29nov-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of 4-Amino-2,6-Dinitrotoluene



R  
U

APROG	FC	Time	Window	AbsResp	SD	Slope	LRSQ	Baseline	RelResp	Id
immobilization	3	114.00	5.00	10348	0.59642	-0.13185	0.58491	YES	0	Baseline
immobilization	3	783.50	5.00	8917	0.68064	-0.15819	0.66203	NO	-1431	ImmobArea
immobilization	3	1361.50	5.00	10763	56.42546	-33.57882	9.60935	NO	414	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    FLOW      5      !  $\mu$ l/min
    ! Mix NHS and EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    ! Activate sensorchip
    INJECT    R1F4 25

    ! Inject 4-A-2,6-DNT onto chip surface
    INJECT    R2D1 25

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT    R1F6 25
END

MAIN
    RACK      1 thermo_b
    RACK      2 thermo_a
    FLOWCELL  3
    APROG     immobilization
END
```

Notebook

Date:  
Operator: Christoph

Purpose: Immobilization of 4-Amino-2,6-Dinitrotoluene  
Results: No immobilization  
Comments:

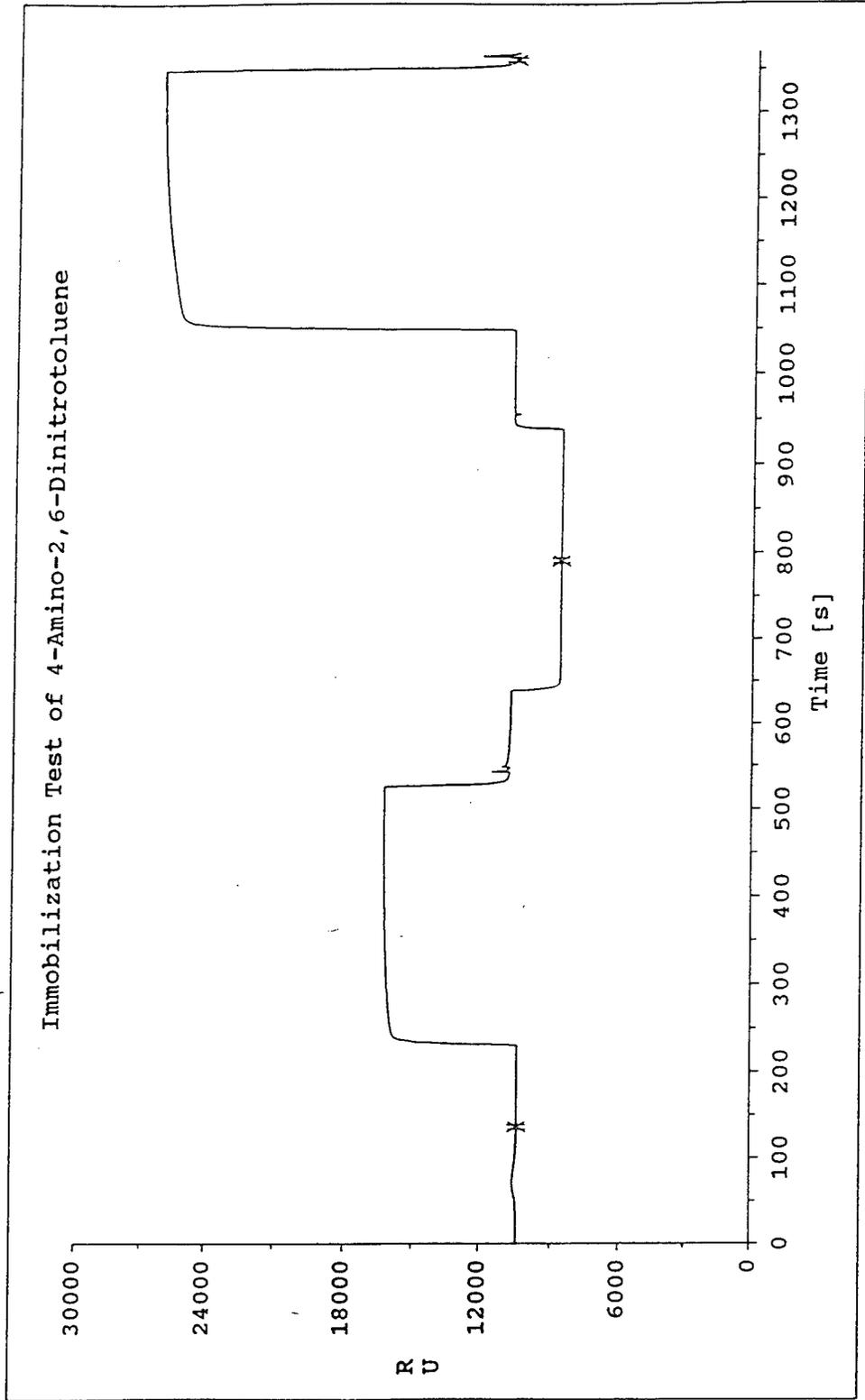
Flow cell: 3  
SensorChip: CM5-DANT/DNAT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: 4-Amino-2,6-Dinitrotoluene  
Volume: 25  $\mu$ l, pH 4.63  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 29nov-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of 4-Amino-2,6-Dinitrotoluene



Program

```
DEFINE APROG immobilization
  ! EDC in R1F1
  ! NHS in R1F2
  ! Empty vial in R1F4
  ! ETHANOLAMINE in R1F6
```

```
FLOW      5      ! µl/min
  ! Mix NHS and EDC
TRANSFER  R1F1 R1F4 70
TRANSFER  R1F2 R1F4 70
MIX       R1F4 115
```

```
  ! Activate sensorchip
INJECT    R1F4 25
```

```
  ! Inject Humic Acid onto chip surface
INJECT    R2D1 25
```

```
  ! Deactivate excess reactive groups with ETHANOLAMINE
INJECT    R1F6 25
```

END

MAIN

```
RACK      1 thermo_b
RACK      2 thermo_a
FLOWCELL  4
APROG     immobilization
END
```

Notebook

Date: 11/30/95  
Operator:

Purpose: Immobilization of Humic Acid  
Results: No immobilization  
Comments:

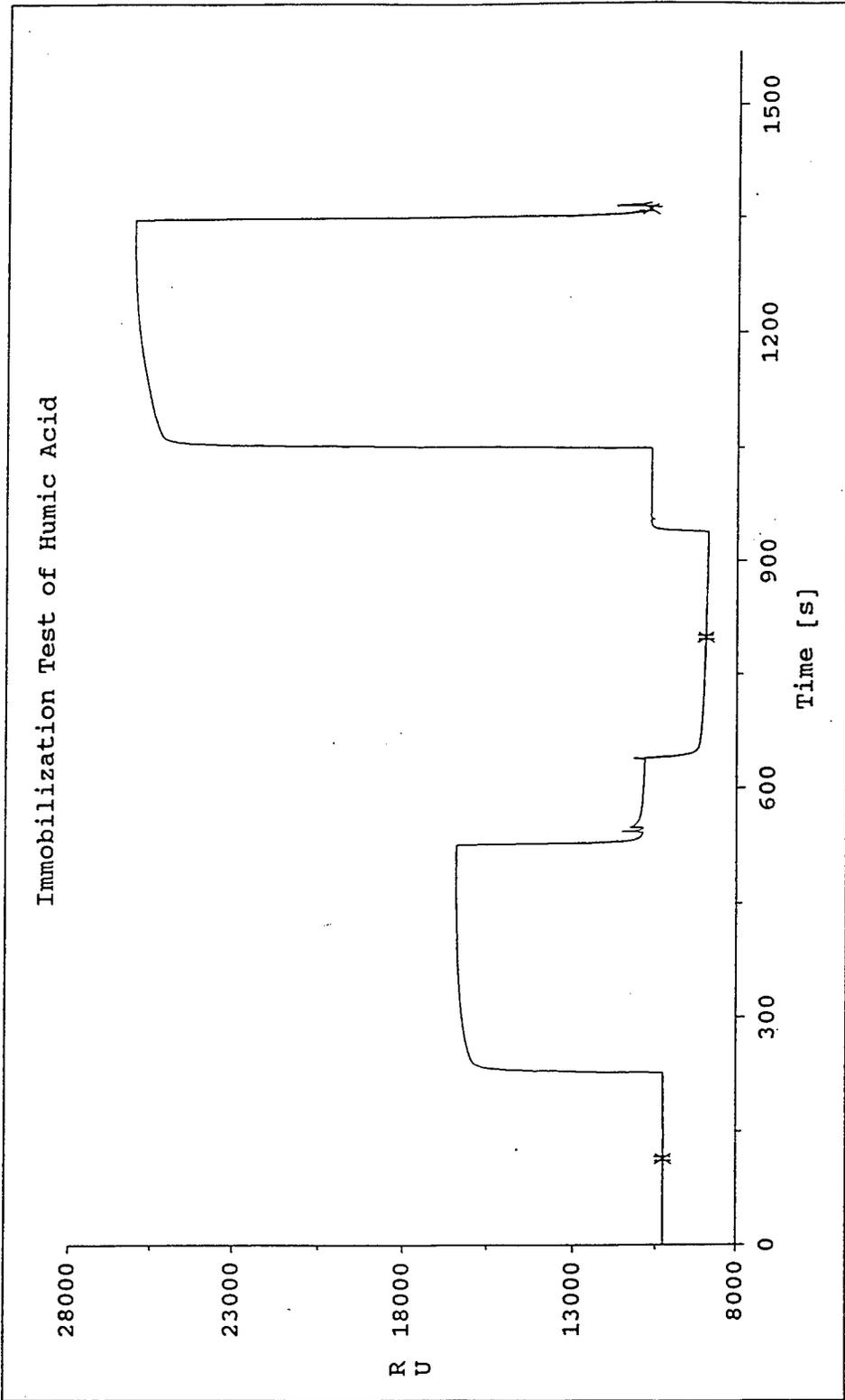
Flow cell: 4  
SensorChip: CM5-DANT/DNAT/Humic-ACID  
EluentBuffer: Sodium Phosphate, pH 7.06  
Regeneration:  
Flow: 5  $\mu$ l/min

~~Immobilized~~ Ligand: Humic Acid  
Volume: 25  $\mu$ l  
Conc: 358  $\mu$ M  
Buffer: Sodium Phosphate, pH 7.1  
Immobilization File: 30nov-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Humic Acid



R  
U

APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
Immobilization	4	114.00	5.00	10260	0.67580	0.02452	0.71107	YES	0	Baseline
immobilization	4	798.50	5.00	8952	1.26702	-0.67821	0.61501	NO	-1308	ImmobArea
immobilization	4	1361.50	5.00	10768	72.90439	-43.48496	11.30190	NO	508	EndSensorgr

Program

```
DEFINE APROG immobilization
  ! EDC in R1F1
  ! NHS in R1F2
  ! Empty vial in R1F4
  ! ETHANOLAMINE in R1F6

  FLOW      5      !  $\mu$ l/min
  ! Mix NHS and EDC
  TRANSFER  R1F1 R1F4 70
  TRANSFER  R1F2 R1F4 70
  MIX       R1F4 115

  ! Activate sensorchip
  INJECT    R1F4 25

  ! Inject Humic Acid onto chip surface
  INJECT    R2D1 25

  ! Deactivate excess reactive groups with ETHANOLAMINE
  INJECT    R1F6 25
END

MAIN
  RACK      1 thermo_b
  RACK      2 thermo_a
  FLOWCELL  4
  APROG     immobilization
END
```

Notebook

Date: 11/30/95  
Operator:

Purpose: Immobilization of Humic Acid  
Results: No immobilization  
Comments:

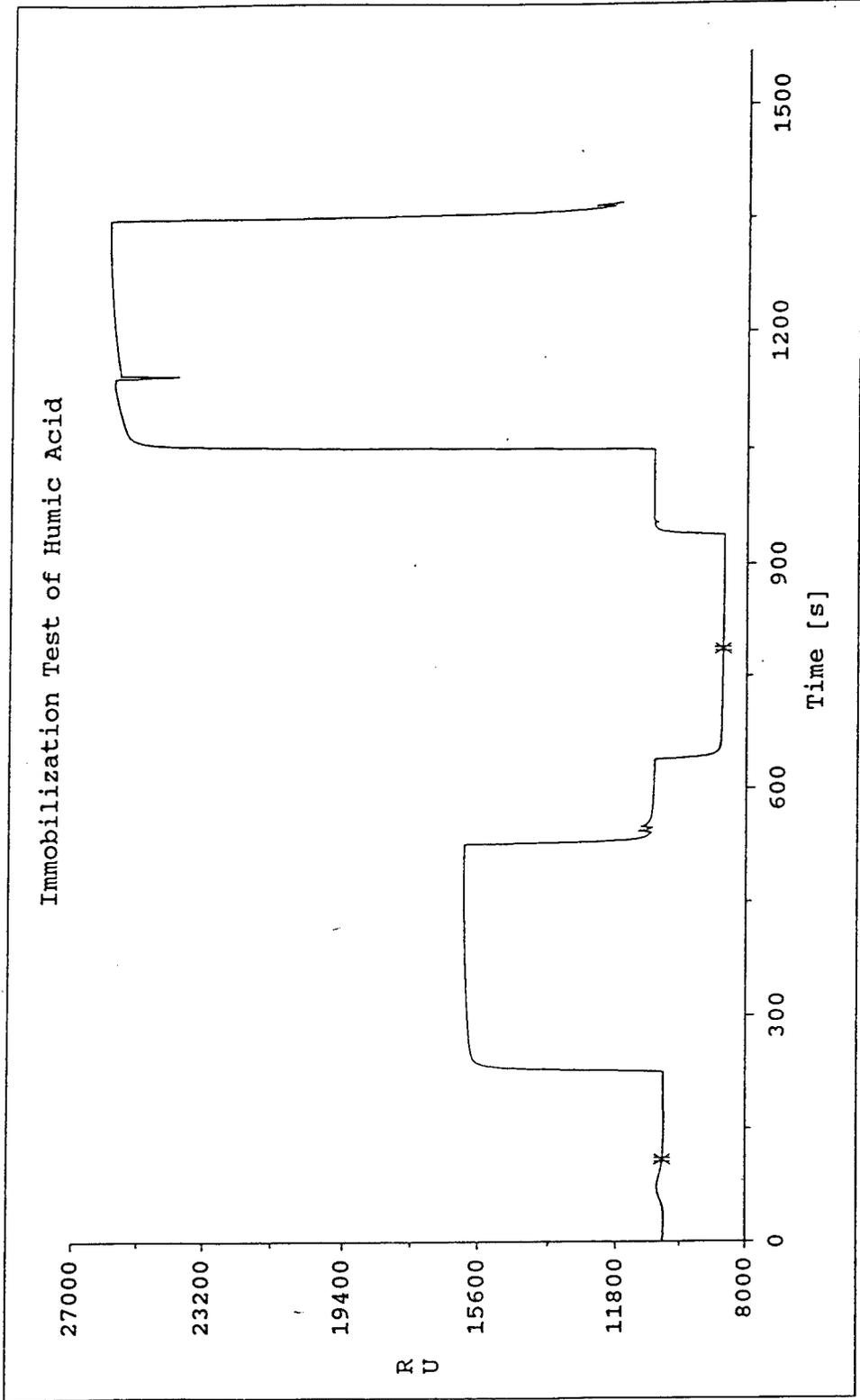
Flow cell: 4  
SensorChip: CM5 DANT/DNAT/HumicAcid  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: Humic Acid  
Volume: 25  $\mu$ l, pH 4.90  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 30nov-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of Humic Acid



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	4	109.00	5.00	10411	3.71359	-2.18885	0.82685	YES	0	Baseline
immobilization	4	786.00	5.00	8679	1.05639	-0.45424	0.78069	NO	-1732	ImmobArea
immobilization	4	1369.50	0.00	11848	#N/A	#N/A	#N/A	NO	1437	EndSensorgr

Program

```
DEFINE APROG immobilization
  ! EDC in R1F1
  ! NHS in R1F2
  ! Empty vial in R1F4
  ! ETHANOLAMINE in R1F6
```

```
FLOW      1      !  $\mu$ l/min
  ! Mix NHS and EDC
TRANSFER  R1F1 R1F4 70
TRANSFER  R1F2 R1F4 70
MIX       R1F4 115
```

```
  ! Activate sensorchip
INJECT    R1F4 25
```

```
  ! Inject Humic Acid onto chip surface
INJECT    R2D1 25
```

```
  ! Deactivate excess reactive groups with ETHANOLAMINE
INJECT    R1F6 30
```

END

MAIN

```
RACK      1 thermo_b
RACK      2 thermo_a
FLOWCELL  4
APROG     immobilization
END
```

Notebook

Date: 12/01/95  
Operator:

Purpose: Immobilization of Humic Acid  
Results: No immobilization  
Comments:

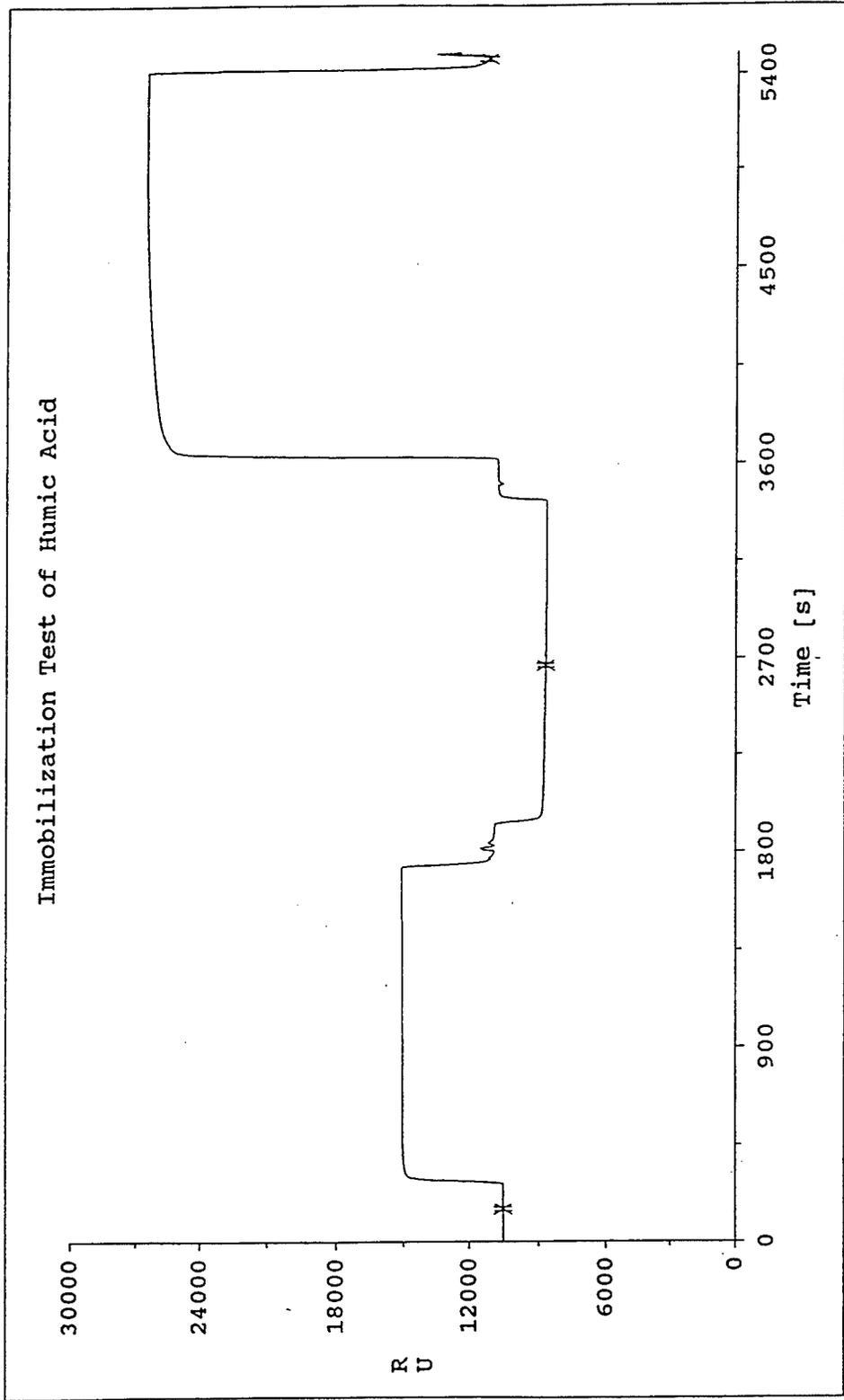
Flow cell: 4  
SensorChip: CM5-DANT/DNAT/Humic Acid  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 1  $\mu$ l/min

Ligand: Humic Acid  
Volume: 25  $\mu$ l, pH 4.90  
Conc: 200  $\mu$ M  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 01dec-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Humic Acid



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	4	154.00	20.00	10512	0.77016	0.06001	0.69500	YES	0	Baseline
immobilization	4	2666.00	20.00	8703	1.10645	-0.10644	0.89798	NO	-1808	ImmobArea
immobilization	4	5472.00	20.00	11232	49.33905	-7.41934	3.69596	NO	721	EndSensorgr

Program

```
DEFINE APROG IMMOB1
    !R1F1 EDC
    !R1F2 nhs
    !R1F4 EMPTY

    FLOW      5
    !MIX NHS AND EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    !ACTIVATE SENSORCHIP
    INJECT    R1F4 50

    !INJECT Recognin C1 ONTO CHIP SURFACE
    INJECT    R2E1 50

    !DEACTIVATE EXCESS REACTIVE GROUPS WITH ETHANOLAMINE
    INJECT    R1F6 40

END

MAIN
    RACK      1 THERMO_b
    RACK      2 THERMO_a
    FLOWCELL  1
    APROG     IMMOB1
    WASH      b

END
```

Notebook

Date: 08/31/95  
Operator:

Purpose: Immobilization of Recognin C1  
Results: ?  
Comments:

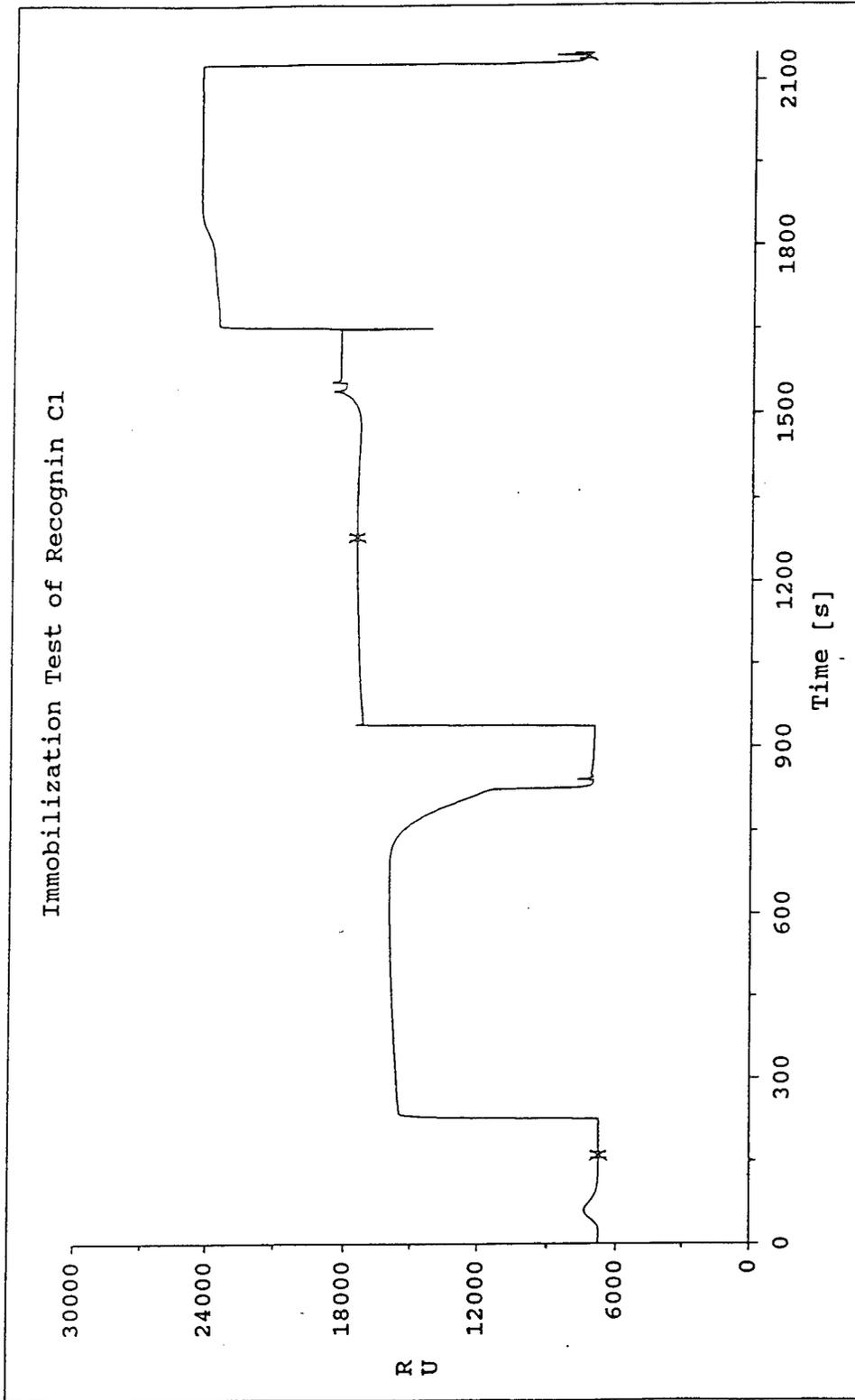
Flow cell: 1  
SensorChip: CM5  
EluentBuffer: Sodium Phosphate, pH 7.1  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: Recognin C1/Sodiumacetate 1:1  
Volume: 50  $\mu$ l  
Conc: 202 mM  
Buffer: H<sub>2</sub>O dest./Sodiumacetate  
Immobilization File: 31aug-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Recognin C1



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	1	161.00	10.00	6788	0.85985	-0.23834	0.35661	YES	0	Baseline
IMMOB1	1	1283.00	10.00	17537	0.30980	0.01547	0.32205	NO	10770	ImmobArea
IMMOB1	1	2144.00	10.00	7716	400.50827	42.61407	395.01242	NO	949	EndSensorgr

Program

DEFINE APROG IMMOB1

!R1F1 EDC

!R1F2 nhs

!R1F4 EMPTY

FLOW 5

!MIX NHS AND EDC

TRANSFER R1F1 R1F4 70

TRANSFER R1F2 R1F4 70

MIX R1F4 115

!ACTIVATE SENSORCHIP

INJECT R1F4 50

!INJECT Lysozyme ONTO CHIP SURFACE

INJECT R2E1 50

!DEACTIVATE EXCESS REACTIVE GROUPS WITH ETHANOLAMINE

INJECT R1F6 40

END

MAIN

RACK 1 THERMO\_b

RACK 2 THERMO\_a

FLOWCELL 1

APROG IMMOB1

WASH b

END

Notebook

Date: 09/27/95  
Operator:

Purpose: Immobilization LYSOZYME  
Results: No immobilization ?  
Comments: Sensorgram graph indicates immobilization  
of a small amount ?

Flow cell: 1  
SensorChip: CM5 LYSO

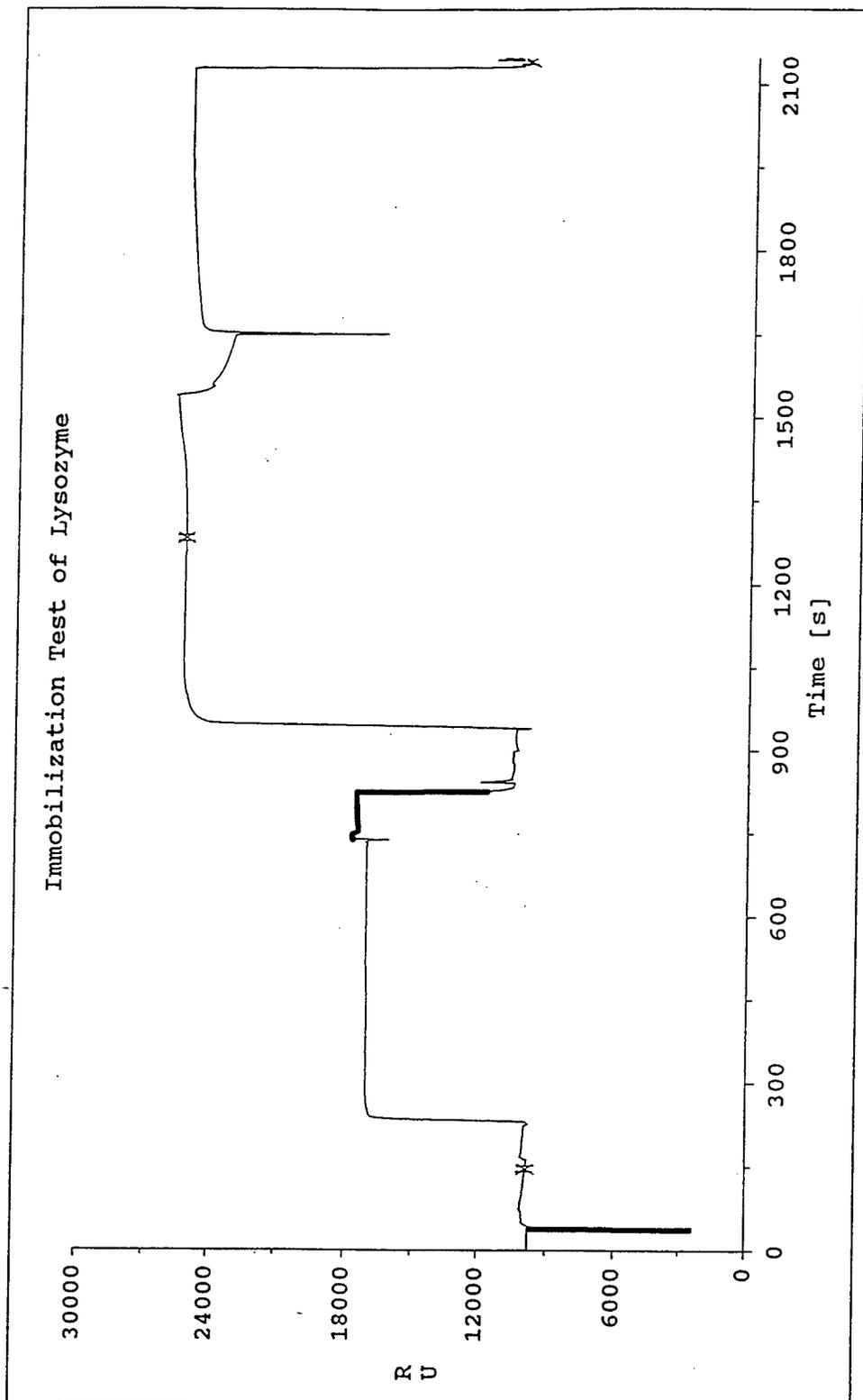
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5 ul/min

Ligand: Lysozyme  
Volume: 50  $\mu$ l  
Conc: 0.2 mg/ml, pH 6.4  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 27sep-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Lysozyme



APROG	FC	TTime	Window	AbsResp	SD	Slope	LRSD	Baseline	ReliResp	Id
IMMOB1	1	148.00	10.00	9882	11.70040	-3.21042	5.11256	YES	0	Baseline
IMMOB1	1	1283.00	10.00	25252	1.42440	0.24132	1.24201	NO	15370	ImmobArea
IMMOB1	1	2144.00	10.00	10497	472.38505	76.08104	420.94611	NO	615	EndSensorgr

Program

```
DEFINE APROG IMMOB1
    !R1F1 EDC
    !R1F2 nhs
    !R1F4 EMPTY

    FLOW          5
    !MIX NHS AND EDC
    TRANSFER     R1F1 R1F4 70
    TRANSFER     R1F2 R1F4 70
    MIX          R1F4 115

    !ACTIVATE SENSORCHIP
    INJECT       R1F4 50

    !INJECT Lysozyme ONTO CHIP SURFACE
    INJECT       R2E1 50

    !DEACTIVATE EXCESS REACTIVE GROUPS WITH ETHANOLAMINE
    INJECT       R1F6 40

END

MAIN
    RACK         1 THERMO_b
    RACK         2 THERMO_a
    FLOWCELL     2
    APROG        IMMOB1
    WASH         b

END
```

Notebook

Date: 09/27/95  
Operator:

Purpose: Immobilization LYSOZYME  
Results: No immobilisation ?  
Comments: Sensogram graph indicates immobilization  
of a small amount ?

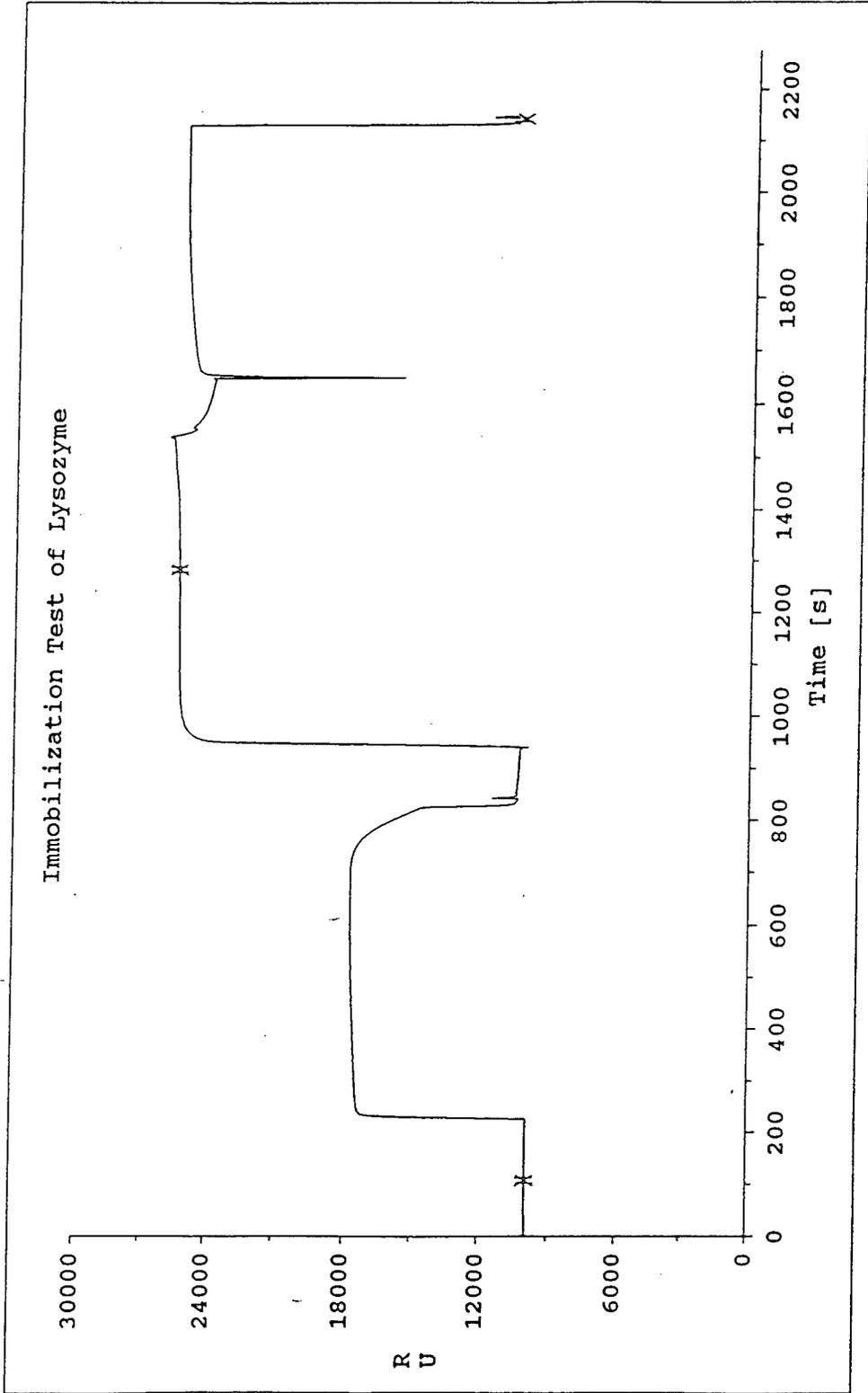
Flow cell: 2  
SensorChip: CM5 LYSO  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5 ul/min

Ligand: Lysozyme  
Volume: 50  $\mu$ l  
Conc: 0.2 mg/ml, pH 6.4  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 27sp-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Lysozyme



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	2	107.00	10.00	9939	0.52123	0.12425	0.33645	YES	0	Baseline
IMMOB1	2	1285.00	10.00	25415	1.17380	0.33846	0.36163	NO	15476	ImmobiArea
IMMOB1	2	2144.00	10.00	10730	409.67310	52.77063	390.44123	NO	791	EndSensorgr

Program

DEFINE APROG IMMOB1

!R1F1 EDC  
!R1F2 nhs  
!R1F4 EMPTY

FLOW 5  
!MIX NHS AND EDC  
TRANSFER R1F1 R1F4 70  
TRANSFER R1F2 R1F4 70  
MIX R1F4 115

!ACTIVATE SENSORCHIP  
INJECT R1F4 50

!INJECT Lysozyme ONTO CHIP SURFACE  
INJECT R2E1 50

!DEACTIVATE EXCESS REACTIVE GROUPS WITH ETHANOLAMINE  
INJECT R1F6 40

END

MAIN

RACK 1 THERMO\_b  
RACK 2 THERMO\_a  
FLOWCELL 4  
APROG IMMOB1  
WASH b

END

Notebook

Date: 09/29/95  
Operator:

Purpose: Immobilization LYSOZYME  
Results: No immobilization ?  
Comments: Sensogram graph indicates immobilization  
of a small amount ?

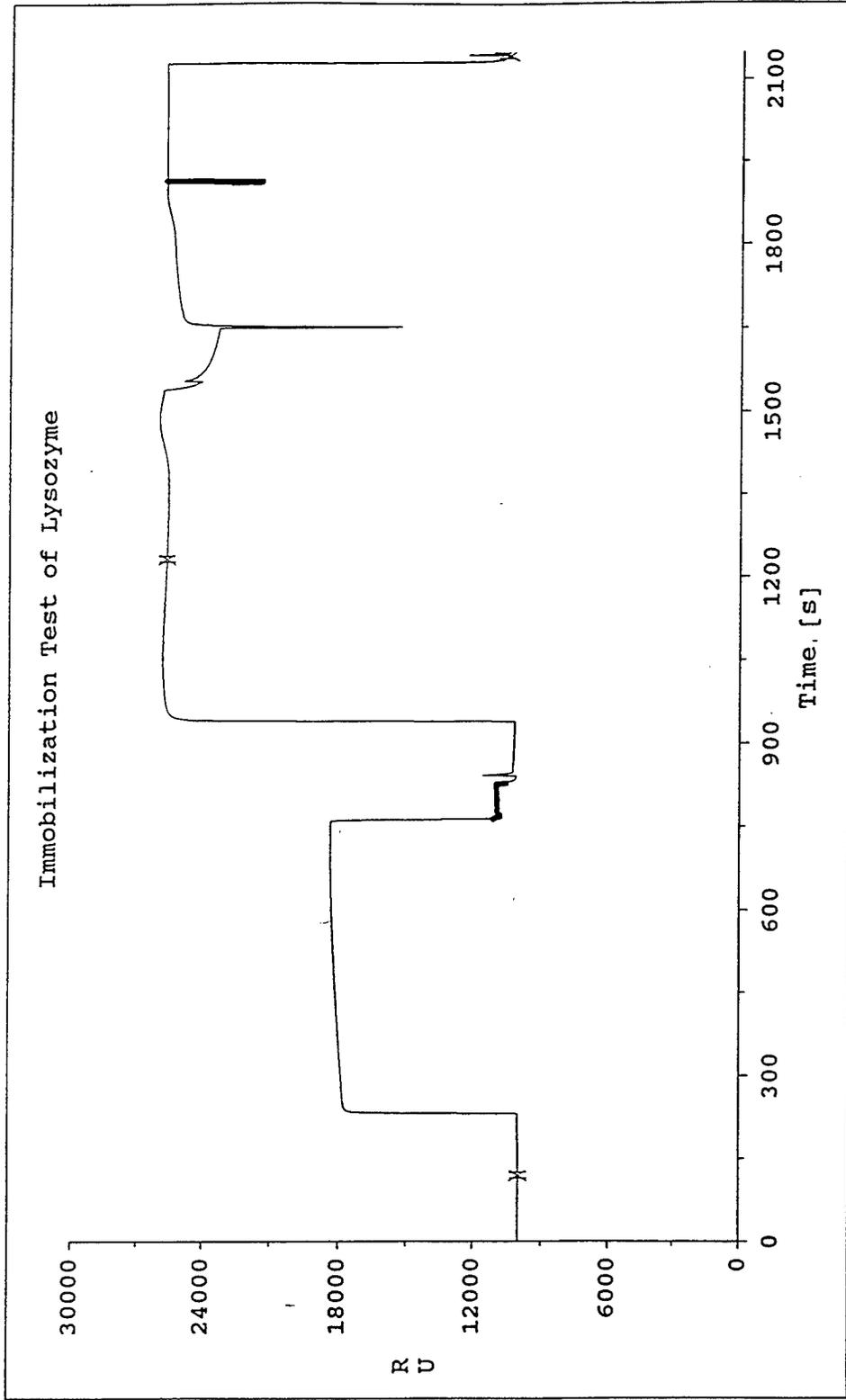
Flow cell: 4  
SensorChip: CM5 LYSO  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: Lysozyme  
Volume: 50  $\mu$ l  
Conc: 1 mg/ml, pH 5.6  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 29sep-1.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of Lysozyme



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	4	121.00	10.00	985	0.49838	-0.02467	0.51821	YES	0	Baseline
IMMOB1	4	1233.00	10.00	25646	3.58558	-1.06484	0.65463	NO	15661	ImmobArea
IMMOB1	4	2144.00	10.00	10871	567.95716	61.80266	558.33092	NO	886	EndSensorgr

Program

```
DEFINE APROG IMMOB1
    !R1F1 EDC
    !R1F2 nhs
    !R1F4 EMPTY

    FLOW      5
    !MIX NHS AND EDC
    TRANSFER  R1F1 R1F4 70
    TRANSFER  R1F2 R1F4 70
    MIX       R1F4 115

    !ACTIVATE SENSORCHIP
    INJECT    R1F4 50

    !INJECT Lysozyme ONTO CHIP SURFACE
    INJECT    R2E1 50

    !DEACTIVATE EXCESS REACTIVE GROUPS WITH ETHANOLAMINE
    INJECT    R1F6 40

END

MAIN
    RACK      1 THERMO_b
    RACK      2 THERMO_a
    FLOWCELL  1
    APROG     IMMOB1
    WASH      b

END
```

Notebook

Date: 09/29/95  
Operator:

Purpose: Immobilization LYSOZYME  
Results: No immobilization ?  
Comments: Sensorgram graph indicates immobilization  
of a small amount ?

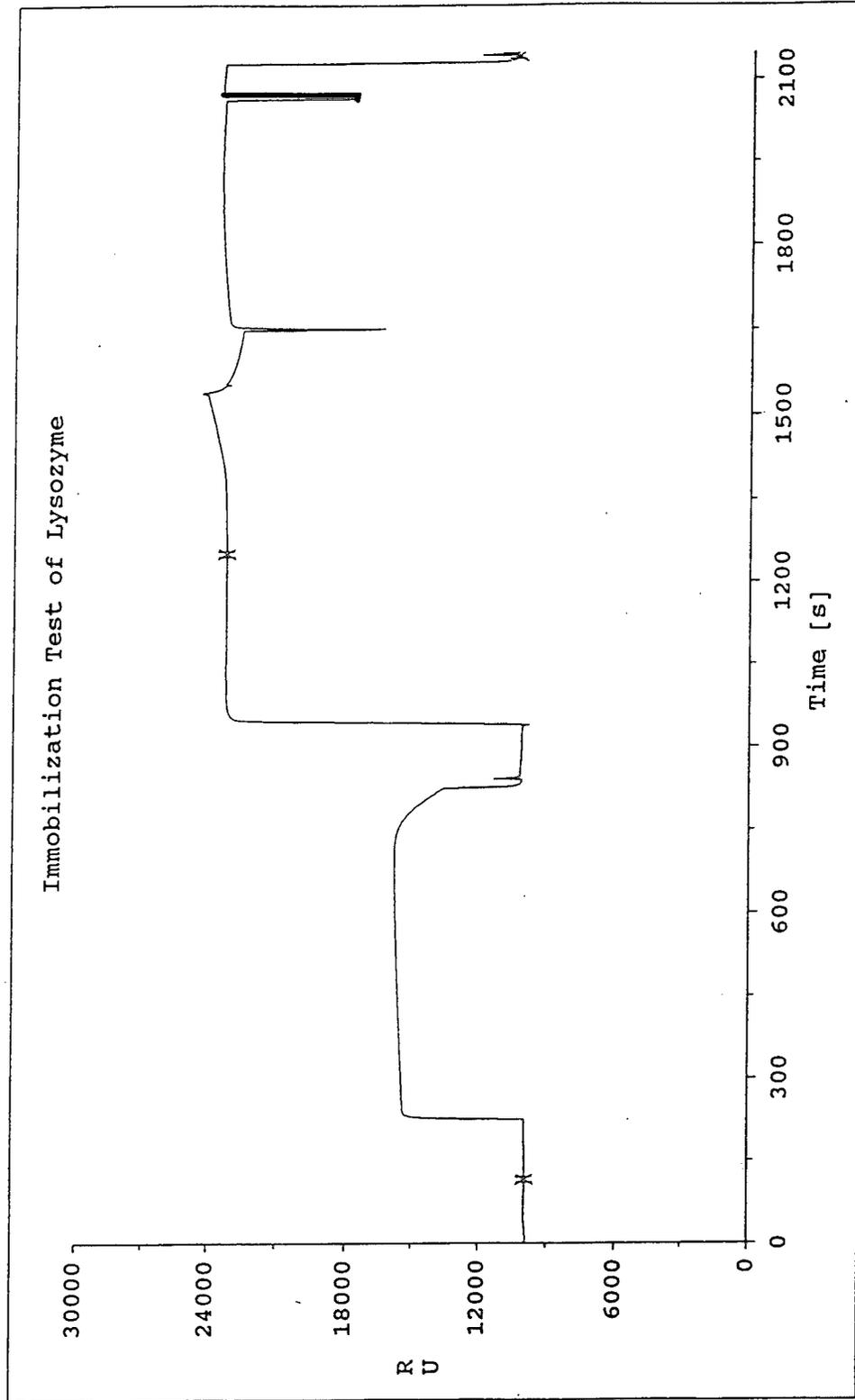
Flow cell: 1  
SensorChip: CM5 LYSO  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

Ligand: Lysozyme  
Volume: 50  $\mu$ l  
Conc: 0.2 mg/ml, pH 4.9  
Buffer: H<sub>2</sub>O dest.  
Immobilization File: 29sep-2.blr  
Comments:

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

# Immobilization Test of Lysozyme



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
IMMOB1	1	116.00	10.00	9926	0.35847	0.00440	0.37755	YES	0	Baseline
IMMOB1	1	1253.00	10.00	23260	0.84067	0.20359	0.52788	NO	13334	ImmobArea
IMMOB1	1	2144.00	10.00	10749	502.14313	60.75004	484.82697	NO	823	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6
```

```
FLOW          5
    ! Mix NHS and EDC
TRANSFER      R1F1 R1F4 70
TRANSFER      R1F2 R1F4 70
MIX           R1F4 115
```

```
    ! Activate sensorchip
INJECT        R1F4 50
```

```
    ! Inject Lysozyme onto chip surface
INJECT        R2E1 50
```

```
    ! Deactivate excess reactive groups with ETHANOLAMINE
INJECT        R1F6 40
```

END

MAIN

```
RACK          1 thermo_b
RACK          2 thermo_a
FLOWCELL      2
APROG         immobilization
WASH          b
```

END

Notebook

Date: 11/09/95

Operator:

Purpose: Immobilization Lysozyme

Results: Immobilization achieved

Comments: Use of new system specific chemicals  
reason for success

Flow cell: 2

SensorChip: CM5 Lyso/DANT

EluentBuffer: HEPES/P20, pH 7.4

Regeneration:

Flow: 5  $\mu$ l/min

Immobilized Ligand: Lysozyme

Volume: 50  $\mu$ l

Conc: 1 mg/ml, pH 5.6

Buffer: H<sub>2</sub>O dest.

Immobilization File: 09nov-1.blr

Comments:

Analyte 1:

Volume:

Conc:

Buffer:

Comments:

Analyte 2:

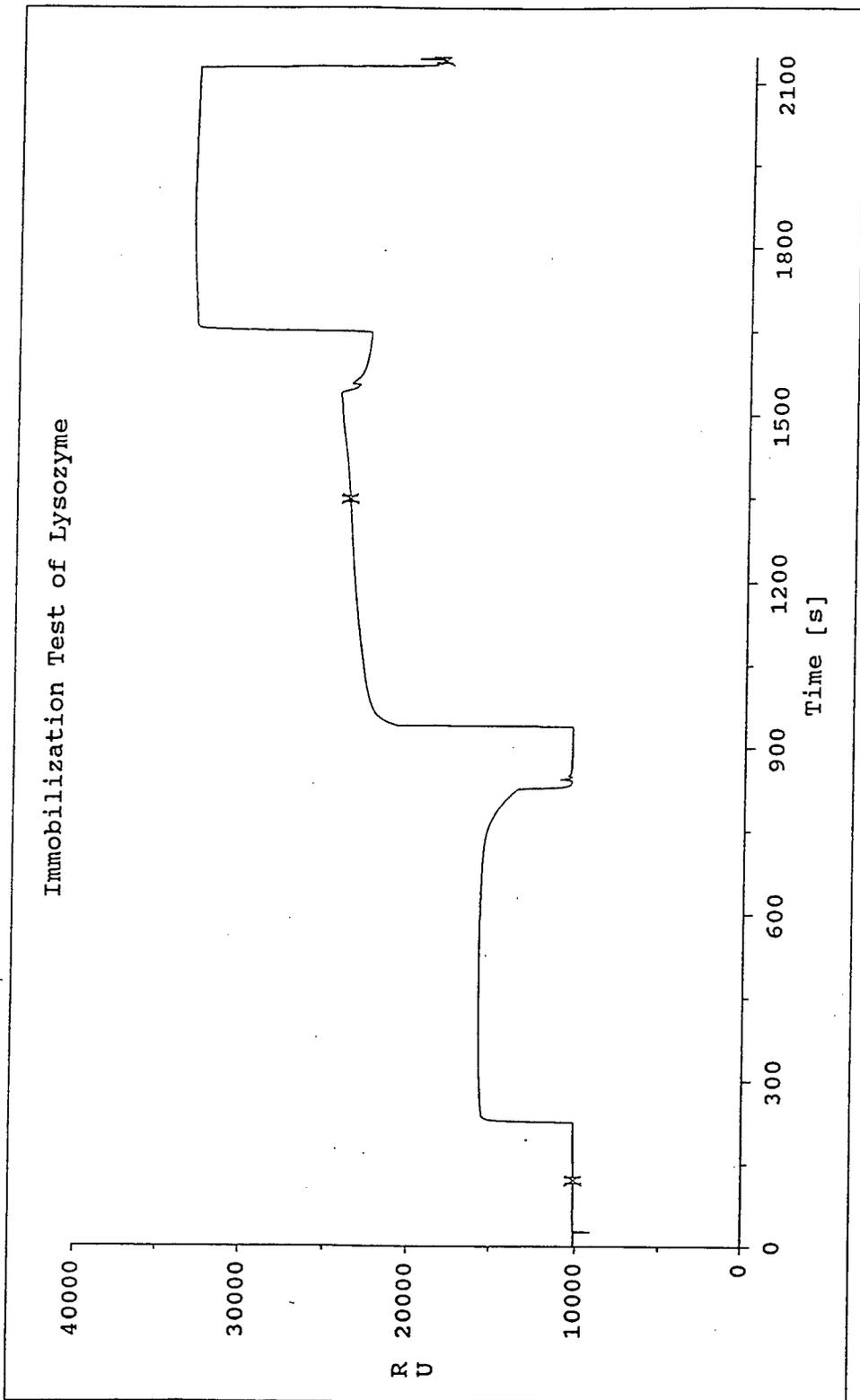
Volume:

Conc:

Buffer:

Comments:

# Immobilization Test of Lysozyme



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
immobilization	2	121.00	10.00	10143	0.64709	-0.03074	0.67357	YES	0	Baseline
immobilization	2	1349.00	10.00	23855	8.00500	2.40257	0.80558	NO	13711	ImmobArea
immobilization	2	2144.00	10.00	18736	483.81284	45.33681	484.72808	NO	8593	EndSensorgr

Program

```
DEFINE APROG immobilization
    ! EDC in R1F1
    ! NHS in R1F2
    ! Empty vial in R1F4
    ! ETHANOLAMINE in R1F6

    CAPTION      Immobilization Test of Lysozyme
    FLOW         5
    ! Mix NHS and EDC
    TRANSFER    R1F1 R1F4 70
    TRANSFER    R1F2 R1F4 70
    MIX         R1F4 115

    ! Activate sensorchip
    INJECT      R1F4 50

    ! Inject ligand onto chip surface
    INJECT      R2E1 50

    ! Deactivate excess reactive groups with ETHANOLAMINE
    INJECT      R1F6 40
END
```

```
MAIN
    RACK        1 thermo_b
    RACK        2 thermo_a
    FLOWCELL    3
    APROG       immobilization
    WASH        b
```

END

Notebook

Date: 11/09/95  
Operator:

Purpose: Immobilization Lysozyme  
Results: Immobilization achieved  
Comments: Use of new system specific chemicals  
reason for success

Flow cell: 3  
SensorChip: CM5 Lyso/DANT  
EluentBuffer: HEPES/P20, pH 7.4  
Regeneration:  
Flow: 5  $\mu$ l/min

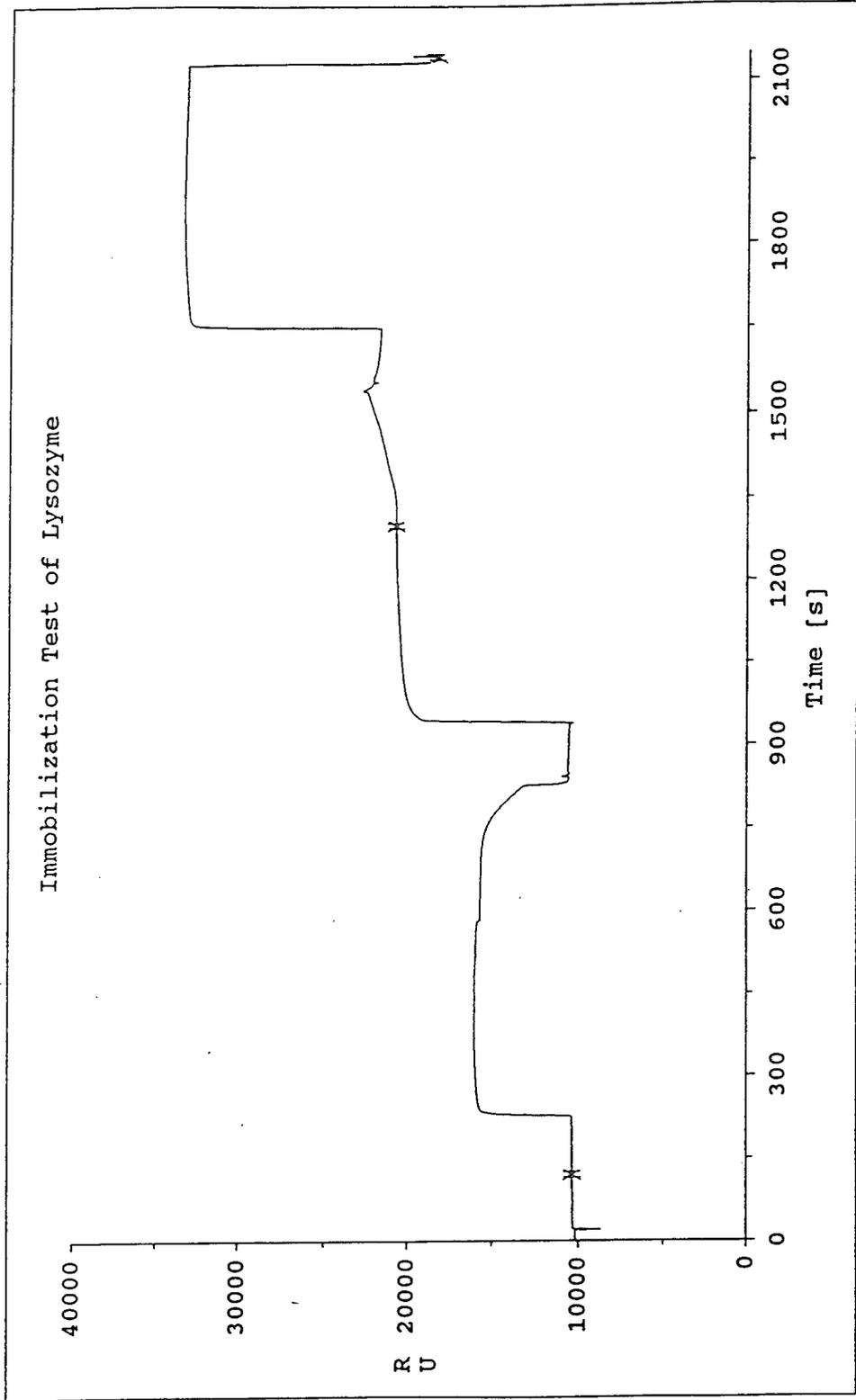
Immobilized Ligand: Lysozyme  
Volume: 50  $\mu$ l  
Conc: 0.2 mg/ml, pH 4.9  
Buffer: H<sub>2</sub>O dest.

Immobilization File: 09nov-2.blr  
Comments: Both activation and immobilization period should be  
shortened (graph strongly inclining and ascending  
at the periods' end respectively)

Analyte 1:  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Immobilization Test of Lysozyme



Interaction Program

```
DEFINE APROG reaction
  PARAM %POS %CONC %ID
  KEYWORD ANALYTE %ID
  KEYWORD CONC(uM) %CONC
  CAPTION Interaction of Humic acid %CONC uM solution with ....
  FLOW      ...
  INJECT    R2E2 ...      !REMOVE NON-SPEC BONDS WITH NaOH
  * INJECT  %POS ...      !INJECT HUMIC ACID
  INJECT    R2F1 ...      !STRIP WITH HCl
```

END

```
DEFINE LOOP INTERACTION
  LPARAM %POS %CONC %ID
  TIMES ...
  R2A4 ... Humic_Acid
  R2A5 ... Humic_Acid
  R2A6 ... Humic_Acid
  R2A7 ... Humic_Acid
```

END

```
MAIN
  RACK      1 THERMO_b
  RACK      2 THERMO_a
  FLOWCELL  ...
  LOOP INTERACTION RANDOM
  APROG     reaction %POS %CONC %ID
  WASH      b
  ENDLLOOP
```

END

Program

```
DEFINE APROG REACT1
  PARAM %POS %CONC %ID
  KEYWORD ANALYTE %ID
  KEYWORD CONC(µM) %CONC

      FLOW          5
      INJECT        R2E2 10      !REMOVE NON-SPEC BONDS WITH NaOH
*   INJECT          %POS 50      !INJECT HUMIC ACID
      INJECT        R2F1 15      !STRIP WITH HCl

END

DEFINE LOOP INTERACTION
  LPARAM %POS %CONC %ID
  TIMES 2
      R2A4  10      Humic_Acid
      R2A5  5       Humic_Acid
      R2A6  2.5    Humic_Acid
      R2A7  1       Humic_Acid

END

MAIN
  RACK          1 THERMO_b
  RACK          2 THERMO_a
  FLOWCELL      1
  LOOP INTERACTION RANDOM
  APROG          REACT1 %POS %CONC %ID
  WASH          b
  ENDLOOP

END
```

Notebook

Date: 07/06/95  
Operator:

Purpose: Test run interaction program  
Results:  
Comments:

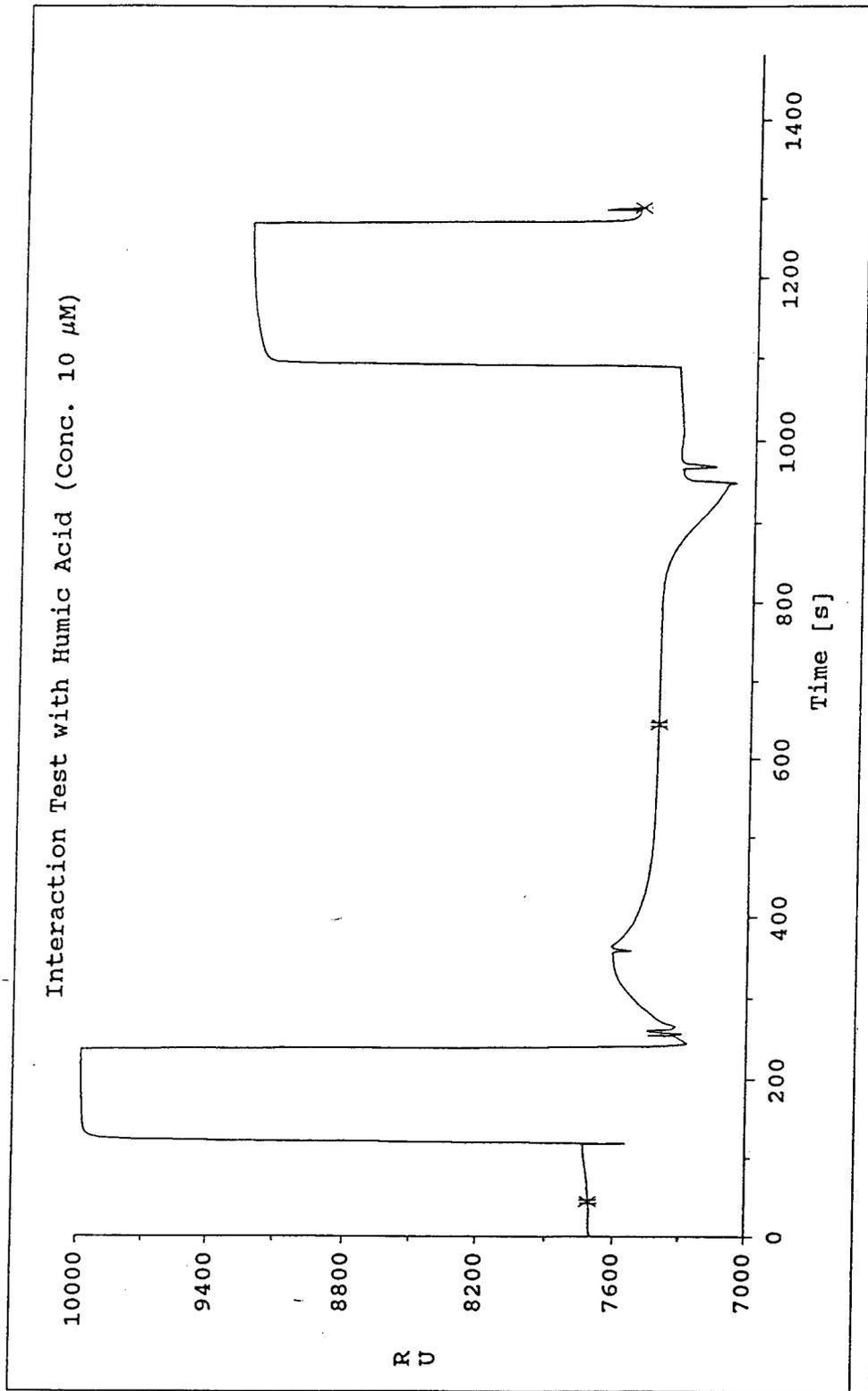
Flow cell: 1  
SensorChip: CM5/DNAT  
EluentBuffer: Sodium phosphate  
Regeneration:  
Flow: 5  $\mu$ l/min

Immobilized Ligand: None  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 1: Humic acid  
Volume: 50  $\mu$ l  
Conc: 1, 2.5, 5, 10  $\mu$ M  
Buffer:  
Comments:  
Interaction File: 06jul.blr

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Interaction Test with Humic Acid (Conc. 10  $\mu\text{M}$ )



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id	ANALYTE	CONC( $\mu\text{M}$ )
REACT1	1	45.00	5.00	7712	0.64347	0.29016	0.45033	YES	0	Baseline	Humic_Acid	10
REACT1	1	64.50	5.00	7421	0.56556	-0.22984	0.44044	NO	-291	InteractArea	Humic_Acid	10
REACT1	1	1288.50	0.00	7532	#N/A	#N/A	#N/A	NO	-180	EndSensorgr	Humic_Acid	10

Program

```
DEFINE APROG REACT1
  PARAM %POS %CONC %ID
  KEYWORD ANALYTE %ID
  KEYWORD CONC(µM) %CONC

      FLOW      5
*   INJECT      %POS 50      !NO REMOVING STEP
   INJECT      R2F1 15      !INJECT HUMIC ACID
                              !STRIP WITH HCl

END

DEFINE LOOP INTERACTION
  LPARAM %POS %CONC %ID
  TIMES 1
      R2A4  200   Humic_Acid
      R2A5  100   Humic_Acid
      R2A6   50   Humic_Acid
      R2A7  10    Humic_Acid

END

MAIN
  RACK      1 THERMO_b
  RACK      2 THERMO_a
  FLOWCELL  4
  LOOP INTERACTION RANDOM
  APROG      REACT1 %POS %CONC %ID
  WASH       b
  ENDLLOOP

END
```

Notebook

Date: 07/28/95

Operator:

Purpose: Test run interaction program  
without removing step

Results:  
Comments:

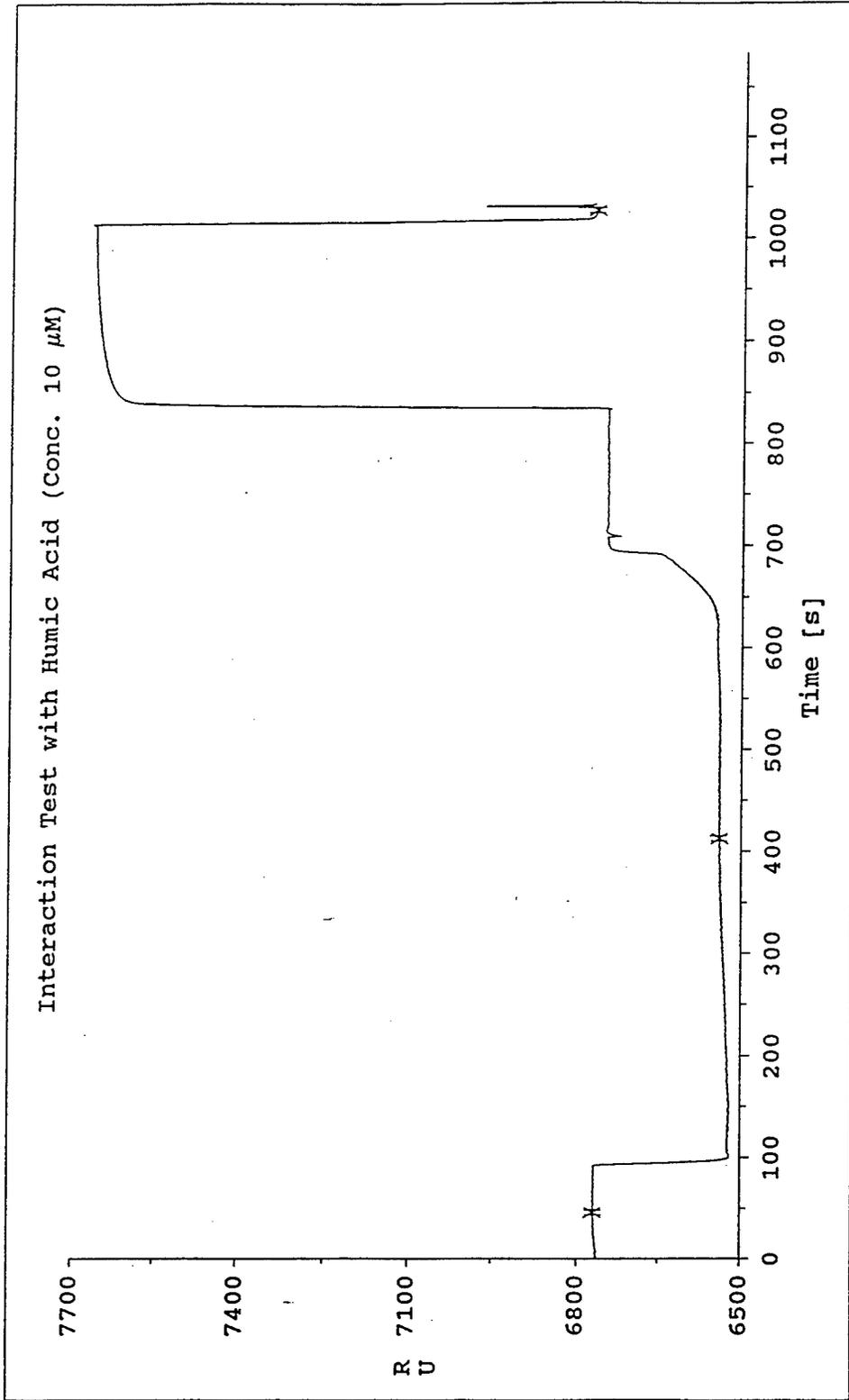
Flow cell: 4  
SensorChip: CM5 DANT  
EluentBuffer: sodium phosphate  
Regeneration:  
Flow: 5  $\mu$ l/min

Immobilized Ligand: None  
Volume:  
Conc:  
Buffer:  
Comments:

Analyte 1: Humic Acid  
Volume: 50  $\mu$ l  
Conc: 200, 100, 50, 10  $\mu$ M  
Buffer: Sodium Phosphate  
Comments:  
Interaction File: 28jul-2.blr

Analyte 2:  
Volume:  
Conc:  
Buffer:  
Comments:

Interaction Test with Humic Acid (Conc. 10  $\mu$ M)



R  
U

APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id	ANALYTE	CONC( $\mu$ M)
REACT1	4	46.50	5.00	6771	0.37689	-0.06548	0.38043	YES	0	Baseline	Humic Acid	10
REACT1	4	412.50	5.00	6539	0.95220	-0.01229	0.68714	NO	-232	InteractArea	Humic Acid	10
REACT1	4	1027.00	5.00	6772	0.86424	-0.45549	0.44269	NO	1	EndSensorgr	Humic Acid	10

**Immobilization and Interaction Tests with the BIA-Core-System**

Compound	In Buffer	pH	Quantity (µl)	Conc.	Flow Rate (µl/min)	Chemicals		Result	Result	pH	Conc. (mM)
						Immob.	Immob.				
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	50	194 µM	5	old	neg.			7.06	10
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	50	194 µM	5	old	neg.			7.06	10
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	50	424 µM	5	old	neg.			7.06	10
2,6-DA-4-NT	H <sub>2</sub> O dest.	6.6	25	200 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	8	50	194 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7	50	194 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	4.4	50	194 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	4.4	25	194 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	H <sub>2</sub> O dest.	4.6	25	200 µM	5	new	neg.			7.4	50
2,6-DA-4-NT	H <sub>2</sub> O dest.	4.6	25	200 µM	2	new	neg.			7.4	50
2,6-DA-4-NT	H <sub>2</sub> O dest.	4.6	25	200 µM	1	new	neg.			7.4	50
4-A-2,6-DNT	H <sub>2</sub> O dest.	6.3	25	200 µM	5	new	neg.			7.4	50
4-A-2,6-DNT	H <sub>2</sub> O dest.	4.6	25	200 µM	5	new	neg.			7.4	50
Humic Acid	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	25	358 µM	5	new	neg.			7.06	10
Humic Acid	H <sub>2</sub> O dest.	4.9	25	200 µM	5	new	neg.			7.4	50
Humic Acid	H <sub>2</sub> O dest.	4.9	25	200 µM	1	new	neg.			7.4	50
Reagin-G1	H <sub>2</sub> O dest.		50	202 mM	5	old	?			7.1	50
Lyszyme	H <sub>2</sub> O dest.	6.4	50	0.2 mg/ml	5	old	neg. ?			7.4	50
Lyszyme	H <sub>2</sub> O dest.	6.4	50	0.2 mg/ml	5	old	neg. ?			7.4	50
Lyszyme	H <sub>2</sub> O dest.	5.6	50	1.0 mg/ml	5	old	neg. ?			7.4	50
Lyszyme	H <sub>2</sub> O dest.	4.9	50	0.2 mg/ml	5	old	neg. ?			7.4	50
Lyszyme	H <sub>2</sub> O dest.	5.6	50	1.0 mg/ml	5	new	pos.			7.4	50
Lyszyme	H <sub>2</sub> O dest.	4.9	50	0.2 mg/ml	5	new	pos.			7.4	50
IAT-Humic Acid	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	50	1-10 µM	5	old	neg.	PigT		7.1	10
IAT-Humic Acid	NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	7.1	50	10-200 µM	5	old	neg.	PigT		7.1	10

# REPORT DOCUMENTATION PAGE

*Form Approved*  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

<b>1. AGENCY USE ONLY (Leave blank)</b>	<b>2. REPORT DATE</b> August 1997	<b>3. REPORT TYPE AND DATES COVERED</b> Final report	
<b>4. TITLE AND SUBTITLE</b> Explosives Conjugation Products in Remediation Matrices		<b>5. FUNDING NUMBERS</b>	
<b>6. AUTHOR(S)</b>  See Reverse			
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  See Reverse		<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>  Technical Report SERDP-97-7	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Corps of Engineers Washington, DC 20314-1000		<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>  Available from National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161.			
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for public release; distribution is unlimited.		<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 words)</b>  During investigations of potential treatment technologies for explosives-contaminated soils, 2,4,6-trinitrotoluene (TNT) has been observed to interact with some component of the matrix in such a way as to preclude extraction with organic solvents. Mass balance studies using radiolabeled TNT reveal that as much as 80 percent of the radioactivity added to tests is still present in the matrix. Therefore, the explosive has been changed to a more complex form. The long-term stability and environmental safety of these uncharacterized conjugates are unknown. Therefore, the objectives of this study were to characterize the conjugates, develop analytical methods for their identification, and determine their potential long-term stability and environmental safety. The approach includes development of hydrolytic methods for releasing identifiable explosives-related products from conjugated matrices, formation of conjugates by covalent linkage with humic acid functional groups using nuclear magnetic resonance, dialysis partitioning of explosives and transformation products with humic acid, surface plasmon resonance as a tool for assessing the interaction with humic acid, microbial degradability of conjugated products, and toxicology of explosives transformation and conjugated products. Results to date indicate that conjugates result from several processes occurring in the matrix. Some of these processes are potentially reversible, while others are more stable.			
<b>14. SUBJECT TERMS</b>  Humic acid                      Soil Immobilization                TNT		<b>15. NUMBER OF PAGES</b> 156	
		<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> UNCLASSIFIED	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> UNCLASSIFIED	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b>	<b>20. LIMITATION OF ABSTRACT</b>

6. (Concluded).

J. C. Pennington  
M. E. Honeycutt  
A. Z. Li  
P. G. Thorne  
D. R. Felt  
C. H. Allersmeier  
A. S. Jarvis  
K. A. Marx  
D. C. Leggett  
C. A. Hayes  
V. A. McFarland  
J. Walker  
B. E. Porter  
D. L. Kaplan  
D. Gunnison  
H. Fredrickson  
K. A. Thom

7. (Concluded).

U.S. Army Engineer Waterways Experiment Station  
3909 Halls Ferry Road, Vicksburg, MS 39180-6199

Department of Chemistry  
University of Massachusetts Lowell  
Lowell, MA 01854

U.S. Army Cold Regions Research and Engineering Laboratory  
Hanover, NH 03755-1290

ASCI Corporation  
1365 Beverly Road  
McLean, VA 22101

Biotechnology Division  
U. S. Army Natick Research, Development and Engineering Center  
Natick, MA 01760-5020

U.S. Department of Interior Geological Survey  
Box 25046 Federal Center  
Denver, Co 80225-0046