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# ENZYMATIC DESULFURIZATION OF COAL

**Final Report (Revised)**

DynaGen, Inc. Report No. 2484
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# ACKNOWLEDGEMENT

# ABBREVIATIONS

| | |
|---|---|
| ACN | Acetonitrile |
| DBT | Dibenzothiophene |
| ECF | Extracellular Fraction |
| EPS | Ethylphenylsulfide |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| ICF | Intracellular Fraction |
| HPLC | High Performance Liquid Chromatography |
| MF | Membrane Fraction |
| TLC | Thin Layer Chromatography |
| UV | Ultraviolet |

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# 1.0    INTRODUCTION

## 1.1    Background

### 1.1.1    The Need for Clean Coal

Anyone interested enough to be reading this report will be familiar with the environmental and human health issues related to emissions from coal and oil powered energy conversion processes. Coal is a U.S. resource which can supply a significant portion of our national energy needs. Present methods of energy recovery, however, release sulfur oxides which, if not captured, are cycled into acids which degrade our environment. At this writing, it appears that Congress will enact a "Clean Air" program which will require significant changes in present power plant operations.

If the full range of U.S. coals is ever to be utilized under "Clean Air" constraints, the issue of coal beneficiation must be addressed. The use of high sulfur coals for power generation would be expected to increase over the next decades, as reserves of high quality oils and coals diminish. This change is occurring at a time when the ecological impact of high sulfur and nitrogen oxide releases is of growing regulatory concern.

Either pre- or post-combustion processes can be utilized for removal of coal-derived sulfur and nitrogen. Post-combustion processing, e.g., flue gas desulfurization, is generally inconvenient and expensive. However, it can provide up to 95% remediation. Precombustion cleaning processes may offer significant advantages over post-combustion processes because the pre-combustion schemes can eliminate problems such as equipment corrosion and acid-forming flue emissions.

Pre-combustion processes exploit both physical and chemical methods. Physical methods involve surface-based methods(e.g. flotation) and density-based methods (e.g. heavy medium cyclone) for separating the pyrite from the coal. Compared to chemical methods, these are generally more economical. The drawback to these physical methods, however, lies in the limited removal of overall sulfur. While effective for removal of pyritic sulfur, organic sulfur remains untouched. Since organic sulfur can make up as much as 50% of sulfur in coal, this is a significant drawback. In addition, physical cleaning is often associated with energy loss; removal of the pyrite results in some removal of pyrite-containing coal. This "coal refuse" from the cleaning process may only contain 10 to 15% pyrite sulfur by weight and have a Btu value as high as 8,000 Btu/lb.[1]

Chemical pre-combustion cleaning methods usually employ an oxidizing agent, e.g., ferric salts, chlorine, or ozone, or reducing agent, e.g., solvent-hydrogen mixtures. While chemical methods can be reasonably effective in removing some of the organic sulfur along with the pyrite, there are some disadvantages which have an impact on the efficiencies

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associated with chemical cleaning operations. Often there are corrosion problems resulting from the use of high concentrations of the chemical agents; high temperature/high pressure operations are energy intensive; and reagent recovery can be costly.[2]

Thus, there remains a need for methods of coal desulfurization which improve upon the traditional processes. Most desirable would be a pre-combustion procedure which could remove at least 99% of the total sulfur in the coal matrix. The method must, however, be cost effective. In addition, the method must not add to the load on the environment indirectly, e.g. by requiring chemicals which are produced at some environmental expense, or which generate excessive heat or cause water contamination. It is anticipated that a bioprocessing method can be employed without exceeding the cost premium now placed on low sulfur coal.[3]

## 1.1.2  Bioprocessing

One means to circumvent the high cost of traditional coal cleaning processes lies in the use of biological methods that should result in much lower capital and operating costs. Indeed, microbial coal desulfurization is under some scrutiny as a viable alternative. Several excellent review articles cover the recent work in this area[4,5,6]. In addition, the publications resulting from annual conferences on the topic of biological coal processing are useful.[7,8] Early interests focused upon microorganisms for pyritic sulfur removal. These microorganisms were "naturally" suited to this task having themselves adapted to sulfur utilization on mine waters (e.g., as with Thiobacillus) or sulfur springs (e.g., as with Sulfolobus). Several more recent efforts have focused upon the adaptation and use of various strains of microorganisms to metabolize and remove the organic sulfur (e.g., those projects at ARCTECH, Inc., the Institute of Gas Technology, and the University of Georgia). These "coal bugs," are now quite well known, and have been shown to have "appetites" for organic sulfur.

Oxidation of sulfur for removal as sulfate has been the most commonly demonstrated mechanism for microbial removal of organic sulfate. While there have been encouraging demonstrations of the feasibility of organic sulfur removal via biological means, these projects by no means represent optimized solutions. Microbial efficiencies and process operations await improvements and these programs continue, for the most part, as research projects. Additional lessons in how microbes could be utilized for coal cleaning are desperately needed to promote further conceptual development. Questions to be considered are presented in Section 1.1.3.

## 1.1.3  Problems in Bioprocessing of Coal for Organic Sulfur Removal

While the studies of bioprocessing methods are very encouraging, many questions remain. Some of these questions are listed below:

- **Wetting of coal**

  Will the biological processing medium have a surface tension similar to that of coal, so that interaction between the bio-catalyst and the coal can occur?

- **Penetration of coal structure**

  Will the biocatalyst be able to access the full matrix of the coal, or will the coal have to be presolubilized or ground incredibly fine?

- **Selective removal of organic sulfur**

  Will the biocatalyst be able to remove sulfur without attacking other high energy bonds in the coal?

- **Cost**

  - How many dollars will bioprocessing cost per ton of coal?
  - What is the speed of the catalyst?
  - What is the lifetime of catalyst including recycling and regeneration capability?
  - Is there a low cost carbon source for bioproduction of the biocatalyst and what are the other factors in unit cost?
  - What is the fate of spent catalyst (dry, burn, dump)?
  - What is the cost of solvent removal/recycling?

- **Generality of treatment**

  - Will the same biocatalyst work on various coal types?

- **Isolation of released sulfur**

  - What will be the form of the released sulfur?
  - How will the released sulfur be isolated from the coal stream?

### 1.1.4  The Impact of This Development Program on Coal Bioprocessing

This program has made several important contributions to the development of coal non-microbial bioprocessing methods. These contributions are summarized below:

**Isolation of One New Organism From a Source Appropriate to the Expected Process**

While many organisms which metabolize coal components are already identified, the isolation of the strain GB-1 from soil at a deep sea thermal vent is considered

important. The organism grows in a high salt, high sulfur environment which could be relevant to a coal desulfurization process where organosulfur compounds and sulfate are present in high concentrations.

### Demonstration that Bioprocessing Need Not be Limited to Use of Whole Organisms

Most bioprocessing schemes have focused on the use of live microorganisms to breakdown (or build up) macromolecular structures. During this program it has been shown that biological catalysts isolated from organisms can operate on coal model compounds. This is important because in real life processing situations, organisms may not be viable.

### Demonstration that Coal Bioprocessing is Not Limited to Aqueous Media

It is known that enzymes (biocatalysts) can function in non-aqueous media. In fact some polymer synthesis schemes have been carried out in organic solvents with enzymes. In this program it was shown that thiophene compounds can be desulfurized by enzymes in organic media.

### Evaluation of Appropriate and Reliable Analytical Techniques

One of the more important aspects of this program was the effort made to develop analytical methods for evaluation of coal bioprocessing. This has not been a trivial matter, as hydrophobic and reactive intermediates and products, many of unknown structure, obscure easy monitoring.

Since these operations exploit live organisms, there is concern as to their maintenance as both viable and selective populations. While ample precedent exists for microbial-based treatment systems, e.g., waste water treatment, there are recognized sensitivities implicit in the use of live systems, such as mutational problems, reactor upsets, substrate variations, etc. A possible solution may lie in the use of enzyme-based cleaning processes, that is, processes which solely use the "active extracts" of the microbes to effect desulfurization. Preliminary studies of this concept are the subject of this report.

## 1.2    Program Outline

The program was an effort to develop clean coal technology based on the use of enzymes rather than live organisms for catalysis. Two enzyme sources were investigated: commercial suppliers and de novo isolation from a novel microbial source. The enzymes were to be utilized in hydrated organic solvents. The enzymes were to be tested first on model compounds and then on coal.

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The overall objective of this program was to investigate the feasibility of an enzymatic desulfurization process specifically intended for organic sulfur removal from coal. Toward that end, a series of specific objectives were defined: (Area 1) establish the feasibility of (bio)oxidative pretreatment followed by biochemical sulfate cleavage for representative sulfur-containing model compounds and coals using commercially-available enzymes; (Area 2) investigate the potential for the isolation and selective use of enzyme preparations from coal-utilizing microbial systems for desulfurization of sulfur-containing model compounds and coals; and (Area 3) develop a conceptual design and economic analysis of a process for enzymatic removal of organic sulfur from coal. Within the scope of this program, it was proposed to carry out a portion of each of these efforts concurrently.

In Area 1, it was proposed to establish the feasibility of the underlying concept for enzymatic desulfurization. That is, is it possible to manipulate enzymes to the extent that these biochemicals can catalyze chemical transformations in a more generic fashion that originally intended by nature? Klibanov's work[9] is now familiar science. The extension of the heretofore acknowledged "stagnant" properties of enzymes is encouraging for the concept of enzymatic processing. The work effort in Area 1 was to address the fundamental aspects of the concept.

It was proposed that both model compounds (dibenzothiophene for aromatic sulfur and ethyl phenyl sulfide for organic sulfide) and coals (e.g., Wyoming sub-bituminous and Illinois bituminous) be used in this Area 1 study. Enzyme studies on model compounds can help to delineate mechanistic feasibilities. Such a program is unlike microbial studies where acclimatization or genetic engineering using model compounds influences subsequent transfer to coal. Standard peroxidase-type enzymes were investigated for their pretreatment potential; and standard hydrolase-type enzymes were investigated for the potential to cleave the prepared organic sulfur.

In Area 2, it was proposed to consider the extraction of select enzyme consortia from coal-utilizing microorganisms for desulfurization. While most of the sulfur-utilizing microorganisms have been adapted to the use of inorganic sulfur, there are a few which have shown metabolic activities effecting the organic sulfur moieties in coal[10]. In these cases, it has been suggested that desulfurization, for the most part, is an extracellular enzymatic process. Obviously, then, acclimated microbial populations can serve as a rich source of candidate enzymes for desulfurization processes. In addition to those microbial populations specifically adapted for sulfur removal are those which have been shown to more generally utilize coal-type substrates as seen in some published demonstrations of microbial-induced liquefaction or gasification (e.g., Cohen's popular article[11]). These systems can also be viewed as acting via a consortium of enzymes each with selective bond-cleaving capabilities. Certainly in aerobic coal transformations, the chemistry is one of oxidation and hydrolysis, while in anaerobic systems, the spectrum of breakdown products implicates similar enzymatic attacks (although of a more complex interaction).

In Area 3, the effort was to consider the preliminary aspects of the bioreactor design. Given that an enzymatic desulfurization process is feasible, is it reasonable? While the Area 1 and Area 2 workscopes address, in a research sense, both the fundamental aspects of (bio)transformations of coal and the potential for selective use of the enzyme aspects of those transformations, the Area 3 workscope was to provide an engineering perspective of the process itself. Ample precedent exists to define reasonable bounds for enzymatic processes. However, the proposed process for enzymatic desulfurization of coal adds its own unique processing constraints for reasonableness. Coal is a complex solid substrate and a multi-enzyme system must be carefully conceived.

## 1.3 Rationale

### 1.3.1 Enzymes

This program was unique in looking at isolation of the biocatalyst from a growth situation. Rather than employing growing cells, the biocatalyst would be an enzyme solution. There are many potential advantages to this approach; these are discussed briefly below:

**Enzymes will not accumulate sulfur:**

Enzymes are catalysts, unchanged by reactants or reaction products. This is in contrast to microorganisms which liberate sulfur from substrate, often for their own internalization. In that latter instance, the sulfur would have been moved from one bound form to another.

**Enzymes will not add significant bulk in processing:**

Enzymes will be derived from whole organisms. They will be present in small amounts, relative to the amount of coal to be processed, and will not hinder the process of isolation of the released sulfate. While the enzyme fractions may be crude, the unwanted portion of the source organisms will be removed and either used for another purpose or discarded as fertilizer. Thus the amount of exogenous material added to the reactor would be relatively small.

**Enzymes will be selective in their activity:**

The question of catalyst specificity has not been sufficiently addressed in considering pre-combustion coal desulfurization. The energy of coal is held primarily in carbon-carbon bonds. The organic matrix also contains oxygen, sulfur, and nitrogen. When coal is used as a fuel, thermal energy is gained through the addition of oxygen to the organic molecules. Complete oxidation breaks all bonds to carbon, adding oxygens to produce carbon dioxide and inorganic oxides and acids such as $H_2SO_4$. The aim of pre-combustion desulfurization is to achieve selective oxidative cleavage of sulfur-

carbon bonds, releasing sulfuric acid. Ideally, this is accomplished without affecting the stability of the carbon-carbon bonds, thus preserving most of the energy content of the coal. A secondary issue is the disruption or "solubilization" of the coal matrix, deemed necessary by some to provide access of the catalyst to the sulfur sites. Little is known about the need for "solubilization" in coal treatment with various catalysts.

**Enzymes will be functional in a variety of media, including organic solvents:**

Microbes function in an aqueous environment. Enzymes can function in water, buffer or aqueous organic solvents. The opportunity to use organic solvents gives a much greater versatility in planning economical product recovery, waste disposal and transport.

### 1.3.2 Microbes

Numerous studies are underway to develop biological processes for the removal of both mineral and organic sulfur from coal. Various research groups are studying strains of bacteria and fungi which can be induced to utilize organic sulfur compounds as feedstocks. Others have been ingenious in selecting sources of microbes, scourering such sites as: coal piles; thermal springs in Yellowstone Park; and soils from around the world. In general, screening has been performed by looking either at growth on coal, oil, or on low molecular weight model compounds.

When considering biological processing methods, it is important to anticipate the limitations which may be caused by activity constraints on the biocatalysts. When likely operating conditions are known in advance, it is logical to seek natural environments as sources for biocatalysts which are similar to those prevailing in a coal processing reactor. In this program, the concept of coal bioprocessing implies a high sulfur, high salt environment. Thus, an area of deep sea thermal vents in the Gulf of California (Guaymas Basin) was chosen as source of potential biocatalysts.[12]

### 1.3.3 Utilization of Organic Solvents

The use of organic media for coal bioprocessing was considered for three reasons.

**Wetting of Hydrophobic Surfaces:**

Coal is not water-soluble and many constituents of coal, especially those containing organic sulfur, are particularly hydrophobic. It is not clear that aqueous media wet the coal surface sufficiently for biocatalytic activity. Organic solvents, on the other hand, do wet coal and may even have a "swelling" effect which would enhance sulfur removal.

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**Manipulation of Enzyme Activity:**

Pioneering work in the applications of organic solvents as media for enzyme reactions was published by Dr. Alexander Klibanov. The first lesson from his work was that enzymes, generally considered as water soluble and water dependent, can function in hydrated or non-hydrated organic solvents.[13] This concept was not simply of academic interest, since one drawback to the use of enzymes in many industrial processes has been the limited solubility of substrates in aqueous media. Additionally, Klibanov showed that enzyme specificity may be changed in such reaction media.[14]

Dr. Klibanov and his co-workers have made it clear that while the range of solvents which may be utilized is large, the number of solvents which may be effective for a specific task could be very limited. Klibanov and his co-workers have now elucidated the importance of the effect of the organic solvent on the enzyme-bound water. Many enzymes require a certain amount of water (essential water) for activity. If the essential water molecules are loosely bound to the enzyme surface, they may be stripped away by hydrophilic or water-miscible solvents, and this stripping can destroy catalytic activity. The extent to which such stripping may be a significant factor will vary depending on the charge distribution on the surface of the enzyme molecule as well as on the electrostatic interaction required for correct formation of an enzyme-(water)-substrate complex, and, further, the need to maintain a critical level of enzyme-bound water in the system.

Prior work with enzymes in organic solvents, targeting coal processing was carried out at Oak Ridge Laboratories by C.D. Scott and co-workers.[15,16] Additionally, work with horseradish peroxidase and DBT, showing molecular changes, had been performed at DynaGen Inc.[17]

**Separation of Hydrophilic Sulfur Products by Extraction:**

The processing schemes proposed have centered on the production of oxidized sulfur (e.g., sulfate). An ideal situation for product isolation would be liquid/liquid extraction of sulfur from organic media.

### 1.3.4 Use of Model Compounds

Model coal compounds are often used to simulate aspects of coal structure for several reasons: coal structures are complex; samples from different sites vary substantially; and methods of coal analysis are quite tedious. Because a large portion of the organic sulfur in higher ranked coals is believed to be thiophenic, this type of compound is often used as a

coal model. The model compounds used in this study are dibenzothiophene (DBT) and ethylphenylsulfide (EPS). The logical progression of sulfur oxidation in DBT, dubbed the "4S" pathway is shown in Figure 1.1.

The "4S" pathway is not simply theoretical. With the model compound dibenzothiophene (DBT) microbial pathways have been identified in which the sulfur atom is sequentially oxidized to sulfoxide, to sulfone, to sulfonate, and finally to sulfuric acid. The mechanisms of this "4S" pathway of DBT oxidation are not known, but it is curious to note that diphenyl products bearing either one or two hydroxyls have been observed. Interestingly, many microbial studies performed with DBT as a substrate have shown that the reaction most frequently observed in microbial oxidative pathways is one in which DBT is oxidized at ring carbons.[18]

Our experimental approach focused on the use of enzymes which catalyze the addition of oxygen to organic compounds. In tailoring the application of these enzymes to coal processing, it was of particular interest to ensure that oxidation occurs at sulfur and not at carbon-carbon bonds. In coal, there are many types of bonds which might be attacked. These might be generalized into four types of oxidation sites: carbon-carbon bonds; carbon-(oxygen or nitrogen) bonds; carbon-sulfur bonds; and sulfur-sulfur bonds. Clearly, the last two types of oxidation sites are the ones desired. The program focus was on maximizing these reactions while eliminating or at least minimizing the others.

In addition to the focus on types of chemical bonds in model compounds, it is also prudent to consider the solubility of the model compound and its oxidation intermediates; and also, if the model is insoluble, its porosity and surface characteristics. The ideal model would be a polymer which would not dissolve in the reaction medium, even if desulfurized, but which would be subsequently soluble in another solvent so that the sulfur states of treated material could be examined readily.

## 1.4    Organizational Changes During this Program

There were many changes in the management and organizational structure of the program. The proposal was written under Dynatech Scientific which was bought by Holometrix and subsequently became DynaGen. At DOE, the Program Manager changed from Mr. Eli George to Mr. David Boron in the fall of 1989. From the time the proposal was accepted by DOE in 1987, the originally proposed Principal Investigator was no longer with the company and Dr. Judith Marquis, of Boston University, was appointed to fulfill this function. Dr. David Odelson, and later Dr. Judith Kitchell, directed the work at DynaGen (Holometrix). In September of 1989, Dr. Marquis left Boston University and Dr. Kitchell was appointed Principal Investigator. Fortunately, good communication among principals maintained continuity during the program.
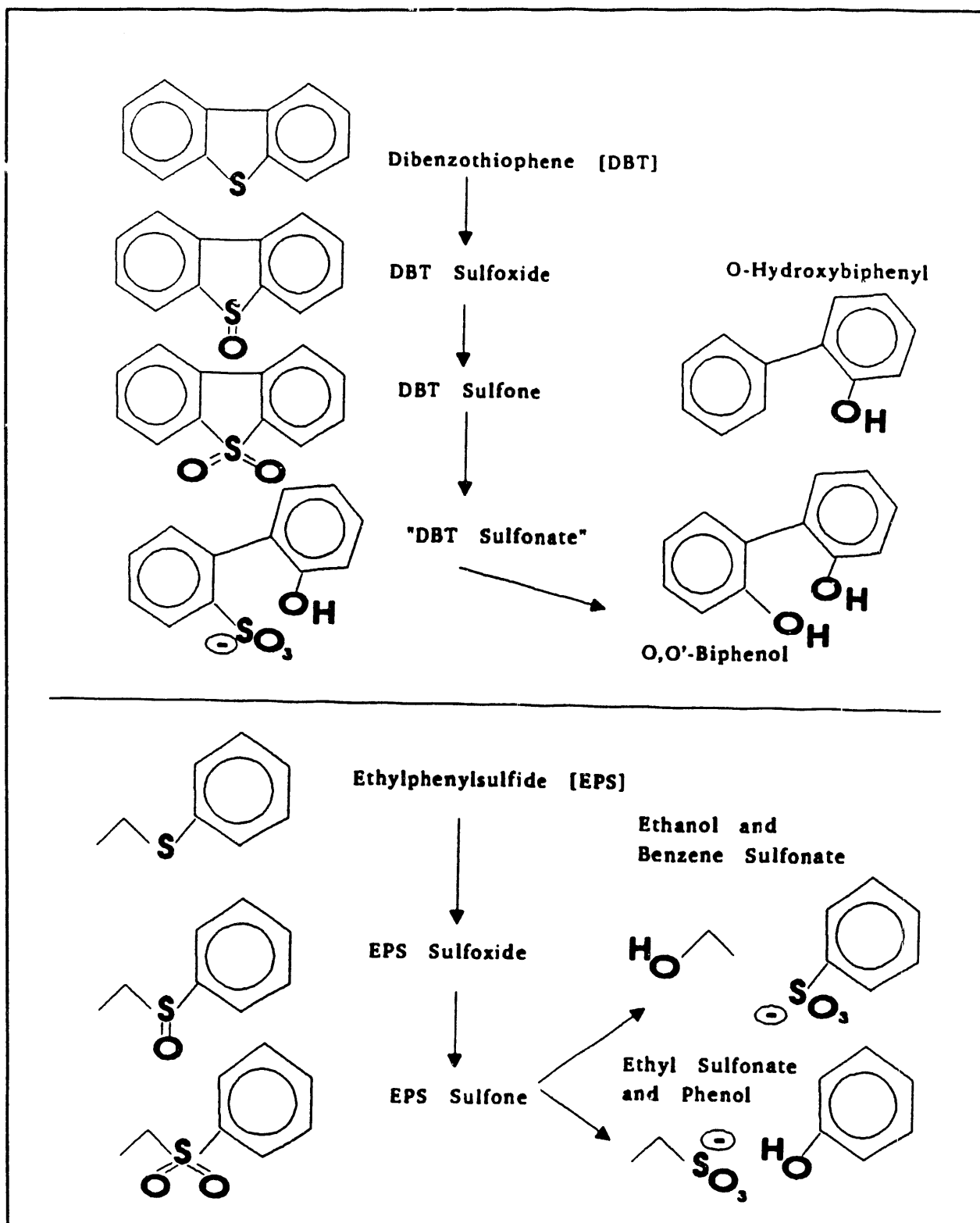
FIGURE 1.1: THE MODEL COMPOUNDS AND THEIR SULFUR OXIDATION
PRODUCTS

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## 2.0   WORK WITH MICROBES AND MICROBIAL ENZYMES

### 2.1   Summary of Work With Microorganisms

Under this program, a subcontract was awarded to Dr. Holgar Jannasch of the Woods Hole Oceanographic Institute, to screen water and sediments from deep sea sites for microorganisms which could oxidize the model compounds DBT and/or EPS. Two promising cultures from Jannasch's work, GB-1 and GB-2, survived in the presence of DBT and were selected for further study.

GB-1 and GB-2 were grown in artificial sea water medium containing DBT and supplemented with 0.05% yeast extract. Within 24 hours of incubation in the presence of DBT, the GB-1 culture medium turned red while the GB-2 culture remained colorless. GB-1 could grow without yeast extract and with DBT as sole carbon source and sole sulfur source. GB-2 did not grow without yeast extract supplementation or without another carbon source. Both cultures are aerobic and do not grow above 28°C. Improved growth was obtained with shaking.

When GB-1 was grown in media containing DBT, "4S" oxidation products were found, specifically DBT sulfoxide and DBT sulfone. When DBT sulfone was used as substrate, it was removed from solution, but no o,o'-biphenol or o-hydroxybiphenyl was observed in the medium. No oxidation products were obtained with DBT sulfoxide as substrate.

### 2.2   Isolation of Microorganisms

Approximately 1-2 g of sediment and water from the Guaymas Basin hydrothermal vents were used as inocula and added to flasks containing modified Lyman and Fleming artificial sea water medium (see Tables 2.1-A&B for composition). The medium was autoclaved after which 2.7 ml $L^{-1}$ of sterile potassium phosphate buffer (750 mM, pH 7.2) and 5 ml $L^{-1}$ of a filter sterilized vitamin solution (Table 2.1-B)[19,20] were added. The pH of the medium was adjusted to 7.2 with sterile 0.1 M NaOH or HCl. Sterile DBT or EPS was added to the medium at a concentration of 0.1% (w/v) as the sole carbon source. DBT was dissolved in acetone or ether and filter-sterilized; EPS was filter-sterilized directly (solvent resistant FP Vericel; porosity, 0.2 $\mu$m; Gelman Sciences, Inc., Ann Arbor, MI). The acetone or ether was evaporated under a stream of sterile nitrogen gas.

Previous results with hydrocarbon-utilizing bacteria isolated from oily Guaymas Basin sediments showed that most, if not all, of these bacteria are mesophilic (growth optima >20°C and <45°C)[16]. For this reason, all enrichment cultures were incubated at 25°C.

Enrichment cultures were checked daily for: (1) turbidity; (2) appearance of colored compounds; (3) change in the appearance of DBT and EPS; and (4) fluorescence

## TABLE 2.1-A: COMPOSITION OF MODIFIED LYMAN AND FLEMING ARTIFICIAL SEA WATER MEDIUM

| Material | Amount/liter | Comments |
|---|---|---|
| NaCl | 23.477 g | |
| $MgCl_2 \cdot 6H_2O$ | 4.981 g | changed from 10.635 |
| $Na_2SO_4$ | 3.917 g | |
| $CaCl_2 \cdot 2H_2O$ | 1.102 g | changed from 1.459 |
| KCl | 0.664 g | |
| $NaHCO_3$ | 0.192 g | |
| KBr | 0.096 g | |
| $H_3BO_3$ | 0.026 g | |
| $SrCl_2 \cdot 6H_2O$ | 0.026 g | changed from 0.040 |
| NaF | 0.0 g | eliminated |
| $NH_4Cl$ | 0.5 g | |
| $KHPO_4$ (buffer), 750 mM, pH 7.2(final concentration 2 mM $PO_4$) | 2.7 ml | |
| Wolfe's Mineral Solution | 5.0 ml | Composition given in Table 2.1-B |
| DAB's Vitamin Solution | 5.0 ml | Composition given in Table 2.1-B |
| Yeast Extract | 0.5 g | |

The pH of the medium was adjusted to ~7.2 aseptically with sterile NaOH or HCl after autoclaving.

# TABLE 2.1-B: COMPOSITION OF WOLFE'S MINERAL SOLUTION (MODIFIED)

**Wolfe's Mineral Solution (Modified)**

| Material | Amount (grams/liter) |
|---|---|
| Nitrilotriacetic Acid (NTA) | 1.5 |
| $MgSO_4 \cdot 7H_2O$ | 3.0 |
| $MnSO_4 \cdot H_2O$ | 0.5 |
| NaCl | 1.0 |
| $FeSO_4 \cdot 7H_2O$ | 0.1 |
| $CoSO_4$ or $CoCl_2$ | 0.1 |
| $CaCl_2 \cdot 2H_2O$ | 0.1 |
| $ZnSO_4$ | 0.1 |
| $CuSO_4 \cdot 5H_2O$ | 0.01 |
| $AlK(SO_4)_2$ | 0.01 |
| $H_3BO_3$ | 0.01 |
| $NaMoO_4$ | 0.40 |

Dissolve NTA with KOH in 500 ml of distilled $H_2O$. pH to 6.5, and add each ingredient in order. Bring to 1 liter after addition of all components.

**DAB's Vitamin Solution (added after autoclaving as sterile stock)**

| Material | Final Concentration in Media (mg/500 ml) | Final Concentration in Media($\mu g\ L^{-1}$) |
|---|---|---|
| Niacin | 5 | 50 |
| Biotin | 2 | 20 |
| Pantothenate | 5 | 50 |
| Lipic (Thioctic) Acid | 5 | 50 |
| Folic Acid | 2 | 20 |
| p-aminobenzoic Acid | 5 | 50 |
| Thiamine ($B_1$) | 5 | 50 |
| Riboflavin ($B_2$) | 5 | 50 |
| Pyridoxine ($B_6$) | 5 | 50 |
| Cobalamin ($B_{12}$) | 5 | 50 |

Filter, sterilize, and store refrigerated in the dark.

under short wave ultraviolet light. Cultures were considered positive if any of the above criteria were met.

Material from positive enrichment cultures was streaked onto similar medium solidified with 1.5% Purified Agar (Difco). Some agar plates also contained various amounts (0.01-0.05%, w/v) of Yeast Extract (Difco). DBT was dissolved in acetone and ether (2%, w/v) and sprayed onto the surface of the agar plates using a nebulizer. Approximately 1.0-1.5 ml was sprayed onto each agar plate. Once separated colonies were apparent, material from these colonies was streaked onto new plates. This was repeated at least three times to ensure purity.

The results were as follows: all enrichment cultures containing EPS (using inocula from all samples sites) were negative for turbidity (growth). In addition, there was never any change in the appearance of the EPS and microscopic examination showed no increase in bacterial numbers.

Although most enrichment cultures containing DBT that were inoculated with Guaymas Basin sediment were negative, one culture containing sediment from an oily core obtained from 1967 "bottom" was positive. The results are shown in Table 2.2. After two weeks of incubation, the culture fluid was red and some of the DBT had disappeared. The culture was slightly turbid and microscopic examination indicated large numbers of a motile, rod-shaped bacterium. There was no fluorescence under short wave ultraviolet light. A sample of red culture fluid was filtered and analyzed spectrophotometrically. The pigment had a visible absorbance maximum at about 392 nm.

The enrichment procedure was repeated using more sediment from the same source and material from the original positive enrichment was streaked onto DBT agar plates as described earlier. As in the first enrichment culture, culture fluid from the second also became reddish and slightly turbid after two weeks. Microscopic examination of the culture showed organisms of similar morphology to those observed in the first enrichment culture. This result suggested that these organisms were distributed throughout the sediment of the sample. Red areas became apparent on the DBT-containing agar plates (with and without yeast extract) in about 11-12 days. However, single colonies grew much more quickly when yeast extract was included. Several colonies were restreaked onto DBT agar plates containing 0.05% (w/v) yeast extract.

In two weeks, colonies formed with reddish zones surrounding them. This red pigment eventually diffused throughout the medium indicating that the pigment was water soluble. In addition, sprayed water-insoluble DBT particles disappeared around the colonies. This and the fact that no reddish color appeared on control agar plates lacking DBT indicated that the red material resulted from a bacterial transformation of DBT. This restreaking was repeated once more.

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## TABLE 2.2: PRELIMINARY GROWTH DATA FOR EIGHT WEEK ENRICHMENT CULTURES - GROWTH EXCLUSIVELY ON ORGANIC SULFUR FEEDSTOCK

| | Sample Designation | DBT | EPS |
|---|---|---|---|
| 1. | 1161 | - | - |
| 2. | 1615 | - | - |
| 3. | 1966 y-o Mat | - | - |
| 4. | 1966 O Mat | - | - |
| 5. | 1967 (Top) | - | - |
| 6. | 1967 (Middle) | - | - |
| 7. | 1967 (Bottom) | * | - |
| 8. | 1971 Inj. Core** | - | - |

In this table, "-" indicates no growth.

\* This enrichment culture turned red after about six weeks. Two weeks later it started turning yellow. It did not fluoresce under wave UV light. Microscopic examination showed viable cells but not a number large enough to indicate an enrichment.

** Inj. Core means that DBT was introduced into the mud in-situ at the Guaymas Basin, thus the incubation period was very long.

Once returned to liquid culture, the strain also produced red pigment from DBT which occasionally precipitated out of the growth medium late in the growth cycle. Several strains were purified and kept. These were designated GB1DBTa, GB1DBTb, GB1DBTc, and GB1DBTd. The organism was rod-shaped, motile, and stained variably with the Gram stain. The KOH test for the determination of the Gram reaction[21] was also equivocable. In all subsequent work only GB1DBTa was used and it was referred to simply as "GB-1".

Smaller colonies which did have clear zones on DBT agar plates and did not produce any pigment were also restreaked to obtain a pure culture. Two strains were obtained and designated GB2DBTa and GB2DBTb. The organism was vibrioid-to-helical in morphology, motile, and Gram-negative. There was no indication that this organism could transform DBT in any way. These two strains were used interchangeably in subsequent work and referred to as "GB-2".

## 2.3    Studies on Microbial Growth and Activity

### Growth of GB-1 and GB-2 Cultures in Liquid Medium

Although it had been established that GB-1 grows in medium with DBT as the sole sulfur source, in those experiments, DBT was the sole carbon source too. In order to see the effect of an additional carbon source, GB-1 was incubated in sulfur-free medium with 0.01% DBT as sole sulfur source and 0.2% sodium succinate as carbon source; and as a control, with 0.01% DBT as carbon and sulfur source. A control of medium with DBT without cells was also included. As noted previously, GB-1 did grow on DBT as sole sulfur source; however, no DBT sulfur-oxidation products were detected in the medium when the cells were grown under such conditions. Supplementation of the sulfur-free medium with succinate did seem to increase growth, but again, no DBT sulfur oxidation products were observed. Addition of 50 $\mu$m $FeSO_4$ to the medium did not improve DBT degradation by GB-1. In artificial sea water medium with yeast extract and DBT, DBT sulfoxide and DBT sulfone were produced by GB-1 from DBT. No spontaneous oxidation of DBT occurred in uninoculated flasks.

GB-2, on the other hand, could not grow with DBT as the sole source of carbon and sulfur. Yeast extract supplementation was required for growth. No DBT oxidation products were detected even with yeast extract or an alternate carbon source present in the medium. Further research was carried out only with the GB-1 culture.

### Preliminary Examination of DBT-Degradation Products Extracted from Growth Media

Cells were grown in artificial sea water medium with DBT and yeast extract. A 1% GB-1 inoculum was used. Preliminary experiments involved extraction of growth media at various times with methylene chloride (with and without acidification prior to extraction). Concentrated extracts were examined for their UV absorption spectra, and it was found that

the spectra were obscured by the media components and the multiplicity of DBT-derived products (Figures 2.1A-C). The absorption maximum of the red-colored compound was at 528 nm (Figure 2.1C). The red-colored compound(s) seem to be cell-bound/cell-associated since by centrifuging the culture and extracting the supernatant, the red compound(s) was lost in the cell pellet. The red color was found not to be pH dependent. Red DBT derived compounds produced by other organisms was previously reported by Kodama.[22]

## Examination of Culture Medium Extract by TLC

Thin-layer chromatography (TLC) was employed for the identification of DBT and its sulfur oxidation products in the media extracts from culture. Cultures grown on DBT (0.01% and 0.05% DBT concentrations were tested) were acidified to pH 2.1 to 2.3 and extracted in an equal volume of methylene chloride. The methylene chloride extract was evaporated to dryness and the material was resuspended in a small volume of methylene chloride. Ten microliters of this was then spotted for each plate.

Three different systems were used:

1. alumina plates (Eastman Kodak 13252) with fluorescence indicator (No. 6063) were used with ethanol:water: ammonium hydroxide (2:15:1) as eluent[23];

2. silica gel plates -60F$_{254}$ (Merck No. 5628) with chloroform:acetone (80:20) as eluent [24]; and

3. silica gel plates -60F$_{254}$ (Merck No. 5628) with benzene:methanol (97:3) as eluent[24].

With System 1, of the four "4S" pathway products, only o,o'-biphenol fluoresced (with a purple fluorescence) in the standard (at an $R_f$ of 0.61). When GB-1 was grown in DBT-medium supplemented with yeast extract and extracted, concentrated, and observed by this system, a compound that fluoresced purple was observed (Figure 2.2 A&B). The $R_f$ of this compound was different ($R_f$ = 0.39), however, from that of o,o'-biphenol (Figure 2.2-B). When two-dimensional TLCs of this sample and biphenol werecompared the $R_f$ values were very close ($R_f$ = 0.26 for biphenol versus 0.27 for the unknown). When the sample was spiked with biphenol, however, it was clear that the fluorescing compound in the sample was different from o,o'-biphenol.
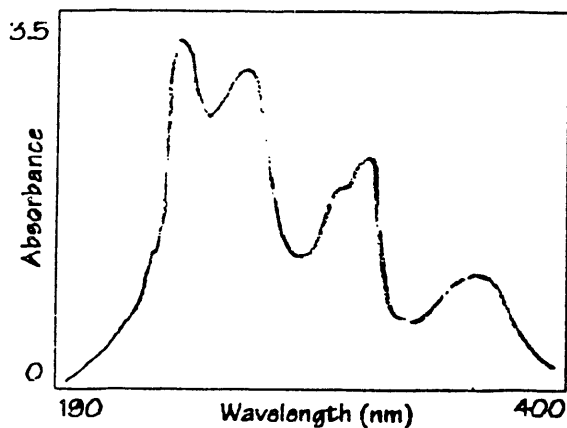
2.1-A - UV absorbance spectrum of 48 hr old GB-1 extracellular fraction, acidified, extracted in methylene chloride, and concentrated 15-fold
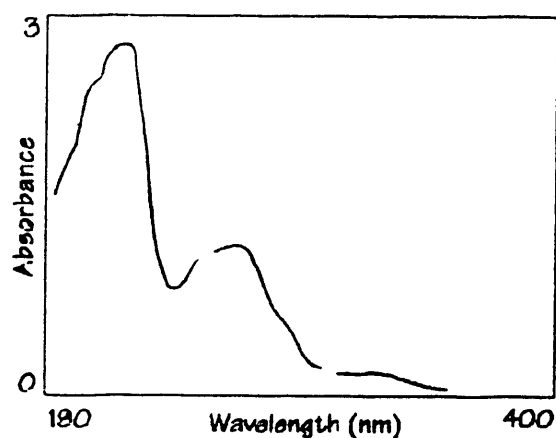
2.1-B - UV absorbance spectrum of 0.05% yeast extract, acidified, extracted in methylene chloride, and concentrated 15-fold
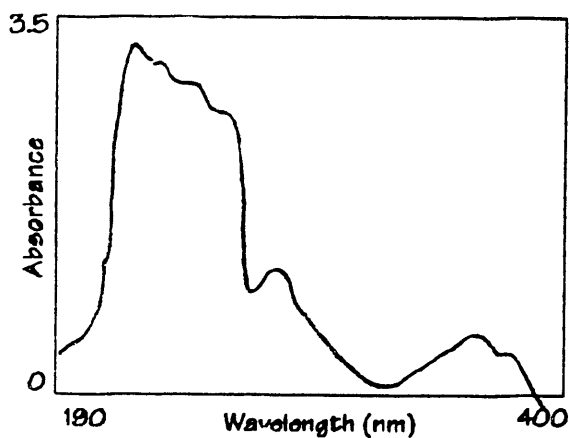
2.1-C - UV absorbance spectrum of 60 hr old GB-1 extracellular fraction, incubated with DBT, acidified, extracted in an equal volume of methylene chloride (no concentration)

FIGURE 2.1-A,B &C: UV ABSORBANCES OF GB-1 ECF EXTRACTS

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(Note: The plate is Kodak alumina 13252 with fluorescent indicator. The eluent is ethanol:water:ammonium hydroxide 2:15:1.)

Sample 1: GB-1 was grown on 0.05% DBT and harvested at 65 hours. After acidification to pH 2.2, the media was extracted with an equal volume of methylene chloride (100 ml). The solvent was reduced to 3 ml before analysis.

Sample 2: GB-1 was grown on 0.01% DBT. The harvesting and extraction conditions are identical to Sample 1.

The spotting order, from right to left was: 1) Sample 2 + biphenol; 2) Sample 1 + biphenol; 3) sample 2; 4) biphenol; 5) Sample 1; 6) DBT; and 7) DBT sulfoxide.
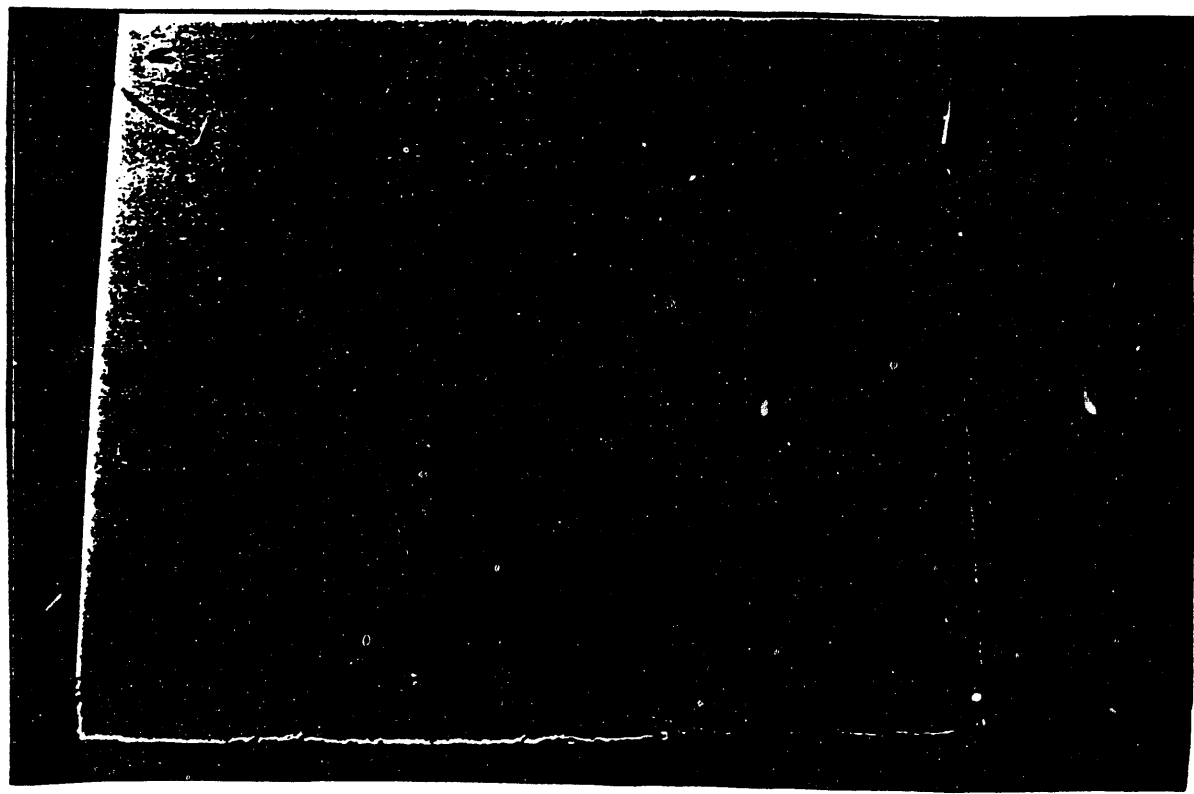


FIGURE 2.2-A: PHOTOGRAPH OF A TLC PLATE ANALYZING GB-1 GROWTH MEDIA - The plate in visible light

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(Note: The plate is Kodak alumina 13252 with fluorescent indicator. The eluent is ethanol:water:ammonium hydroxide 2:15:1.)

Sample 1: GB-1 was grown on 0.05% DBT and harvested at 65 hours. After acidification to pH 2.2, the media was extracted with an equal volume of methylene chloride (100 ml). The solvent was reduced to 3 ml before analysis.

Sample 2: GB-1 was grown on 0.01% DBT. The harvesting and extraction conditions are identical to Sample 1.

The spotting order, from right to left was: 1) Sample 2 + biphenol; 2) Sample 1 + biphenol; 3) sample 2; 4) biphenol; 5) Sample 1; 6) DBT; and 7) DBT sulfoxide.
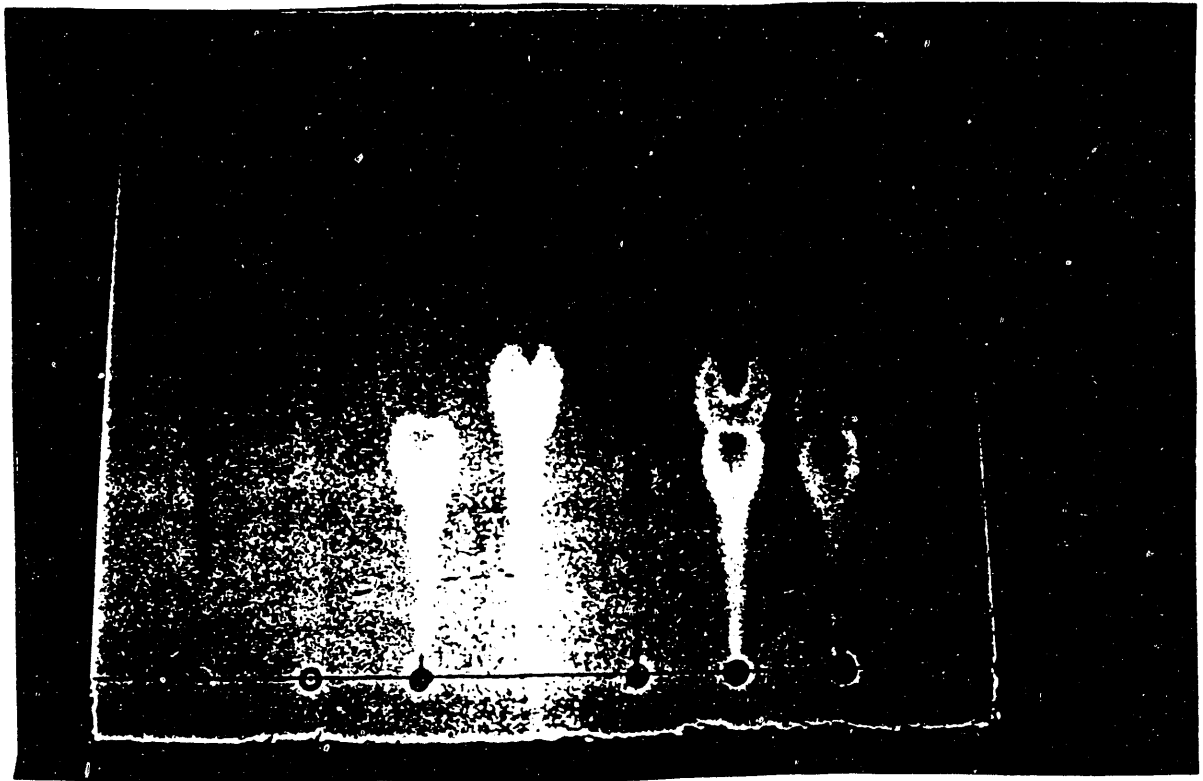


FIGURE 2.2-B: PHOTOGRAPH OF A TLC PLATE ANALYZING GB-1 GROWTH MEDIA - The plate in UV light

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On silica gel plates with chloroform:acetone (80:20) as eluent (System 2), all four "4S" pathway oxidation products were visible under UV light. However, DBT sulfoxide and o,o'-biphenol had very similar $R_f$ values by this method (see Table 2.3). In this system, the GB-1

TABLE 2.3: $R_f$ VALUES OF DBT AND ITS SULFUR OXIDATION PRODUCTS BY TLC

| Compound | System 1* | System 2* | System 3* |
|----------|-----------|-----------|-----------|
| DBT | | 0.87 | 0.78 |
| DBT Sulfoxide | | 0.63 | 0.12 |
| DBT Sulfone | | 0.79 | 0.56 |
| o,o'-biphenol | 0.61 | 0.64 | 0.22 |

* Refer to the text for eluents used in the three systems.

culture medium extract discussed in the previous paragraph had a fluorescent spot at an $R_f$ similar to that of the DBT sulfoxide/biphenol. Other compounds in the mixture were observable with visible light. No compounds were observed with GB-2 cultures on TLC using either of these systems.

On silica gel plates with benzene:methanol (97:3) as eluent (System 3), better separation was observed between DBT sulfoxide and o,o'-biphenol in the standard (Table 2.3). It appeared that GB-1 growing on DBT produced compounds of interest and a more quantitative analytical method was required.

### Examination of Culture Medium Extracts by HPLC

To increase sensitivity and accuracy, HPLC analysis of media extracts was employed. The HPLC conditions were a modification of the method of Wyza[25] (described in Section 5.4).

For analysis of samples by HPLC, GB-1 was grown with shaking in 100 ml volumes in medium containing 0.05% and 0.01% DBT. For each time point, a whole bottle of cells and medium was harvested. The bottle contents were acidified to pH 2.1 to 2.3 and then extracted with an equal volume of methylene chloride. The methylene chloride was evaporated to dryness and the residual material was resuspended in 2 to 3 ml of methylene chloride.

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Figures 2.3-A and 2.3-B present HPLC chromatograms of "4S" standards. Unfortunately, although good resolution of the "4S" oxidation products was obtained with this system, extraneous peaks were observed in the vicinity of the DBT peak even with injection of solvent alone.

As seen in Figures 2.4 and 2.5, GB-1 grown in 0.05% DBT produces DBT sulfoxide and some DBT sulfone. The apparent quantities of these compounds vary as a function of time of incubation. Figures 2.6 and 2.7 show similar chromatograms from a sample derived from a culture grown with 0.01% DBT. From Figures 2.6 and 2.7, it is possible to see the decrease in DBT concentration as a function of time of incubation.

Figure 2.8 shows a chromatogram of GB-2 which was grown in the medium of the same composition with 0.05% DBT. The peaks obtained with GB-1 extracts are not observed in GB-2 extracts, indicating that they are indeed DBT oxidation products and not medium artifacts.

## Separation of Components of Culture Medium Extracts by Column Chromatography

GB-1 was grown on 0.01% DBT for 7 days in 100 ml of artificial sea water medium with 0.05% yeast extract. The culture medium was then acidified and extracted in an equal volume of methylene chloride. The methylene chloride was evaporated and the extract was redissolved in 2 ml of methylene chloride.

To separate the products of microbial degradation of DBT in the culture extract, a silica gel column (1 cm x 20 cm) was prepared from a slurry of 6.65 g of silica gel G-60 (90-230 mesh ASTM) in 50 ml of methylene chloride. After the column had equilibrated, 1 ml of the concentrated culture extract was applied, and about 40 ml of methylene chloride was passed through the column and approximately 1 ml fractions were collected. After elution with methylene chloride, about 25 ml of ethanol was passed through the column and collected. The fractions were analyzed by TLC first using chloroform:acetone [80:20] as the eluent and then some of the fractions were analyzed using hexane:methylene chloride [50:50] as the eluent[21].

Fractions which appeared similar by TLC were pooled and further analyzed by HPLC. The early methylene chloride fractions (Group A) from the silica gel column contained DBT (retention time of 18.46 minutes on HPLC), while several following fractions (Group B) contained a pink compound that appeared on the HPLC chromatogram at a retention time of 9.21 minutes. These were followed by fractions
which contained a compound with a retention time (22.38 minutes) slightly longer than that of DBT (Group C). When the same silica gel column was further eluted (with 5 ml of ethanol), a red colored fraction was collected which exhibited several bands on TLC, including one corresponding to that of DBT sulfoxide. This fraction also contained several

Note: HPLC Method A (Section 5.4)
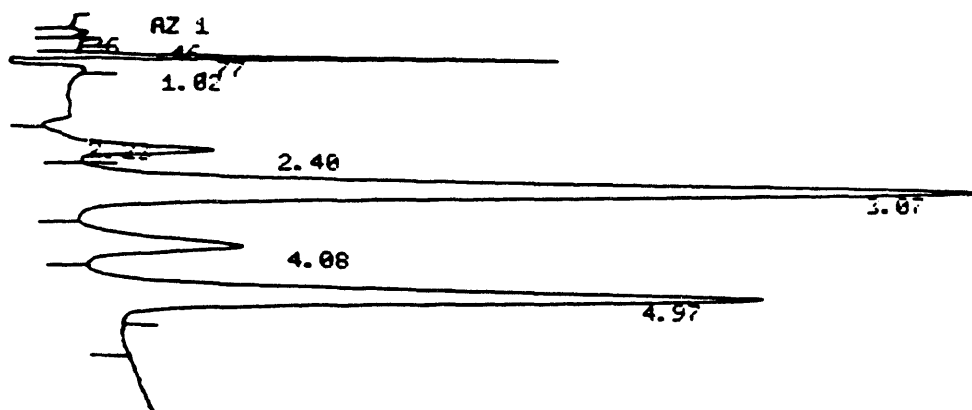


Standards of DBT "4S" oxidation products in methylene chloride. Methylene chloride, DBT sulfoxide, biphenol, and DBT sulfone elute at 2.4, 3.07, 4.08, and 4.97 minutes respectively.

Refer to Section 5.4 for description of method (A).

FIGURE 2.3-A: HPLC CHROMATOGRAM OF A STANDARD CONTAINING DBT SULFOXIDE, DBT SULFONE, AND O,O'-DIPHENOL IN METHYLENE CHLORIDE

Peaks labeled on chromatogram:
- Methylene Chloride
- Biphenol
- DBT-Sulfone
- DBT

| PEAK# | AREA% | RT | AREA BC |
|---|---|---|---|
| 1 | 0.232 | 0.28 | 8135 02 |
| 2 | 1.27 | 0.42 | 44585 03 |
| 3 | 1.765 | 0.77 | 61957 02 |
| 4 | 1.484 | 0.94 | 52089 03 |
| 5 | 1.16 | 2.34 | 40708 01 |
| 6 | 2.266 | 3.95 | 79531 02 |
| 7 | 5.191 | 4.86 | 182203 03 |
| 8 | 5.39 | 11.47 | 189212 02 |
| 9 | 8.928 | 12.3 | 313405 02 |
| 10 | 21.224 | 13.87 | 745024 02 |
| 11 | 13.761 | 15.6 | 483060 02 |
| 12 | 37.33 | 17.9 | 1310382 03 |
| TOTAL | 100. | | 3510291 |
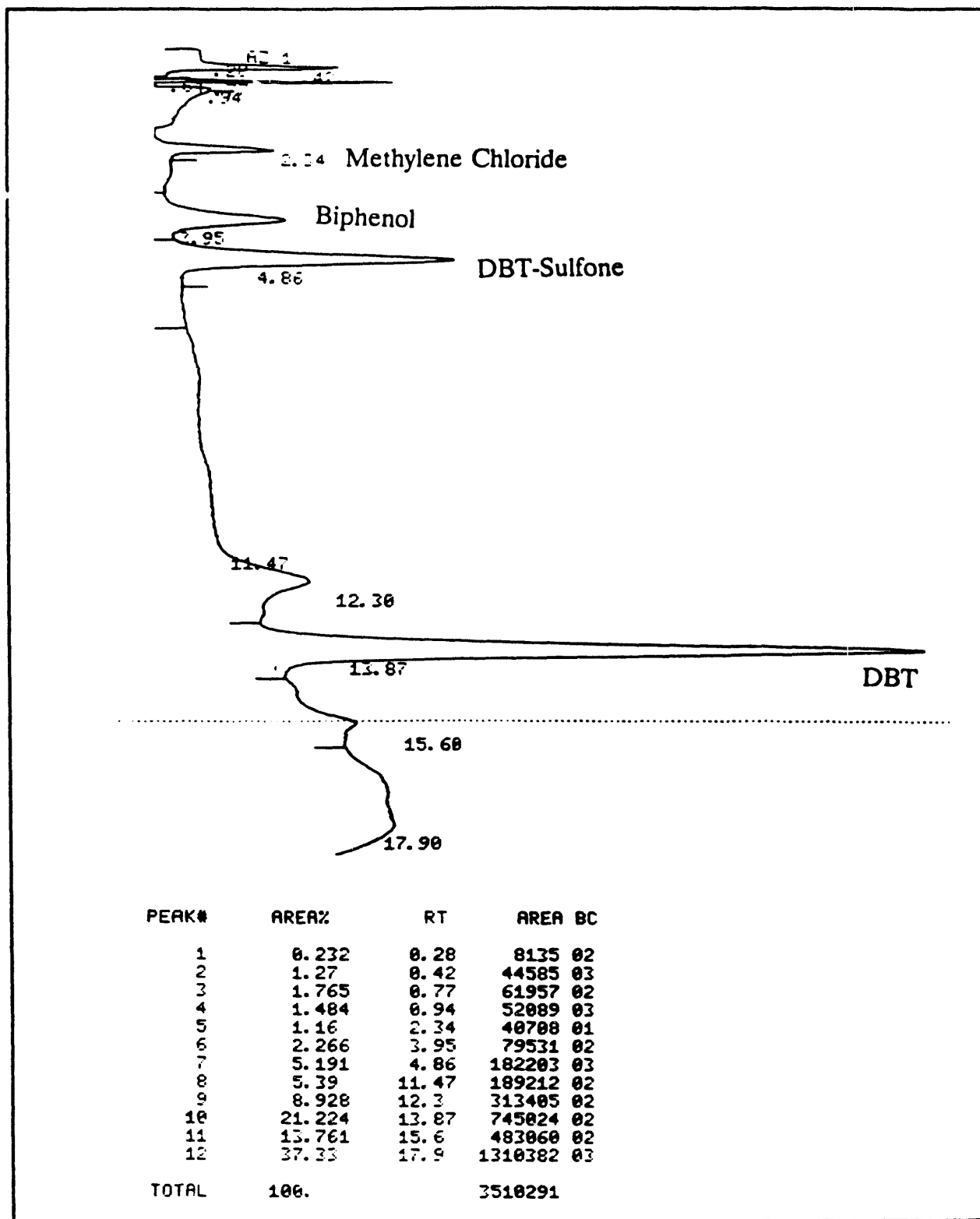
FIGURE 2.3-B: HPLC CHROMATOGRAM OF A STANDARD CONTAINING DBT,
DBT SULFONE AND O,O'-BIPHENOL IN METHYLENE CHLORIDE

24
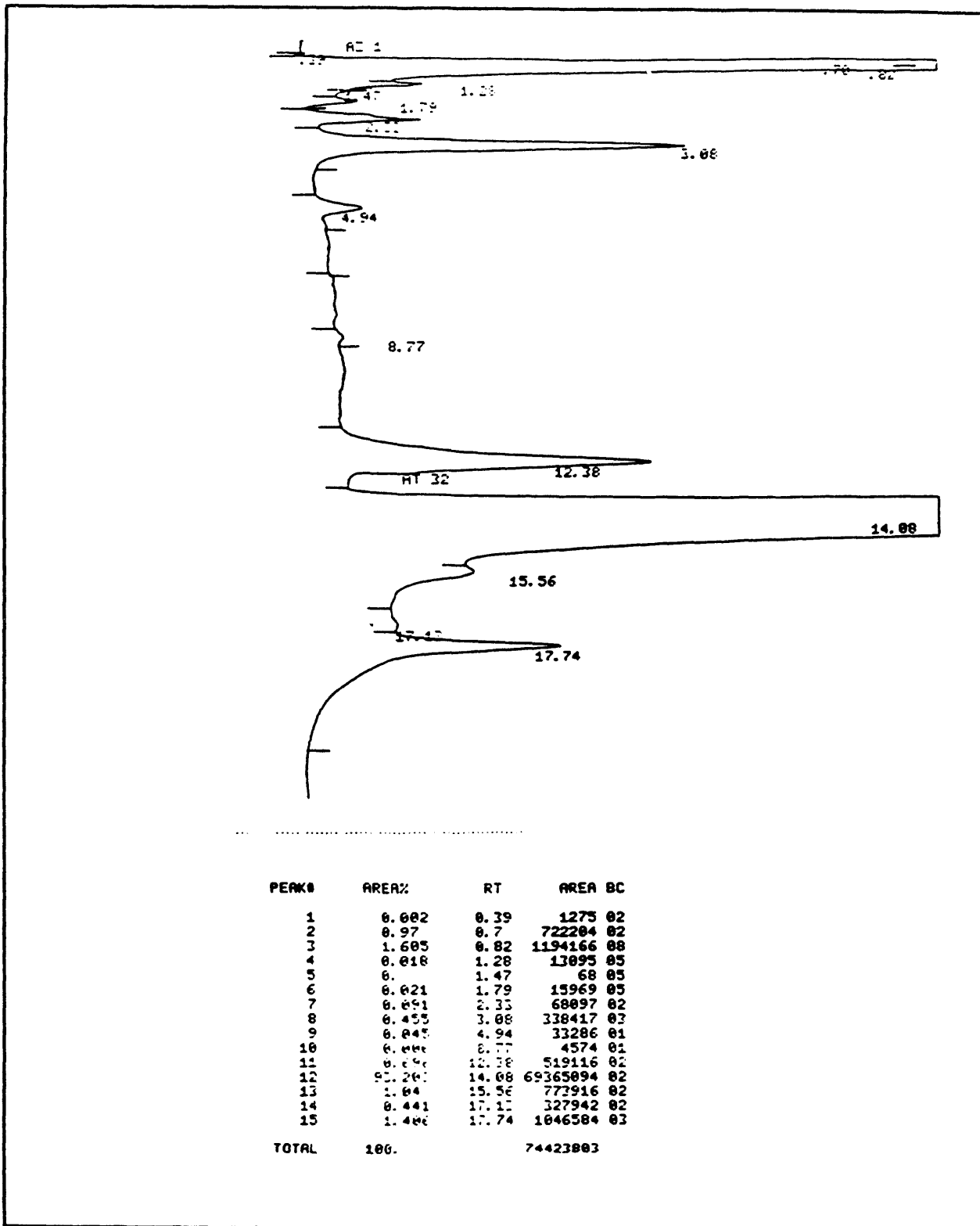
FIGURE 2.4: HPLC CHROMATOGRAM OF GB-1 GROWTH MEDIUM WITH 0.05%
DBT HARVESTED AT 65 HOURS

| PEAK# | AREA% | RT | AREA | BC |
|-------|-------|------|----------|----|
| 1 | 0.002 | 0.39 | 1275 | 02 |
| 2 | 0.97 | 0.7 | 722204 | 02 |
| 3 | 1.605 | 0.82 | 1194166 | 08 |
| 4 | 0.018 | 1.28 | 13095 | 05 |
| 5 | 0. | 1.47 | 68 | 05 |
| 6 | 0.021 | 1.79 | 15969 | 05 |
| 7 | 0.091 | 2.33 | 68097 | 02 |
| 8 | 0.455 | 3.08 | 338417 | 03 |
| 9 | 0.045 | 4.94 | 33286 | 01 |
| 10 | 0.006 | 8.77 | 4574 | 01 |
| 11 | 0.696 | 12.38 | 519116 | 02 |
| 12 | 93.267 | 14.08 | 69365094 | 02 |
| 13 | 1.04 | 15.56 | 773916 | 02 |
| 14 | 0.441 | 17.12 | 327942 | 02 |
| 15 | 1.406 | 17.74 | 1046584 | 03 |
| TOTAL | 100. | | 74423803 | |

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Peaks labeled on chromatogram: AZ 1, 1.12, 1.65, 2.17, 4.68, 5.28, 6.44, 7.10, 9.66, 12.30, 13.98, 15.95, 17.74

| PEAK# | AREA% | RT | AREA BC |
|---|---|---|---|
| 1 | 0.144 | 0.44 | 137958 02 |
| 2 | 0.521 | 0.63 | 500966 02 |
| 3 | 0.148 | 0.92 | 141992 02 |
| 4 | 0.369 | 1.12 | 354947 02 |
| 5 | 0.114 | 1.44 | 109824 02 |
| 6 | 0.166 | 1.65 | 159479 02 |
| 7 | 0.258 | 1.97 | 248003 02 |
| 8 | 0.326 | 2.17 | 312820 02 |
| 9 | 1.138 | 3.08 | 1093210 03 |
| 10 | 0.098 | 4.68 | 94585 02 |
| 11 | 0.165 | 5.28 | 158565 02 |
| 12 | 0.357 | 6.44 | 343257 02 |
| 13 | 0.071 | 7.1 | 68345 03 |
| 14 | 0.003 | 9.66 | 2604 01 |
| 15 | 0.836 | 12.3 | 803583 02 |
| 16 | 89.958 | 13.98 | 86427400 02 |
| 17 | 4.029 | 15.95 | 3871029 02 |
| 18 | 1.298 | 17.74 | 1246986 03 |
| TOTAL | 100. | | 96075553 |

FIGURE 2.5: HPLC CHROMATOGRAM OF GB-1 GROWTH MEDIUM WITH 0.05% DBT HARVESTED AT 113 HOURS
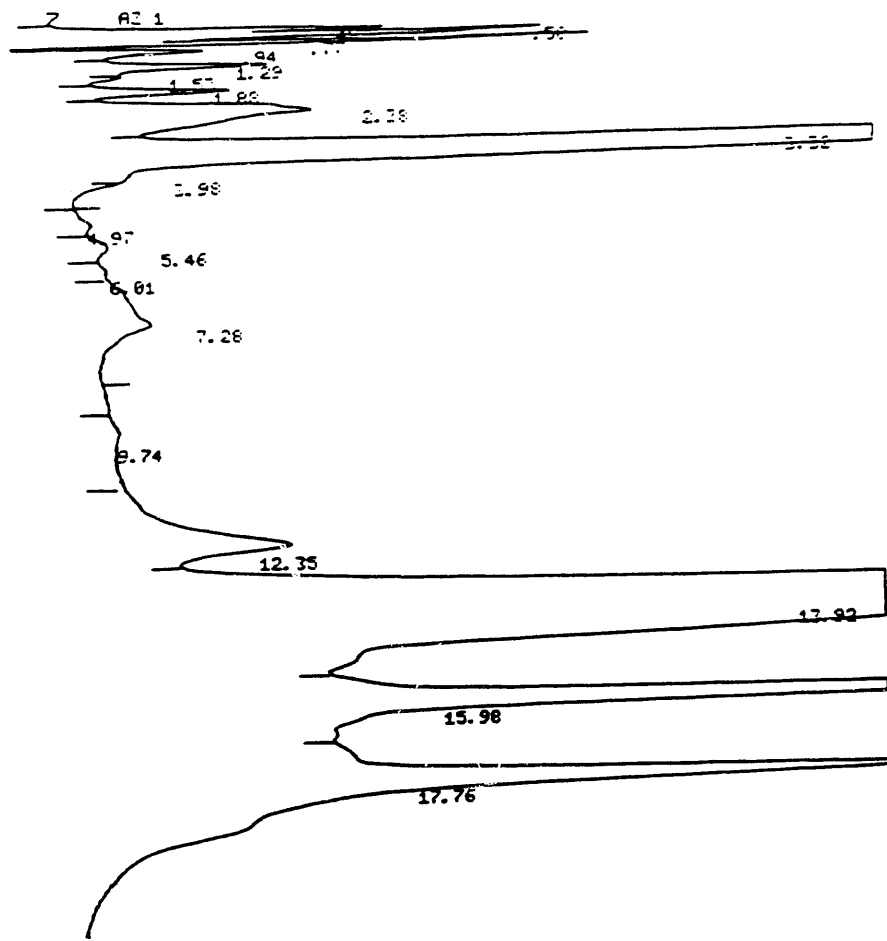
| PEAK# | AREA% | RT | AREA BC | |
|---|---|---|---|---|
| 1 | 0.2 | 0.48 | 91329 | 02 |
| 2 | 0.383 | 0.58 | 174615 | 02 |
| 3 | 0.256 | 0.77 | 116784 | 02 |
| 4 | 0.329 | 0.94 | 149954 | 02 |
| 5 | 0.497 | 1.29 | 226286 | 02 |
| 6 | 0.216 | 1.53 | 98436 | 02 |
| 7 | 0.393 | 1.88 | 179247 | 02 |
| 8 | 1.076 | 2.38 | 490402 | 02 |
| 9 | 5.319 | 3.32 | 2423327 | 02 |
| 10 | 0.082 | 3.98 | 37407 | 03 |
| 11 | 0.025 | 4.97 | 11202 | 02 |
| 12 | 0.06 | 5.46 | 27338 | 02 |
| 13 | 0.044 | 6.01 | 20045 | 02 |
| 14 | 0.293 | 7.28 | 133694 | 03 |
| 15 | 0.08 | 9.74 | 36329 | 02 |
| 16 | 0.743 | 12.35 | 338690 | 02 |
| 17 | 82.64 | 13.92 | 37652043 | 02 |
| 18 | 3.677 | 15.98 | 1675285 | 02 |
| 19 | 3.685 | 17.76 | 1678988 | 03 |
| TOTAL | 100. | | 45561401 | |

FIGURE 2.6: HPLC CHROMATOGRAM OF GB-1 GROWTH MEDIUM WITH 0.01% DBT HARVESTED AT 113 HOURS

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| PEAK# | AREA% | RT | AREA | BC |
|---|---|---|---|---|
| 1 | 0.429 | 0.51 | 96216 | 02 |
| 2 | 0.924 | 0.59 | 207141 | 02 |
| 3 | 0.428 | 0.77 | 95880 | 02 |
| 4 | 0.714 | 0.94 | 160109 | 02 |
| 5 | 1.317 | 1.24 | 295210 | 02 |
| 6 | 0.829 | 1.83 | 185819 | 02 |
| 7 | 0.497 | 2.17 | 111476 | 02 |
| 8 | 1.168 | 2.35 | 261868 | 02 |
| 9 | 5.137 | 3.28 | 1151416 | 03 |
| 10 | 0.017 | 4.98 | 3826 | 02 |
| 11 | 0.071 | 5.48 | 15948 | 03 |
| 12 | 0.195 | 7.04 | 43616 | 02 |
| 13 | 0.274 | 7.26 | 61320 | 02 |
| 14 | 0.021 | 7.74 | 4773 | 03 |
| 15 | 0.157 | 10.72 | 35210 | 02 |
| 16 | 0.501 | 11.44 | 112353 | 02 |
| 17 | 1.356 | 12.35 | 304005 | 02 |
| 18 | 62.062 | 13.92 | 13911057 | 02 |
| 19 | 2.837 | 14.54 | 635932 | 02 |
| 20 | 0.829 | 15.07 | 185731 | 02 |

FIGURE 2.7: HPLC CHROMATOGRAM OF GB-1 GROWTH MEDIUM IN 0.01% DBT HARVESTED AT 161 HOURS

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| PEAK# | AREA% | RT | AREA | BC |
|---|---|---|---|---|
| 1 | 0.027 | 0.47 | 54209 | 02 |
| 2 | 0.017 | 0.59 | 34722 | 02 |
| 3 | 0.035 | 0.76 | 70058 | 02 |
| 4 | 0.047 | 0.92 | 95446 | 02 |
| 5 | 0.19 | 1.35 | 381777 | 02 |
| 6 | 0.07 | 1.84 | 140557 | 02 |
| 7 | 0.063 | 2.35 | 126389 | 02 |
| 8 | 0.044 | 2.64 | 88768 | 02 |
| 9 | 0.074 | 3.17 | 149516 | 03 |
| 10 | 0.015 | 6.46 | 29621 | 02 |
| 11 | 0.151 | 9.76 | 304831 | 02 |
| 12 | 1.193 | 12.67 | 2403410 | 02 |
| 13 | 98.074 | 14.51 | 197559675 | 03 |
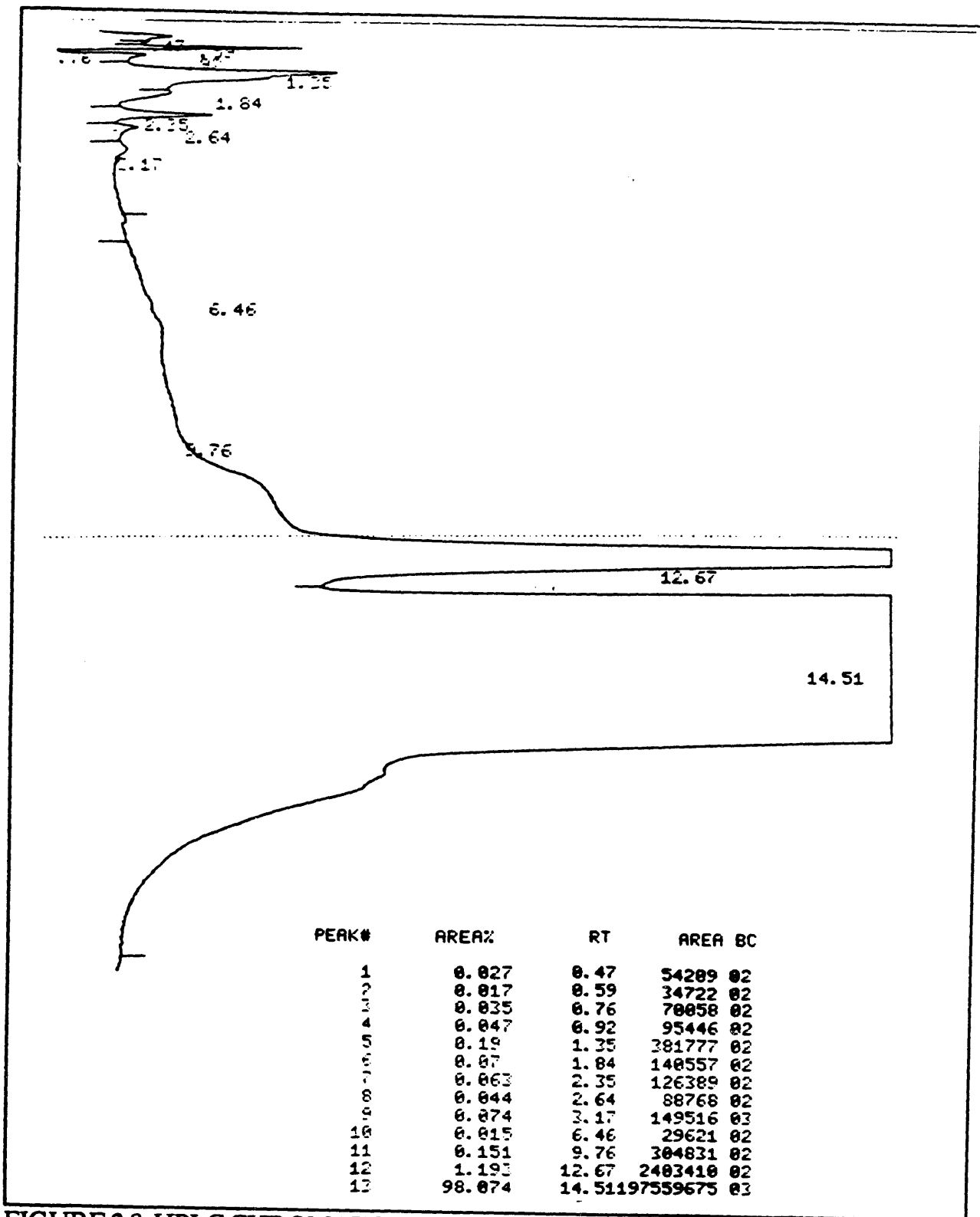
FIGURE 2.8: HPLC CHROMATOGRAM OF GB-2 GROWTH MEDIUM WITH 0.05%
DBT HARVESTED AT 120 HOURS

other components that were not identified. The next two ethanol fractions contained a compound that gave a blue color with Gibbs' reagent, indicative of the presence of a phenolic compound.

The fraction from Group B (containing the compound of a retention time of 9.21 minutes by HPLC) was also analyzed by GC-mass spectroscopy. The two analyses are shown in Figure 2.9. As can be seen in this instance, far greater information was obtained from the HPLC chromatogram than from the GC-MS. It is clear that by the HPLC, one can see several peaks (including one for DBT at retention time of 18.46 minutes, one for DBT sulfoxide at a retention time of 1.73 minutes, and that for an unknown at retention time of 9.21 minutes). However, the GC-MS revealed only the DBT peak and was not useful in establishing an identity for the unknown compound. The further use of GC-MS is discussed in Section 5.

## 2.4   Discussion of Microbial Work

The coal desulfurization process will ultimately result in the formation of high levels of sulfates in solution. It was, therefore, logical to seek enzymes which would function efficiently in such a high salt environment. The goal was to search a high salt environment for microorganisms which could oxidize the model compound, dibenzothiophene (DBT). The second goal was to identify and obtain the enzyme fraction(s) responsible for the activity. As mentioned in Section 1.3, an enzyme system has several advantages over a whole cell system. The third goal was to obtain microbial enzyme fractions which could carry out the desulfurization of coal.

The work of microbial isolation was carried out by Dr. Jannasch at Woods Hole, and his colleague, Dr. Dennis Bazylinski. They utilized a submarine to collect soils from various oceanic thermal vent areas. A variety of microbes are present in such locations, including both thermophiles and mesophiles. It had been hoped that thermophilic bacteria would be found which acted on organic sulfur.

GB-1, an isolate obtained from the Guaymas Basin, was the only isolate that could oxidize DBT. The growth conditions for GB-1 were determined and optimized. In artificial sea water medium with 0.05% yeast extract, GB-1 could oxidize DBT at 24°C and pH 7.0, to produce DBT sulfoxide and DBT sulfone and a red colored compound(s) after 4-7 days with shaking. Furthermore, it was determined that the GB-1 culture supernatant also yielded DBT sulfoxide and DBT sulfone and the red compound(s) from DBT. The red-colored compound(s) was separated from the culture medium by column chromatography; it was, however, not identified.

Further work with the GB-1 culture was aimed at obtaining and identifying the enzyme fractions responsible for the oxidation of DBT and testing them for activity against DBT as well as its "4S" oxidation products in aqueous solution and in organic solvent.

30
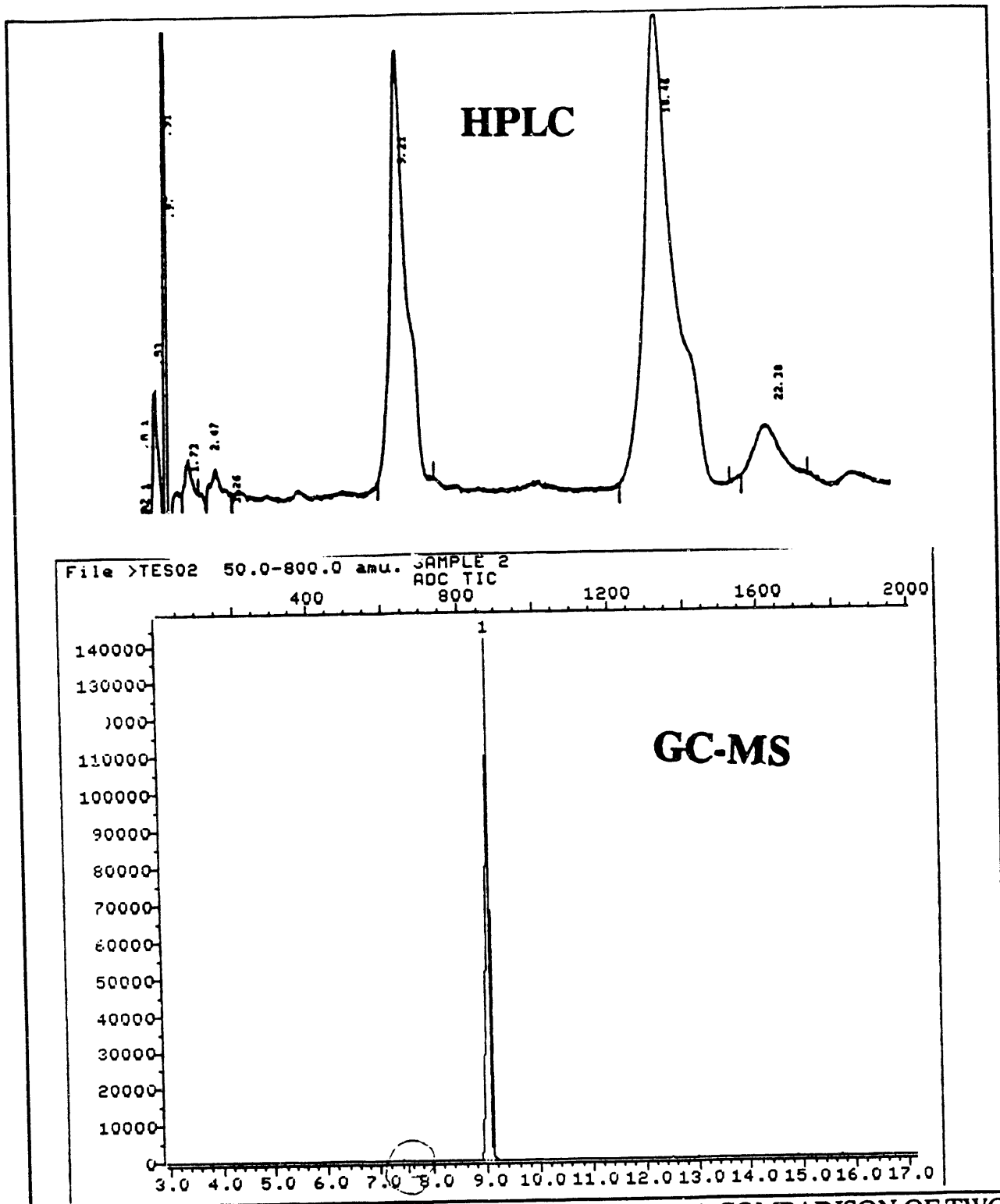
FIGURE 2.9: HPLC AND GC-MS CHROMATOGRAMS: A COMPARISON OF TWO METHODS OF ANALYSIS OF A PARTIALLY PURIFIED EXTRACT OF GB-1 GROWTH MEDIUM

# 3.0  WORK WITH MICROBIAL ENZYMES FROM GB-1

## 3.1  Summary of Work with Microbial Enzymes

GB-1 was grown in 2 liters of artificial sea water containing 0.54 mM DBT for 3 days. The culture medium was fractionated with cell lysing and three fractions were obtained: extracellular fraction [ECF]; intracellular fraction [ICF]; and membrane fraction [MF]. This work is described in Section 3.2. The ECF, ICF, and MF were assayed for their activity against DBT in the aqueous phase as well as in acetonitrile and ethyl acetate. The ECF was also assayed in DMF.

When neat ECF was incubated with DBT, DBT sulfoxide and some DBT sulfone were produced; red colored compounds were also observed but not identified. When the ECF was incubated with either DBT sulfoxide or DBT sulfone, no known DBT oxidation products were found.

The concentrated ECF was also tested for activity against DBT, and after 2 days of incubation at 24°C, DBT sulfoxide and an unknown which eluted between DBT sulfone and o,o'-biphenol on HPLC were detected. It is possible that either the ability to form DBT sulfone was lost during the concentration step (perhaps due to loss of essential co-factors) or that any DBT sulfone formed was further oxidized to the unidentified product which was observed.

Concentrated ECF was also incubated with DBT in 90% solvent. The three solvents tested were acetonitrile, ethyl acetate, and dimethylformamide. At the end of 7 days of incubation at 24°C, DBT sulfoxide and DBT sulfone were observed in 90% acetonitrile and in DMF. Incubation of ECF with DBT in ethyl acetate did not yield any DBT oxidation products.

The ICF was tested for activity against DBT. Both DBT sulfoxide and DBT sulfone were found after 5 days. DBT sulfone was also observed in ICF controls to which no DBT had been added. It was shown that when the cells were harvested from the DBT-containing medium, some DBT was carried over and isolated with the ICF. This DBT was apparently converted to DBT sulfone in ICF controls under the assay conditions.

The ICF was incubated in 90% acetonitrile for 7 days with DBT. DBT sulfoxide and DBT sulfone were found. When DBT sulfone was used as substrate, these products plus small amounts of o,o'-biphenol were found. These compounds were not found in the controls . When concentrated ICF was used, the assay time was shortened to 24 hours and DBT sulfoxide, DBT sulfone, o,o'-biphenol, and o-hydroxy biphenyl were found. Again, some oxidation products were found in the ICF controls at 24 hours. Concentrated ICF incubated with DBT sulfone produced o,o'-biphenol, and o-hydroxy biphenyl.

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The MF showed no activity against DBT in aqueous solution or in 90% acetonitrile.

## 3.2    Fractionation of GB-1

Initially, experiments with GB-1 whole cells were performed. Activity against DBT was demonstrated by using HPLC to detect the presence of DBT sulfoxide and DBT sulfone in culture medium after incubation with GB-1 for a few days at 24°C. In order to determine where the active agents of this catalysis resided, the culture media and cells were fractionated into extracellular, intracellular and membrane fractions. The fractionation procedure is outlined in Figure 3.1 and is described below.

GB-1 was grown in artificial sea water medium with 0.54 mM DBT at 25°C with shaking. At the end of 72 hours, the cells were harvested by centrifugation in the cold at 9,000 rpm for 15 minutes. The supernatant was considered the extracellular fraction [ECF].

The pellet was washed in 10 mM Tris buffer, pH 7.0, and resuspended in 50 ml of the same buffer containing 0.5 ml of a 100 mM ethanolic solution of phenylmethy'-sulfonylfluoride (PMSF), a protease inhibitor. This suspension was sonicated on ice for 3-4 minutes using 15 second bursts at 1 minute intervals. The sonicate was examined microscopically to confirm cell lysis and then ultracentrifuged at 35,000 rpm for 1 hour at 4°C. The supernatant was collected and termed the intracellular fraction [ICF]. The pellet was washed with Tris buffer and centrifuged again, then the supernatant was discarded. The pellet was resuspended in ~5.0 ml of 10 mM Tris buffer, pH 7.0, and labelled as the membrane fraction (MF).

The ECF was initially dialysed and then lyophilized. GB-1 ECF was dialyzed against 10 mM potassium phosphate buffer, pH 6.8. The dialysis tubing used was a Spectropor membrane tubing with a molecular weight cut off of 6,000 to 8,000 from Spectrum Medical Industries, Inc. Prior to dialysis, an ethanolic solution of the protease inhibitor phenylmethylsulfonylfluoride (PMSF) was added to a final concentration of 1 mM. Dialysis was done in the cold over 6 hours with a buffer change every 2 hours.

The dialyzed medium was then lyophilized. For the lyophilization, the material was frozen. Due to the inefficiency of the lyophilization process, the material thawed and was refrozen a few times before lyophilization was complete. A BioRad assay for determination of protein in the lyophilized material revealed very low protein content. It appeared that either a loss of low molecular weight proteins had occured during dialysis, or protein denaturation had occured due to the freeze-thaw cycles during lyophilization. Lyophilization without dialysis was also not successful. Subsequently, concentration of ECF was carried out by ultrafiltration as described below.

Ultrafiltration was a more successful method of concentration. An Amicon Model 8050 ultrafiltration cell (50 ml volume) was used with a Pharmacia Omega Series 43 mm ultrafiltration membrane with a 10,000 molecular weight cut-off. Ice cold water was

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        ┌─────────────────────────────┐
        │  CELLS GROWN WITH 0.5 Mm DBT │
        │        FOR 72 HOURS         │
        └─────────────────────────────┘
                     │
                     ▼
        ┌─────────────────────────────┐
        │          CENTRIFUGE         │
        │      9,000 RPM FOR 15 MIN   │
        └─────────────────────────────┘
              │                    │
              ▼                    ▼
```

**CELLS GROWN WITH 0.5 Mm DBT FOR 72 HOURS**

**CENTRIFUGE**
9,000 RPM FOR 15 MIN

**PELLET**
WASH IN 10.0 mM TRIS, pH 7.0

**SUPERNATANT**
*CONCENTRATE BY ULTRAFILTRATION*

**EXTRACELLULAR FRACTION (ECF)**

**PELLET**
RESUSPEND IN 10 mM TRIS; 1mM PMSF

**SONICATE**
15 SEC BURSTS AT 1 MIN INTERVALS

**ULTRACENTRIFUGE**
1 HOUR, 35,000 RPM

**PELLET**
*WASH IN 10 mM TRIS*

**SUPERNATANT**
CONCENTRATE BY ULTRAFILTRATION

**INTRACELLULAR FRACTION (ECF)**

**MEMBRANE FRACTION (MF)**

FIGURE 3.1: PRELIMINARY FRACTIONATION OF GB-1 FOR ISOLATION OF SULFUR-OXIDIZING ENZYMES

constantly passed through a tubing wrapped around the ultrafiltration cell using a peristaltic pump. Nitrogen was used to pressurize the cell to ~20 psi. The fraction with molecular weight greater than 10,000 accumulated within the ultrafiltration cell and was labelled as concentrated extracellular fraction. In this manner, about 1.8 liters of culture supernatant was concentrated to 47 ml. The ECF-concentrate, ICF, and MF were distributed into 4 ml aliquots in vials and frozen at -20°C until used.

The ICF was concentrated with a 43 mm Amicon ultrafiltration membrane (Diaflo No. 5YC05) with a molecular weight cut-off of 500. This low cut-off was used assuming that the enzyme(s) that brings about DBT or DBT sulfone oxidation might need a co-factor(s) of low molecular weight. The ICF was kept cold during the concentration step by pumping ice-cold water through tubing surrounding the filtration chamber. Thus, 26 ml of ICF was concentrated to 3.4 ml.

## 3.3    Activity of Fractions vs Model Compounds

### 3.3.1    Extracellular Fraction (ECF)

#### ECF - pH Studies in Aqueous Solution

During the initial growth experiments, GB-1 was grown without DBT in medium supplemented with yeast extract. After 48 hours and 96 hours of incubation, the cultures were centrifuged and extracellular fractions were collected. The activities of these ECFs were tested at 25°C against 0.1% DBT at pH 5.0, 6.0, 7.0, and 8.0. GB-1 ECF at pH 6.0 and 7.0 turned red in color within 24 hours of incubation at 25°C with DBT.

#### ECF in Aqueous Solution - Activity Against DBT

GB-1 cells were grown in artificial sea water medium with 0.05% yeast extract and no DBT. The inoculum was also from cells grown under the same conditions. Cells were grown at 25°C with shaking and at the end of 70 hours, they were centrifuged at 10,000 rpm for 20 minutes. The cell pellet was discarded and part of the ECF was boiled for 5 minutes. The two fractions of boiled and unboiled ECFs were incubated with 0.01% DBT (0.54 mM) for 1 week at 25°C with shaking, simulating the conditions used with whole cells. The media were extracted and concentrated and examined by HPLC. In the extract from the heat-treated ECF, no DBT oxidation products were seen, however, DBT sulfoxide (0.0023 mM), and DBT sulfone (0.00007 mM) were detected in the extract from the ECF that had not been heat treated. The residual DBT concentration was 0.23 mM. This gave conclusive evidence that the formation of "4S" oxidation products were biologically catalyzed.

ECFs that had been obtained after allowing growth of GB-1 in DBT-free medium for 65 hours, 90 hours, and 114 hours were incubated with each of 0.01% DBT, 0.01%DBT sulfoxide, and 0.01% DBT sulfone. While all the three ECFs oxidized DBT to DBT

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sulfoxide and DBT sulfone, no oxidation products were observed with DBT sulfoxide and DBT sulfone as substrates. No o,o'-biphenol was detected.

The levels of DBT sulfoxide and DBT sulfone obtained in the above experiments of DBT oxidation by GB-1 ECFs were much lower than in previous experiments. This was attributed to the fact that previously the inocula had been from media containing DBT and with repeated transfers in media without DBT, the cells probably no longer produced as much DBT-oxidation enzyme(s) as they might in media containing DBT. It was therefore considered necessary to use an inoculum which was grown with DBT in the medium.

### Concentrated ECF in Aqueous Solution - Activity Against DBT

For the assay in the aqueous phase, 1 ml each of the concentrated ECF was incubated with 1 mg of DBT in 8 ml screw cap tubes. The tubes were capped and incubated horizontally at 25°C in a shaker. Control tubes included fractions incubated without DBT, and Tris buffer and medium concentrated by ultrafiltration (as done to obtain the ECF) incubated with DBT.

DBT sulfoxide and a small amount of an unknown were observed as a result of DBT oxidation by concentrated ECF in aqueous phase (Figure 3.2). No other DBT oxidation products were observed.

### Concentrated ECF in Organic Solvent - Activity Against DBT

Assays in organic solvent were carried out as follows: a DBT solution was prepared in organic solvent so that when all components were added the final DBT concentration was 0.05 mM. To 0.9 ml of this solution was added 0.1 ml of ECF; the tubes were capped and incubated as described before. Controls without DBT and of buffer and medium concentrate with DBT were also included.

An additional experiment included incubation of ECF in 50% acetonitrile with a final DBT concentration of 0.1 mM. Tubes were incubated as before with shaking at 25°C. Controls included ECF in 50% acetonitrile without DBT.

Results by both GC and HPLC are given qualitatively in Table 3.1. The samples had been dried and resuspended for analysis and some material was apparently lost in this process. Thus, the quantities measured were variable. Because the Tris buffer seemed to affect the GC retention times, HPLC data for these samples were more reliable.

The assays of extracellular fractions in 90% acetonitrile and in 50% dimethylformamide showed the presence of small amounts of DBT sulfoxide and DBT sulfone. In 90% ethyl acetate no DBT oxidation products were seen. The controls also occasionally showed very small levels of DBT sulfoxide which may be residual from the initial cell growth on DBT.

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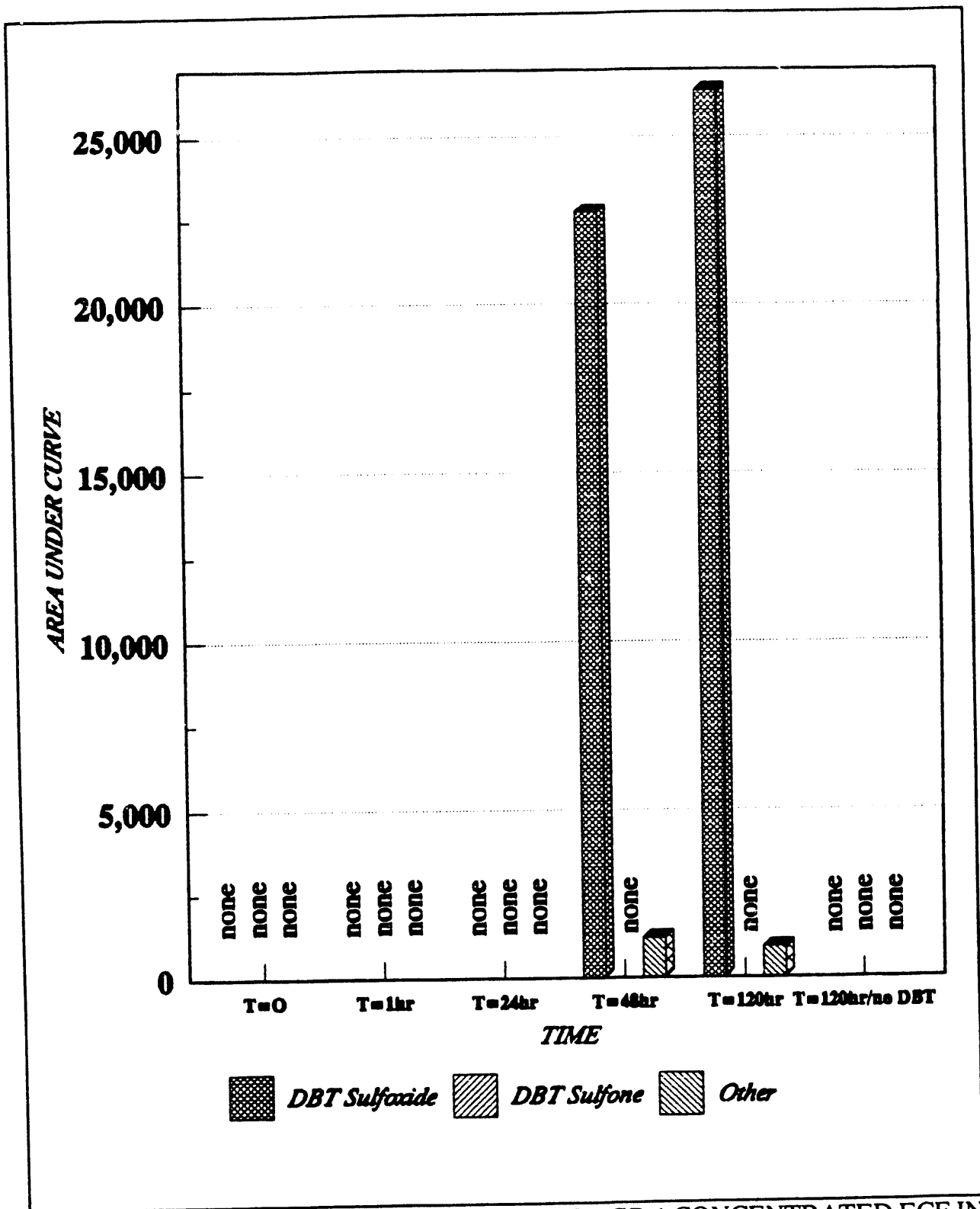
FIGURE 3.2: HPLC ANALYSIS OF ACTIVITY OF GB-1 CONCENTRATED ECF IN AQUEOUS MEDIUM VS DBT

TABLE 3.1: GC AND HPLC ANALYSIS OF GB-1 ECF ACTIVITY IN ACETONITRILE, ETHYL ACETATE, AND DIMETHYLFORMAMIDE VS DBT

| Solvent | Initial DBT (mM) | Method | o,o'-biphenol | DBT | DBT* Sulfoxide | DBT* sulfone | DBT** Oxide/One | o-hydroxy biphenyl |
|---|---|---|---|---|---|---|---|---|
| 90% Acetonitrile | 0.05 | GC | - | + | NA | NA | + | + |
| | | HPLC | - | + | + | + | NA | + |
| 90% Acetonitrile | 0.0 | GC | - | - | NA | NA | + | + |
| | | HPLC | - | - | + | - | NA | - |
| 90% Ethyl Acetate | 0.05 | GC | not done | not done | NA | NA | not done | not done |
| | | HPLC | - | + | - | - | NA | + |
| 90% Ethyl Acetate | 0.0 | GC | not done | not done | NA | NA | not done | not done |
| | | HPLC | - | - | - | - | NA | - |
| 50% Dimethyl-formamide | 5.43 | GC | not done | not done | NA | NA | not done | not done |
| | | HPLC | - | + | + | + | NA | - |

+  means that the compound was observed
-  means that it was not observed
*  Measured by HPLC only
** measured by GC only, since DBT-sulfoxide and DBT-sulfone are not resolved by GC
NA  Method not Applicable
ECF  Extracellular Fraction

### 3.3.2 Intracellular Fraction

#### GB-1 ICF in Aqueous Solution - Activity Against DBT

For the assay in the aqueous phase, 1 ml each of the ICF was incubated with 1 mg of DBT in 8 ml screw cap tubes. The tubes were capped and incubated horizontally at 25°C in a shaker. Control tubes included fractions incubated without DBT, and Tris buffer incubated with DBT. Aliquots taken after 7 days of incubation were filtered and injected onto the HPLC. ICF incubated with DBT showed the presence of DBT sulfoxide and DBT sulfone (Figure 3.3). No oxidation products were observed in the Tris and DBT control. However, the ICF control (without DBT) showed traces of DBT sulfone (not detected at the 0 time point), indicating that DBT/DBT sulfoxide present in the ICF is oxidized to DBT sulfone during the course of the experiment.

#### GB-1 ICF in Organic Solvent - Activity Against DBT

Assays in organic solvent were set-up as follows: a DBT solution was prepared in organic solvent so as to give a final DBT concentration of 0.05 mM. To 0.9 ml of this solution was added 0.1 ml of ICF and the tubes were capped and incubated with shaking at 25°C. Controls without DBT and of buffer with DBT were also included. An additional experiment included incubation of ICF in 50% acetonitrile with a final DBT concentration of 0.1 mM. Controls included ICF in 50% acetonitrile without DBT. Tubes were incubated as before with shaking at 25°C.

The results are summarized in Table 3.2. Results by both GC and HPLC are given qualitatively. As before, since the Tris buffer seems to affect the GC retention times, HPLC data were more reliable for these samples. The intracellular fraction oxidized DBT to DBT sulfoxide, and o-hydroxy biphenyl in 90% acetonitrile. DBT sulfoxide and DBT sulfone were also detected in 90% ethyl acetate. The controls also showed very small levels of sulfoxide or sulfone which appear to be produced from the residual DBT present from the initial cell growth on DBT.

#### GB-1 ICF in Organic Solvent - Activity Against DBT Sulfone

Sample tubes for the assay were prepared as shown in the table in the next section. Aliquots were removed and filtered through a 0.22 μ Nylon-66 (Rainin No. 38-159) filter. There were two tubes for each sample set: GB-1 ICF with 0.05 mM DBT sulfone, GB-1 ICF only, and 0.05 mM DBT sulfone only. The solvent was 90% acetonitrile with Tris buffer in the case of samples containing ICF, and distilled water in the DBT sulfone controls. After 4 days of incubation at 24°C with shaking, samples were filtered and filtrates were analyzed by HPLC as obtained and also by evaporation to dryness and resuspension

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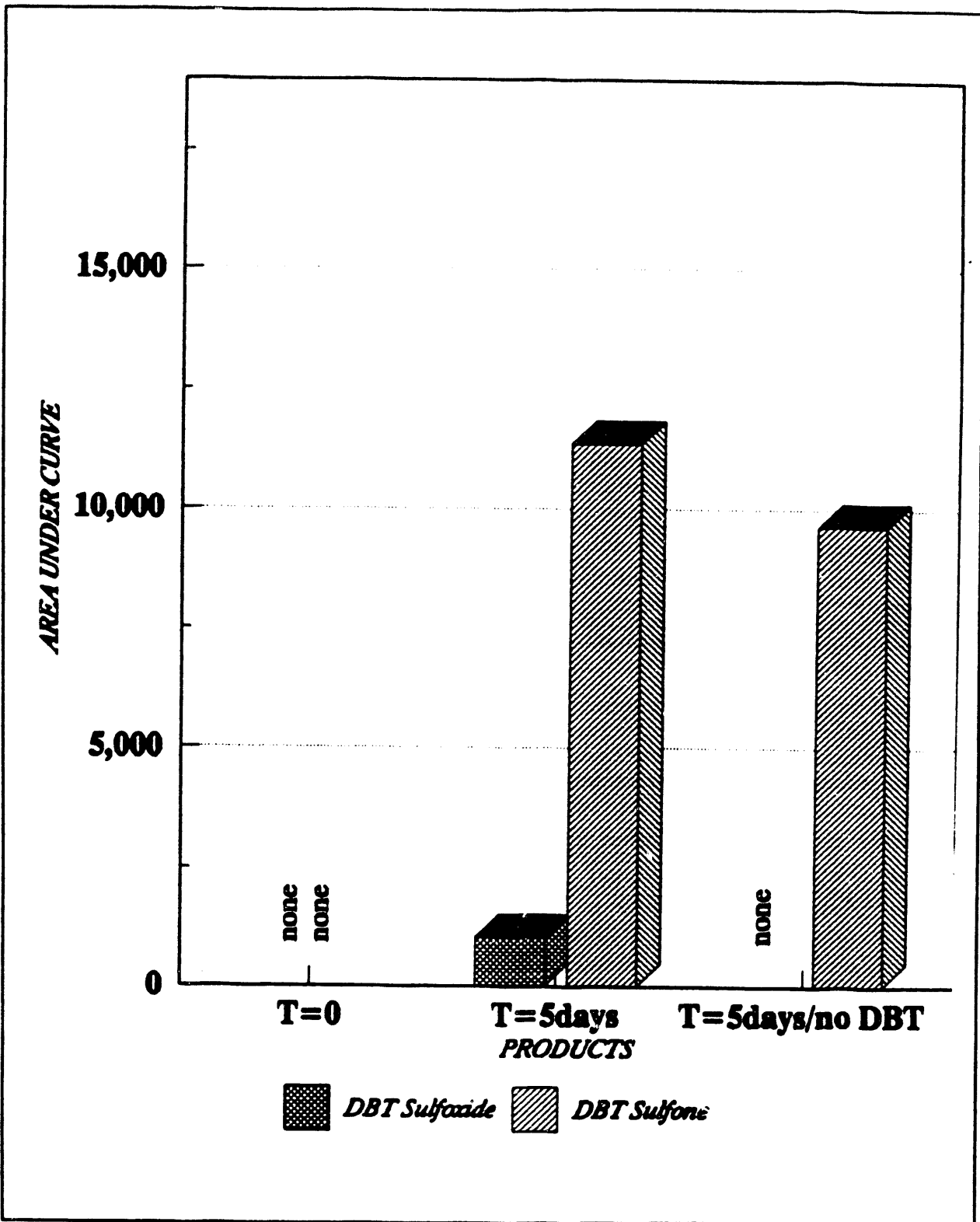
FIGURE 3.3: HPLC ANALYSIS OF ACTIVITY OF GB-1 ICF IN AQUEOUS MEDIUM VS DBT

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## TABLE 3.2: GC AND HPLC ANALYSIS OF GB-1 ICF ACTIVITY IN ACETONITRILE AND ETHYL ACETATE VS DBT

| Solvent | Initial DBT (mM) | Method | o,o'-Biphenol | DBT | DBT* Sulfoxide | DBT* Sulfone | DBT** Oxide/One | o-Hydroxy Biphenyl |
|---|---|---|---|---|---|---|---|---|
| 90% Acetonitrile | 0.05 | GC | - | + | NA | NA | - | + |
|  |  | HPLC | - | + | + | - | NA | + |
| 90% Acetonitrile | 0.0 | GC | - | - | NA | NA | - | + |
|  |  | HPLC | - | - | + | - | NA | - |
| 90% Ethyl Acetate | 0.05 | GC | not done | not done | NA | NA | not done | not done |
|  |  | HPLC | - | + | + |  | NA | + |
| 90% Ethyl Acetate | 0.0 | GC | not done | not done | NA | NA | not done | not done |
|  |  | HPLC | - | - | + |  | NA | - |
| 90% Tris/ Acetonitrile | 0.0 | GC | *** | *** | *** | *** | *** | *** |
|  |  | HPLC | *** | *** | *** | *** | *** | *** |
| 90% Tris/ | 0.0 | GC | *** | *** | *** | *** | *** | *** |

+   means that the compound was observed
-   means that it was not observed
*   Measured by HPLC only
**  measured by GC only, since DBT-sulfoxide and DBT-sulfone are not resolved by GC
NA  Method not Applicable
ECF Extracellular Fraction

in a smaller amount of solvent to effect a 20-fold concentration. For GC analysis, these aliquots were also evaporated to dryness and then derivatized in a volume of Tri-Sil/BSA so as to effect a 20-fold concentration.

HPLC analysis of reaction media indicated the presence of a very slight amount of o,o'-biphenol in the ICF/sulfone samples, whereas none was present in either set of controls. However, the HPLC analysis of the concentrated sample did not detect any o,o'-biphenol while it did detect small amounts of o-hydroxybiphenyl. The control samples did not show evidence of either product. The GC analysis of concentrated aliquots from this sample set indicated the presence of o,o'-biphenol in one sample but not the other and also showed peaks that possibly indicated the presence of o-hydroxybiphenyl. GC analysis of a concentrated DBT sulfone control also indicated the possible presence of o,o'-biphenol, but was not conclusive.

Based on the HPLC analysis which suggested the presence of o,o'-biphenol and o-hydroxybiphenyl in the Day 4 aliquot filtrates from the ICF with DBT sulfone assays, coupled with the lack of any indication of these compounds in either control set, a portion of one of the filtrates from this sample set was taken to Boston University Medical School for GC/MS analysis. The sample was concentrated 15-fold by evaporation to dryness and derivatized with Tri-Sil/BSA prior to analysis. The GC/MS analysis confirmed the presence of o,o'-biphenol and o-hydroxybiphenyl in this sample. A copy of these data is included in Appendix B. No controls were analyzed by GC/MS.

There was some uncertainty raised due to the apparent loss of material during evaporation of solvent. This difficulty is described in Section 5.5. Therefore, the concentration of substrates and products present in these assay samples could not be determined.

### GB-1 ICF/DBT Sulfone Assays and Comparison of Two Lots of GB-1 ICF

There were two reasons for conducting this experiment. First, it was of interest to repeat the assays of the GB-1 ICF against DBT sulfone in 90% acetonitrile with a larger number of samples. The second purpose was to compare the potencies of two different lots of GB-1 ICF.

In general, the procedure for the assays in all sample sets was that the 0.99 mM DBT sulfone solution (in acetonitrile) and acetonitrile were added to screw cap test tubes as shown below:

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| Sample Set | 0.99 mM DBT Sulfone (ml) | ICF added (ml) | Acetonitrile (ml) | Distilled H₂O (ml) |
|---|---|---|---|---|
| 1 | 0.252 | 0.500 | 4.248 | 0.0 |
| 2 | 0.0 | 0.500 | 4.500 | 0.0 |
| 3 | 0.252 | 0.0 | 4.248 | 0.500 |
| 4 | 0.252 | 0.500 | 4.248 | 0.0 |
| 5 | 0.0 | 0.500 | 4.500 | 0.0 |

The indicated ICF solution (or distilled water in the case of Sample Set No. 3) was then added to each tube. The tubes were mixed by gentle inversion and a 1 ml aliquot was removed and filtered for a 0 hour time point. All filtrates were refrigerated until analysis. After the initial aliquots were removed, the assay tubes were agitated by shaking at 24°C. Another 1 ml aliquot was removed and filtered after this first 24 hour period, and the remainder of the assay sample was filtered after 4 days. These filtrates were similarly refrigerated until analysis.

Initial analysis of some Day 4 aliquot filtrates by GC and HPLC yielded inconclusive results (data not shown). Some species detected by GC analysis were not detected by HPLC analysis and vice versa. There was not good agreement between methods as to concentrations of the same materials found to be present in the same samples. Difficulties in analysis due to losses during sample concentration may have contributed to this problem.

### Concentrated ICF in Organic Solvent - Activity Against DBT and DBT Sulfone

Previous experiments with GB-1 ICF were assayed after 4 days of incubation with substrate. It was expected that concentration of the ICF would shorten the reaction time needed to see conversion of substrate. Thus, a 1 day incubation experiment was carried out with concentrated ICF.

ICF was concentrated two-fold by ultrafiltration using a 500 molecular weight cut off (Amicon filter, Diaflo No. 5YC05). ICF-conc. was incubated separately with 0.1 mM DBT and 0.1 mM DBT sulfone in 90% acetonitrile. Controls included ICF incubated without substrate and each of the substrates incubated without ICF. After 24 hours of incubation at 24°C with shaking, the samples were filtered through 0.2 μ Nylon filters and refrigerated in vials until tested. One hundred microliters (100 μl) of each sample was concentrated by drying in reactor vials at room temperature without letting the sample become completely dry. Samples were analyzed by HPLC and GC. The results of these analyses are shown in Tables 3.3A and B.

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From the GC, there was evidence of formation of o,o'-biphenol from DBT sulfone. No products in these samples were observed on the HPLC, although the DBT sulfone controls had a peak close to the retention time of o,o'-biphenol. The DBT sulfone or o-OH-biphenyl seen in the ICF controls were probably due to the formation of these compounds from the DBT already present in the ICF. In ICF incubated with DBT, DBT sulfone, o,o'-biphenol, as well as DBT sulfoxide, were detected by HPLC while DBT sulfone/sulfoxide and a peak close to the retention time of o-OH-biphenyl were detected by the GC. While the DBT controls showed some DBT sulfone, the samples with ICF and DBT had larger DBT sulfone peaks.

### GB-1 ICF in 98% Aqueous Dimethylformamide - Activity Against DBT [0.1 mM] and DBT Sulfone [0.1mM]

To determine whether concentrated GB-1 ICF has activity against DBT in aqueous rather than predominantly organic media, the following assays were performed. A 5 mM solution of DBT was prepared in a water miscible solvent, dimethylformamide. A small volume of this was then mixed with GB-1 ICF to make a final concentration of 0.1 mM DBT per sample tube. Single control samples were mixed and immediately refrigerated. Samples (0 time point controls and DBT controls without ICF) were incubated in triplicate for 8 hours at 24°C with vigorous agitation in a wrist action shaker. These samples were also refrigerated until analysis. The set up of tubes for the assay were as described below:

| Sample Set | Time of Sample (hrs) | GB-1 ICF (ml) | 5 mM DBT in DMF (ml) | DMF (ml) | 10 mM Tris pH 7 (ml) |
|---|---|---|---|---|---|
| 1 | 8 | 0.490 | 0.010 | 0.0 | 0.0 |
| 2 | 8 | 0.0 | 0.010 | 0.0 | 0.490 |
| 3 | 8 | 0.490 | 0.0 | 0.010 | 0.0 |
| 4 | 0 | 0.490 | 0.010 | 0.0 | 0.0 |
| 5 | 0 | 0.0 | 0.010 | 0.0 | 0.490 |
| 6 | 0 | 0.490 | 0.0 | 0.010 | 0.0 |

Each sample and control vial was acidified by the addition of 2 drops of 0.1 N HCl. Three mls of methylene chloride was added to each vial and the samples were vortexed for ~30 seconds before the aqueous and organic layers were allowed to separate. The organic (methylene chloride) layer was transferred to a silanized storage vial and the volume of the transferred extract was measured. The extract was concentrated by transferring 200 µl at a time to a 1 ml silanized "Reacti Vial" and allowing the solvent to evaporate to dryness at room temperature. After a total of 1,000 µl of each sample

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TABLE 3.3-A: GC AND GC-MS ANALYSIS OF ASSAYS OF CONCENTRATED GB-1 ICF WITH DBT SULFONE OR DBT

| Sample | Method | DBT* Sulfoxide/ DBT Sulfone (mM) | o,o'- Biphenol (mM) | o-Hydroxy Biphenyl (mM) | DBT (mM) |
|---|---|---|---|---|---|
| Conc. ICF and DBT Sulfone in 90% Acetonitrile | GC | 0.065 ±0.021 | 0.0009 ±0.0009 | 0.006 ±0.0085 | 0.028 ±0.31 |
| | GC-MS | 0.047 ±0.054 | 0.0 ±0.0 | 0.0017 ±0.0013 | 0.022 ±0.0085 |
| Conc. ICF in 90% Acetonitrile (1 sample) | GC | 0.012 | 0.0 | 0.002 | 0.013 |
| | GC-MS | 0.040 | 0.0 | 0.0012 | 0.014 |
| DBT Sulfone in 90% Acetonitrile | GC | 0.13 ±0.0057 | 0.0 ±0.0 | 0.0 ±0.0 | 0.0015 ±0.002 |
| | GC-MS | 0.078 ±0.051 | 0.0 ±0.0 | trace ±0.0064 | 0.0057 |
| Conc. ICF and DBT in 90% Acetonitrile | GC | 0.006 ±0.0014 | 0.0 ±0.0 | ? | 0.095 ±0.0071 |
| | GC-MS | 0.053 ±0.030 | 0.0 ±0.0 | 0.0018 ±0.00057 | 0.070 ±0.097 |
| DBT in 90% Acetonitrile | GC | 0.0007 ±0.0042 | 0.0 ±0.0 | 0.0 ±0.0 | 0.08 ±0.014 |
| | GC-MS | 0.029 ±0.037 | 0.000089 ±0.000089 | 0.0014 ±0.0018 | 0.028 ±0.022 |

* Since the GC program does not separate DBT sulfoxide from DBT sulfone, the reported concentrations are for the combined concentrations were calculated based on a standard containing only DBT sulfone.

? A peak with retention time close to that of o-OH-Biphenyl was detected.

# TABLE 3.3-B: HPLC ANALYSIS OF ASSAYS OF CONCENTRATED GB-1 ICF VS DBT SULFONE OR DBT

| Sample | DBT Sulfoxide (mM) | DBT Sulfone (mM) | o,o'-Biphenol (mM) | o-Hydroxy Biphenyl (mM) | DBT (mM) |
|---|---|---|---|---|---|
| Conc. ICF and DBT Sulfone in 90% Acetonitrile | ± ± | 0.055 0.057 | 0.0 0.0 | 0.0 0.0 | 0.00 0.007 |
| Conc. ICF in 90% Acetonitrile (1 sample) | 0.0 | ± | 0.0 | 0.0 | 0.005 |
| DBT Sulfone in 90% Acetonitrile | ± ± | 0.047 0.047 | X X | 0.0 0.0 | 0.0001 0.0 |
| Conc. ICF and DBT in 90% Acetonitrile | 0.003 ± | 0.002 0.0004 | 0.0002 0.0002 | 0.0 ND | 0.067 0.07 |
| DBT in 90% Acetonitrile | 0.0 0.0 | 0.0003 0.00005 | 0.0 0.0 | 0.0 0.0 | 0.057 0.035 |

Samples were collected after 24 hours of shaking at 24°C.
+ = Small peaks incorrectly integrated on the HPLC.
X = A peak with retention time close to that of o,o'-diphenol was detected by HPLC analysis.

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had been dried, each sample was redissolved in 15 µl of acetonitrile prior to analysis by HPLC. Initially, it had been planned to analyze these samples by gas chromatography but interferences from residues leached from the silanized vials by the methylene chloride precluded the efficient use of GC for this analysis (see Section 5.5.2). The HPLC analysis results are summarized in Table 3.4.

These results show that the only identifiable product in the ICF/DBT 8 hour sample was DBT sulfoxide and this was also present in the Time = 0 sample in approximately the same concentration. A similar amount was detected in one of the three 8 hour samples that had only DBT in dimethylformamide added to Tris buffer. It does not appear that any significant reaction is occurring in these samples. The presence of DBT sulfoxide in the control samples (Set 3), which had no DBT but only ICF and dimethylformamide added, is most likely due to DBT in the ICF itself.

The same lot of GB-1 ICF was next tested for activity against DBT sulfone. Sample tubes were set up as follows:

| Sample Set | Time of Sample (hrs) | GB-1 ICF (ml) | 5 mM DBT Sulfone in DMF (ml) | DMF (ml) | 10 mM Tris pH 7 (ml) |
|---|---|---|---|---|---|
| 1 | 8 | 0.450 | 0.010 | 0.0 | 0.0 |
| 2 | 8 | 0.0 | 0.010 | 0.0 | 0.450 |
| 3 | 8 | 0.450 | 0.0 | 0.010 | 0.0 |
| 4 | 0 | 0.450 | 0.010 | 0.0 | 0.0 |
| 5 | 0 | 0.0 | 0.010 | 0.0 | 0.450 |
| 6 | 0 | 0.450 | 0.0 | 0.010 | 0.0 |

These ICF/DBT sulfone assay samples were stored, acidified, extracted, and dried as were the GB-1 ICF/DBT assay samples. One sample from Set No. 2 was lost. The dried, concentrated DBT sulfone assay samples were also analyzed by HPLC but there was a slight difference. In addition to the UV detector, a fluorescence detector was added in series. It was hoped that use of the fluorescence detector might better detect the expected products, o-hydroxybiphenyl and o,o'-biphenol. However, under the conditions used, only the o,o biphenol and DBT sulfoxide and DBT sulfone could be detected by the fluorescence detector. The HPLC results are summarized in Table 3.5.

TABLE 3.4: HPLC ANALYSIS OF GB-1 ICF ASSAYS IN 98% AQUEOUS DIMETHYLFORMAMIDE VS DBT

| Sample Set No. | Time (hr) | Sample Type | DBT Sulf-oxide (mM) | DBT Sulfone (mM) | o,o'-Bi phenol (mM) | o-Hydroxy Biphenyl (mM) | DBT (mM) | Other peak at 4-5 Min RT |
|---|---|---|---|---|---|---|---|---|
| 1 | 8 | ICF/ | | 0.006 | | | 0.096 | + |
| | 8 | DBT | | 0.008 | | | 0.12 | + |
| | 8 | | | 0.000 | | | 0.10 | - |
| 2 | 8 | DBT/ | | 0.000 | | | 0.12 | - |
| | 8 | TRIS | | 0.017 | | | 0.13 | - |
| | 8 | | | 0.000 | | | 0.112 | + |
| 3 | 8 | ICF | | 0.000 | | | 0.002 | + |
| | 8 | | | 0.000 | | | 0.001 | + |
| | 8 | | | 0.000 | | | 0.002 | + |
| 4 | 0 | ICF/ TRIS | | 0.010 | | | 0.098 | + |
| 5 | 0 | DBT/ TRIS | | 0.000 | | | 0.11 | + |
| 6 | 0 | ICF | | 0.000 | | | 0.002 | + |

+    Peak present

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## TABLE 3.5: HPLC ANALYSIS OF GB-1 ICF ASSAYS IN 2% AQUEOUS DIMETHYLFORMAMIDE VS DBT SULFONE

| Set No. | Time (hr) | Sample Type | DBT Sulfoxide (mM) | DBT Sulfone (mM) | o,o'-Bi phenol (mM) | o-Hydroxy Biphenyl (mM) | DBT (mM) |
|---|---|---|---|---|---|---|---|
| 1 | 8 | ICF/ DBT Sulfone | 0.0045 ±0.0035 | 0.058 ±0.004 | 0.00004 ±0.00005 | 0.00006 ±0.00009 | 0.00098 ±0.00006 |
| 2 | 8 | DBT Sulfone/ TRIS | 0.0050 ±0.0030 | 0.051 ±0.0060 | 0.00020 ±0.00038 | 0.00014 ±0.00011 | 0.0001 ±0.0000 |
| 3 | 8 | ICF | 0.0050 ±0.0048 | 0.00066 ±0.00041 | 0.00001 ±0.00002 | 0.00007 ±0.00009 | 0.0013 ±0.00026 |
| 4 | 0 | ICF/ DBT Sulfone | 0.0087 ±0.0024 | 0.062 ±0.0082 | 0.0000 ±0.0 | 0.00008 ±0.00008 | 0.00088 ±0.0000 |
| 5 | 0 | DBT Sulfone/ TRIS | 0.0038 ±0.0032 | 0.060 ±0.0022 | 0.00005 ±0.00005 | 0.00010 ±0.00012 | 0.0001 ±0.00007 |
| 6 | 0 | ICF | 0.0042 ±0.0041 | 0.0005 ±0.00036 | 0.00004 ±0.00008 | 0.0001 ±0.00017 | 0.00085 ±0.00015 |

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### 3.3.3    Membrane Fraction

For the assay in the aqueous phase, 1 ml each of the MF were incubated with 1 mg of DBT in 8 ml screw cap tubes. The tubes were capped and incubated horizontally at 25°C in a shaker. Control tubes included fractions incubated without DBT, and Tris buffer incubated with DBT. The tubes were harvested after 7 days. No DBT oxidation products were observed.

Assays in organic solvent were set-up as follows: a DBT solution was prepared in organic solvent so as to give a final DBT concentration of 0.05 mM. To 0.9 ml of this solution was added 0.1 ml of MF, and the tubes were capped and incubated as described before. Controls without DBT and of buffer with DBT were also included. Results are depicted in Table 3.6.

An additional experiment included incubation of MF in 50% acetonitrile with a final DBT concentration of 0.1 mM. Tubes were incubated as before with shaking at 25°C. Controls included MF in 50% acetonitrile without DBT.

No DBT oxidation products were observed on incubation of MF with DBT, even after 7 days in both aqueous solution and in organic solvent.

### 3.4    Activity vs Coal

To determine the desulfurization capabilities of GB-1 and its enzyme extracts on microbial depyritized coal, the following experiments were done:

### 3.4.1    Incubation of GB-1 Whole Cells with Coal

A GB-1 seed culture was grown in medium with 0.5 mM DBT for 72 hours and a 10% inoculum was added to several flasks containing 40 ml fresh medium and coal. Prior to addition to the medium, the coal was made homogeneous by grinding the clumps, followed by ball-milling for 12 minutes. It was then carefully weighed and added so as to make a 5% (wt./v) coal slurry. The flasks were incubated at 24°C on a Burrell wrist action shaker. For each time point, the contents of one flask were harvested. Flasks were harvested on Days 1, 2, 4, 8, and 15 (duplicate flasks for Day 15). 0 hour controls with and without cells, as well as 15 day controls without cells, were included.

For each time point, the contents of the flask were suction filtered through preweighed Whatman No. 1 filter paper in a Buchner funnel and then washed with 40 ml of diluted HCl. This was followed by a 2 x 40 ml wash with distilled water. The filter paper with coal was transferred to a centrifuge tube and allowed to dry overnight at 50°C. The tube and its contents were weighed again. There was loss of 0.19 to 0.66 g of coal in the different samples. This was due to two factors: (i) the coal could not be wetted well with

## TABLE 3.6: HPLC AND GC ANALYSIS OF GB-1 MF IN ACETONITRILE AND ETHYL ACETATE VS DBT

| Solvent | Initial DBT (mM) | Method | DBT (mM) | Bi-phenol (mM) | DBT* Sulf-oxide | DBT* Sulfone | DBT** Oxide /One | o-Hydroxy Biphenyl |
|---|---|---|---|---|---|---|---|---|
| 90% Aceto-nitrile | 0.05 | GC | - | + | | | + | - |
| | | HPLC | - | + | + | - | | - |
| 90% Aceto-nitrile | 0.00 | GC | not done | not done | | | not done | not done |
| | | HPLC | - | - | + | - | - | - |
| 90% Ethyl Acetate | 0.05 | GC | not done | not done | | | not done | not done |
| | | HPLC | - | - | + | - | | - |
| 90% Ethyl Acetate | 0.00 | GC | not done | not done | | | not done | not done |
| | | HPLC | - | - | + | - | | - |

+     means that the compound was observed
-     means that it was not observed
*     HPLC only
**     GC only
MF     Membrane Fraction

water and during the washing step, it was not possible to retrieve all the coal; and (ii) during the 15-day experiment, there were increased losses with time due to splashing that occurred during shaking. The tubes were then flushed with a stream of nitrogen gas and sealed tightly. These were then sent to DOE for analysis of sulfur content. The total sulfur analyses were performed on a LECO SC-32 sulfur analyzer. The instrument was checked by running an NBS reference standard both before and after the coal analyses. The results are presented in Table 3.7. The sample of untreated coal (Table 3.8) had a sulfur content of 3.2%. All the samples exposed to 40 ml of aqueous media (Table 3.7) had essentially the same sulfur content (3.0%). Perhaps washing of the coal with medium removed some residual pyritic sulfur. It is clear that under the conditions tested, there was no significant decrease in sulfur content of the coal due to incubation with GB-1 whole cells.

### 3.4.2 Incubation of GB-1 Concentrated ICF with Coal

The ICF was prepared from GB-1 as previously described. The ICF was concentrated about two-fold by ultrafiltration using a 500 molecular weight cut-off membrane (Diaflo 5YC05). A 5% coal slurry was prepared in 90% acetonitrile and 10% ICF-CONC. The total volume was 40 ml. Flasks were incubated with shaking at 24°C and harvested at the end of 1, 4, and 16 hours (duplicate flasks for 16 hour) of incubation. 0 hour controls with and without ICF and 16 hour controls without ICF were included. The samples were processed as described in the above section except that in this case the coal was not washed with dilute acid but was washed with 3 x 40 ml rinses of distilled water. In this case, the total loss of coal was 0.2 g to 0.25 g for the different samples. The sulfur analyses were done as described in the previous section. The results of this experiment are presented in Table 3.8. There were three samples from the ICF experiment which were exposed to 36 ml of acetonitrile and 4 ml of water. All of these had 3.4% sulfur. The other five samples were exposed to 36 ml of acetonitrile and 4 ml ICF. These had 3.3% sulfur. The data do not indicate assay loss of sulfur due to the action of GB-1 ICF alone.

### 3.5 Identification of GB-1

The method used to identify GB-1 was that of Biolog (Biolog, Inc., Hayward, CA). This method is based on the ability of a microorganism to utilize (oxidize) a preselected panel of 95 different carbon sources. One well contains no carbon source and serves as a negative control or reference well. The necessary nutrients and biochemicals are prefilled and dried into the 96 wells of the plate. The principle of the test is that in wells that contain a chemical that is oxidized, there is a burst of respiration and the cells reduce a tetrazolium dye forming a purple color. Other wells, including the reference well, remain colorless. The test yields a pattern of purple wells which constitutes a "metabolic fingerprint" of the capacities of the inoculated organism. The pattern is entered into a computer with a database of biochemical patterns for a large number of gram-negative species. A match reveals the most likely identity for the organism.

## TABLE 3.7: GB-1 WHOLE CELL ASSAY WITH COAL - RESULTS OF SULFUR ANALYSES

| Sample | Weight of Aliquot | %S | Average Value (%) |
|---|---|---|---|
| NBS 2.0% S, 171 | 0.245 | 1.88 | |
| standard | 0.125 | 1.92 | 1.90 |
| 0 time blank | 0.129 | 2.97 | |
| with cells | 0.129 | 2.94 | |
| | 0.172 | 2.93 | 2.95 |
| 0 time blank | 0.133 | 2.90 | |
| with cells | 0.127 | 2.94 | |
| | 0.128 | 2.85 | 2.90 |
| 1 day sample | 0.141 | 2.93 | |
| | 0.151 | 3.12 | |
| | 0.135 | 3.00 | 3.02 |
| 2 day sample | 0.164 | 3.05 | |
| | 0.151 | 2.98 | |
| | 0.163 | 2.95 | 2.99 |
| 4 day sample | 0.179 | 3.17 | |
| | 0.136 | 2.85 | |
| | 0.133 | 2.96 | 2.99 |
| 8 day sample | 0.141 | 3.06 | |
| | 0.140 | 3.02 | |
| | 0.164 | 2.97 | 3.02 |
| 15 day sample | 0.150 | 3.00 | |
| | 0.144 | 2.95 | |
| | 0.155 | 3.03 | 2.99 |
| 15 day sample | 0.152 | 3.03 | |
| | 0.172 | 2.90 | |
| | 0.137 | 3.00 | 2.98 |
| 15 day blank | 0.133 | 3.03 | |
| (- cells) | 0.145 | 3.05 | |
| | 0.113 | 2.87 | 2.98 |
| 15 day blank | 0.143 | 2.94 | |
| (- cells) | 0.133 | 3.01 | |
| | 0.141 | 3.01 | 2.99 |
| NBS 2% S standard | 0.157 | 1.83 | |

## TABLE 3.8: GB-1 CONCENTRATED ICF ASSAY WITH COAL -RESULTS OF SULFUR ANALYSES

| Sample | Weight of Aliquot (g) | %S | Average Value (%) |
|---|---|---|---|
| NBS 2% S standard | 0.171 | 1.97 | |
| | 0.196 | 2.01 | 1.99 |
| 0 time blank | 0.157 | 3.42 | |
| (coal + ACN + water) | 0.140 | 3.35 | |
| | 0.145 | 3.30 | 3.36 |
| 0 time blank | 0.159 | 3.25 | |
| (coal + ACN + ICF) | 0.162 | 3.36 | |
| | 0.159 | 3.18 | 3.26 |
| 1 hour sample | 0.150 | 3.30 | |
| (coal + ACN + ICF, | 0.138 | 3.27 | |
| incubated for 1 hour) | 0.160 | 3.25 | 3.27 |
| 4 hour sample | 0.142 | 3.30 | |
| (coal + ACN + ICF, | 0.145 | 3.29 | |
| incubated for 4 hours) | 0.212 | 3.29 | 3.29 |
| 16 hour sample | 0.145 | 3.23 | |
| (coal + ACN + ICF, | 0.152 | 3.28 | |
| incubated for 16 hours) | 0.145 | 3.31 | 3.27 |
| 16 hour sample | 0.152 | 3.27 | |
| (coal + ACN + ICF, | 0.145 | 3.36 | |
| incubated for 16 hours) | 0.172 | 3.28 | 3.30 |
| 16 hour blank | 0.136 | 3.29 | |
| (coal + ACN + water, | 0.128 | 3.47 | |
| incubated for 16 hours) | 0.166 | 3.29 | 3.35 |
| 16 hour blank | 0.165 | 3.38 | |
| (coal + ACN + water, | 0.135 | 3.36 | |
| incubated for 16 hours) | 0.133 | 3.42 | 3.39 |
| Untreated coal | 0.150 | 3.23 | |
| | 0.173 | 3.18 | |
| | 0.124 | 3.16 | 3.19 |
| NBS 2% S standard | 0.165 | 2.03 | |

The test is performed in the following manner. GB-1 cells were grown in artificial sea water medium with yeast extract which was solidified by the addition of 1.5% agar (Difco). Single colonies were picked and plated as a mat on another agar plate. After overnight incubation, growth from the plate was rolled onto a cotton swab and immersed into 0.8% sterile saline and twirled against the inside surface of the tube. The tube was mixed to get a uniform suspension. The inoculum density was adjusted so as to fall within the turbidity standards. (Two turbidity standards were prepared using Phillip's[R] Milk of Magnesia (81 mg/ml suspension of $Mg(OH)_2$) - a low standard with 48 $\mu$l of the suspension in 20 ml of water and a high standard with 54 $\mu$l of the suspension in 20 ml of water.) The OD was read at 590 nm. One hundred and fifty microliters of the cell suspension was inoculated into the microplate. The plates were run in duplicate and incubated at 24°C overnight. The pattern of purple wells was described to Biolog and, with their assistance, a low probability identification given to GB-1 was Enterobacter species. The identification was considered very poor.

To confirm these results, the procedure was repeated again in duplicate. The closest identity was again Enterobacter species, and was again labelled a "poor" identification. The next identity was Agrobacterium species. The database has a large number of Pseudomonas species and the GB-1 pattern was definitely not identified as a Pseudomonas. Obviously it is possible that the database did not include this particular bacterial genus, which is from an unusual source, and could therefore not provide an identity match.

### 3.6    Discussion of GB-1 Enzyme Work

GB-1 whole cells were fractionated into extracellular fraction (ECF), intracellular fraction (IF), and membrane fraction (MF). These were tested for activity against DBT in aqueous solution as well as in organic solvent. The membrane fraction did not oxidize DBT under either set of conditions. The ECF and ICF oxidized DBT under various conditions, producing small amounts of DBT sulfoxide, DBT sulfone, as well as the mono- and di-hydroxy biphenyls. Evidence is shown in various figures and in appendix D.

While some of the ECF/ICF controls incubated without the model compound also showed the presence of products, it should be noted that the products were not seen in the time zero controls. It seems apparent that the ECF/ICF had some of the substrate already present (residual from the growth medium), which they subsequently oxidized to product under the conditions of incubation.

To have a low cost desulfurization process, it would be necessary to use crude fractions as opposed to purified enzymes. The disadvantages with working with crude fractions are several-fold. Crude fractions have many enzymes and optimal conditions for each of their activities could be very different. One enzyme might produce a desirable end-product which could subsequently be degraded by another enzyme(s). This could be one reason for not being able to detect some of the intermediates of the "4S" pathway and also for not detecting the phenols which are perhaps broken down further. Different enzymes require specific cofactors and it is possible that under the conditions of obtaining and processing the fractions, key cofactors were lost or destroyed.

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The advantage of using crude fractions is that for complicated pathways such as those involved in the desulfurization process, it is seldom that only one enzyme is responsible for the entire process. Given the complications of assaying for substrates and products, it would be very difficult to work with a single purified enzyme and determine whether it is capable of causing partial or complete desulfurization. Furthermore, several enzymes would have to be identified and purified and optimal conditions determined for each of them before they could be used together in one process.

The work with GB-1, so far, has been with the "crude" enzyme fractions. The feasibility of GB-1 enzyme fractions to oxidize DBT has been demonstrated repeatedly, to varying degrees, depending on: (i) the fraction used; (ii) whether tested in aqueous or organic phase; and (iii) the kind of organic solvent used. The activity against DBT of ECF and ICF in organic solvent is of significance because for a substrate like coal, it might become important to use enzymes in organic solvent in order to increase the wetting of the coal and thereby make it more accessible to breakdown by enzymes. In addition, it has been demonstrated that although some loss of activity occurs on concentrating the fractions, the concentrated fractions can bring about measureable DBT oxidation in less than 24 hours.

Some of the major problems in this work have been in relation to assaying the samples and losses during evaporation and concentration. This resulted in perhaps not being able to detect some of the product(s) and also in errors in the quantitation of the compounds. Further research will have to focus on a good assay system for such compounds and on further optimization of the assay conditions under which the GB-1 fractions could be tested. It would also be useful to process and concentrate the fractions differently, perhaps by ammonium sulfate precipitation. As mentioned earlier, GB-1 has been shown to oxidize DBT to o,o'-biphenol and this activity remains to be optimized and exploited for use against coal.

# 4.0    WORK WITH COMMERCIALLY AVAILABLE ENZYMES

## 4.1    Summary of Work with Commercial Enzymes

Kinetic studies were designed to evaluate the ability of model compounds DBT and EPS and their sulfur oxidation products to inhibit activity of three enzymes, horseradish peroxidase, laccase, and sulfatase against standard substrates. Horseradish peroxidase activity vs. antipyrine was inhibited to some extent by DBT, to a lesser extent by DBT sulfoxide, and essentially not at all by DBT sulfone and biphenol. A similar pattern was seen in the EPS series, where increasing sulfur oxidation state resulted in less favorable binding to enzyme. These patterns held true also when the DBT and EPS series were evaluated as inhibitors of laccase activity vs syringaldazine. Sulfatase activity against p-nitrocatecholsulfate, on the other hand, was inhibited by small amounts of all of the sulfur containing model compounds, but not at all by the end product, biphenol. This kinetic work is described in Section 4.2 and discussed in Section 4.5.1.

Both laccase and horseradish peroxidase were studied extensively in hydrated organic solvents, e.g., dimethylformamide, acetonitrile, ethyl acetate, isopropyl ether, heptane, hexane, dioxane, benzene and toluene. Analytical methods were a dominant issue, as the amounts of products were generally very low and the appearance of unknowns was common. Difficulties were encountered in product concentration and in product identification partially because of the instabilities of the compounds and partially because of the mixed media from which they were isolated and the contaminations added by enzymes. These difficulties and the analytical approaches utilized are discussed in detail in Section 5.

Assays of horseradish peroxidase (with and without hydrogen peroxide) were carried out in organic solvents hydrated with distilled water or buffer with DBT as substrate. While in assays with peroxide, definite changes in DBT occurred as shown by UV spectroscopy, GC, GC-MS, and HPLC, the level of conversion was low and could be attributed to the peroxide. Products included oxidation at sulfur plus other unknown compounds. Without peroxide, no detectable sulfur oxidation occurred. The horseradish peroxidase assays are described in Section 4.3 and discussed in Section 4.5.2.

Laccase assays against DBT were also carried out in hydrated organic solvents, as with horseradish peroxidase, and it was shown by UV spectroscopy that DBT was altered. Analysis of reaction mixtures led to the conclusion that the products were not sulfur oxidation intermediates. No attempt was made to identify products other than the desired sulfur oxidation products. The laccase assays are described in Section 4.4 and discussed in Section 4.5.2.

## 4.2    Enzyme Kinetic Studies

### 4.2.1    Rationale

Although there had been limited prior work with laccase, horseradish peroxidase, and sulfatase with model coal compounds as substrate, it was of interest to demonstrate that the model compounds did indeed interact with the enzymes. In addition, as several steps of oxidation were required for sulfur removal, it was of interest to learn if any "intermediates" in the pathway to full sulfur removal, or final products would inhibit the enzyme activity. Because assays of activity against the sulfur-containing compounds were not well established, the approach of investigating the potency of the model compounds and sulfur oxidation intermediates as inhibitors of normal enzyme activity against known substrate was chosen.

The enzymes laccase, horseradish peroxidase and sulfatase were treated as if they followed Michaelis-Menton kinetics in interactions with "standard" substrates. This assumption has not been docum᷉ ᷉ed, however. For the reader not familiar with enzyme kinetics, the following simpl᷉᷉᷉ ᷉escription is given: in Michaelis-Menton kinetics, the enzyme is assumed to have one substrate binding site; rates are established for substrate binding, changes in substrate/enzyme conformation, covalent changes in substrate, and release of products from the enzyme. For each enzyme/substrate combination, under set conditions, there is a maximum overall rate of catalysis, and this is called $K_{cat}$ or $V_{max}$. Assuming that the concentration of enzyme is low compared with the concentration of substrate, the actual rate depends on the concentration of substrate until $K_{cat}$ is reached. The concentration of substrate at which a rate equal to 1/2 of $K_{cat}$ is called $K_m$.

These kinetics can be perturbed when another compound added to the reaction interacts with the enzyme. In some cases the second compound enters the same binding site as the "standard" substrate. If this second compound can reversibly enter the substrate binding site, it will compete with substrate and, for a given concentration of substrate, the observed rate will drop. In this situation the second compound is a **competitive inhibitor** of the reaction of enzyme with "standard" substrate.

The normal kinetics of the "standard" substrate with enzyme can also be perturbed by a second compound which binds reversibly to the enzyme at a site other than the substrate binding site. Such binding can also cause a decrease in enzyme activity, and this decrease cannot be overcome by increasing the amount of standard substrate. Such inhibition is called **noncompetitive inhibition**. The type of inhibition can be determined by graphing the results of assays at various substrate and inhibitor concentrations in certain ways. One type of graph used for this purpose is the **Lineweaver-Burke** graph.

If assays are carried out with various concentrations of the second compound, the concentration which causes the rate to drop by 50% , called the $I_{50}$ can be determined. The smaller the $I_{50}$, the more potent the inhibition. A far more complete discussion of enzyme kinetics can be found in Allan Fersht's excellent book.[26]

The relevance of the above discussion to the present work is that when it is not known if or how a specific enzyme will act on a new substrate, and how expected catalysis products will affect the kinetics of the proposed reaction, the new compounds can be added to reactions carried out with "standard" substrates, and the types of perturbations in observed rate can reveal the extent and types of interactions which may occur. In this case it was possible to study the interactions of the three enzymes with the model coal compounds DBT and EPS, and also with the desired sulfur oxidation intermediates and (in the case of DBT) the final reaction product. In these studies, the DBT and EPS compounds were considered inhibitors; any catalytic activity which occurred on these compounds would cause greatly complicated kinetics. The studies performed did not consider these pathways and thus the results are considered preliminary and qualitative.

## 4.2.2    Experimental

The relative potency of the organic sulfur compounds as inhibitors of the model coal compounds and their stepwise sulfur oxidation products, as inhibitors of horseradish peroxidase, laccase, and sulfatase in standard conditions of assay were measured. The assay procedures for each enzyme (i.e., the defined substrates, buffers, and reagents that are conventionally used for determining specific activity of each enzyme) are described below.

**Horseradish Peroxidase:** In both the $I_{50}$ and kinetic determinations, horseradish peroxidase was assayed in the presence of hydrogen peroxide as cofactor and 4-aminoantipyrine in phenol as substrate/indicator in 25 mM Tris buffer, pH 6.0. The total reaction volume was 1.5 ml including organic solvent. Blanks were run without $H_2O_2$ and without enzyme. Activity was measured at 510 nm. Enzyme was added to a complete reaction mixture at t = 0 and at t = 5 minutes, the activity was read over a period of three minutes reaction time.

**Laccase:** For both the $I_{50}$ and kinetic determinations, laccase activity was assayed with the substrate syringaldazine in organic solvent and the enzyme in a buffer of 0.1 M sodium phosphate, pH 6.5. A blank was run without enzyme. Activity was measured at 530 nm. Enzyme was added at t = 0 and t = 8 minutes, the activity was read over a period of three minutes reaction time.

**Sulfatase:** Sulfatase activity was found to be especially sensitive to temperature, so all assays were run at a constant temperature of 37°C. For both the $I_{50}$ and kinetic determinations, sulfatase activity was assayed with p-nitrocatechol sulfate as substrate in a buffer of 0.2 M sodium acetate, pH 5.0. Parallel assays were set up to contain substrate, buffer, organic solvent, inhibitor, and enzyme or an equal volume of buffer. In the assays without enzyme (blanks), 5 ml of 1 N NaOH were added at t = 0 to stop the reaction. Blanks and enzyme assays were incubated in a water bath for 30 minutes. At t = 30 minutes, the enzyme assays were stopped with 5 ml of 1 N NaOH. The t = 0

blanks were zeroed at 515 nm, and the activity of the enzyme assays were read as single points.

For all three enzymes, dose-response curves were developed with at least five different substrate concentrations, and the data were evaluated by linear regression analysis of double-reciprocal (Lineweaver-Burke) plots of activity vs. substrate concentration. The following organic sulfur compounds were examined: DBT (Aldrich), DBT sulfoxide (K&K Biochemicals), DBT sulfone (Lancaster Biochemicals), EPS (Aldrich), ethylphenylsulfoxide (Lancaster Biochemicals), ethylphenylsulfone (Lancaster Biochemicals), and o,o'-biphenol (Aldrich).

## 4.2.3 Results

The data presented in Tables 4.1 and 4.2-A-C summarize the inhibition of three different enzymes by organic sulfur compounds and the development of kinetic constants to describe the quantitative interaction between enzyme(s) and inhibitor(s).

As indicated in the legends to the tables, these kinetic measurements were carried out in ratios of organic solvent (dimethylformamide) to aqueous buffer that provided maximal solubility of both the enzyme and the organic sulfur compound (i.e. inhibitor) while retaining the expected enzyme activity against the standard substrates. While that is generally in the range of 85:15 (dimethylformamide:buffer) for laccase and sulfatase, it was found that horseradish peroxidase required ratios closer to 50:50 for measurable activity.

The data in Table 4.1 demonstrate that for each series of organic sulfur compounds (i.e., DBT and EPS and their respective oxidized products, DBT sulfoxide, DBT sulfone, o,o'-biphenol, EPS sulfoxide, and EPS sulfone) there are some significant differences in ability to inhibit enzyme activity against conventional substrates. Horseradish peroxidase activity vs. antipyrine was inhibited to some extent by DBT, to a lesser extent by DBT sulfoxide, and essentially not at all by DBT sulfone and biphenol. A similar pattern was seen in the EPS series where increasing sulfur oxidation state resulted in less binding to enzyme. These patterns held true also with the DBT and EPS series and laccase. Sulfatase activity against p-nitrocatecholsulfate, on the other hand, was inhibited by small amounts of all of the sulfur containing model compounds, but not at all by the end product, biphenol. If one accepts the $I_{50}$ as a reasonable indication of binding of organic sulfur compound to the enzyme one can draw certain conclusions that are applicable to the proposed use of the enzymes for oxidation or desulfurization of the sulfur compounds.

The thermodynamic nature of the interactions between the organic sulfur compounds and each enzyme was examined by running experiments with a single concentration of inhibitor (close to the $I_{50}$) over a range of substrate concentrations. This permitted plotting of conventional double-reciprocal plots. The results are shown in Tables 4.2A-C. However, there are at least some indications that binding of the organic sulfur compounds may occur outside the catalytic site for the conventional

TABLE 4.1: DETERMINATIONS OF $I_{50}$ FOR ORGANIC SULFUR COMPOUNDS AND RELATED DERIVATIVES: EFFECTS ON OXIDATIVE AND HYDROLYTIC ENZYMES

| Substrate | $I_{50}$ (mM) | | |
|---|---|---|---|
| | HRP | Laccase | Sulfatase |
| DBT | $2.8\pm1.2$ | $1.5\pm0.47$ | $0.80\pm0.30$ |
| DBT Sulfoxide | $8.1\pm0.2$ | $8.6\pm2.6$ | $0.32\pm0.03$ |
| DBT Sulfone | >30 | >20 | $0.54\pm0.03$ |
| o,o'-Biphenol | >40 | >20 | >30 |
| EPS | $4.2\pm0.2$ | $3.9\pm4.3$ | $0.34\pm0.09$ |
| EPSulfoxide | $8.8\pm1.5$ | $8.8\pm3.5$ | $0.31\pm0.05$ |
| EPSulfone | >30 | >20 | $0.64\pm0.05$ |

The $I_{50}$ is the concentration of inhibitor that reduces enzyme activity by 50% under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

Data are presented as the mean $\pm$ standard deviation of four experiments.

## TABLE 4.2-A: KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS: INHIBITION OF HORSERADISH PEROXIDASE

| Substrate | $K_m$ (mM) | $V_{max}$ (delta O.D./min.) |
|---|---|---|
| Control | 1.9±0.25 | 0.08±0.009 |
| DBT | 0.8±0.21 | 0.06±0.04 |
| EPS | 0.8±0.26 | 0.03±0.009 (NC) |
| Control | 6.1±2.0 | 0.15±0.04 |
| DBT sulfoxide | 6.6±1.0 | 0.13±0.16 |
| EPSulfoxide | 7.9±0.59 | 0.18±0.06 |

$K_m$ and $V_{max}$ were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

Horseradish peroxidase was purchased from Sigma Chemical Company (P-3912) as a suspension in 2.0 M $(NH_4)_2SO_4$, pH 7.0.

Data are presented as the mean ± standard deviation of four experiments.

NC = apparent noncompetitive inhibition

# TABLE 4.2-B: KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS: INHIBITION OF LACCASE

| Substrate | $K_m$ (mM) | $V_{max}$ (delta O.D./min.) |
|---|---|---|
| Control | 0.05±0.03 | 0.12±0.05 |
| DBT | 0.35±0.001 | 0.52±0.04 |
| EPS | 0.22±0.13 | 0.33±0.15 (NC) |
| DBT sulfoxide | 0.08±0.07 | 0.14±0.08 |
| EPSulfoxide | 0.45±0.38 | 0.53±0.38 |

$K_m$ and $V_{max}$ were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

Laccase was purchased from Sigma Chemical Company (L-5510) from Pyricularia oryzae.

## TABLE 4.2-C: KINETIC CONSTANTS FOR ORGANIC SULFUR COMPOUNDS: INHIBITION OF SULFATASE

| Substrate | $K_m$ (mM) | $V_{max}$ (delta O.D./min.) |
|---|---|---|
| Control | $0.28 \pm 0.03$ | $0.48 \pm 0.02$ |
| DBT | $0.83 \pm 0.44$ | $0.49 \pm 0.08$ (C) |
| EPS | $1.1 \pm 0.55$ | $0.59 \pm 0.19$ (C) |
| DBT sulfoxide | $0.48 \pm 0.22$ | $0.29 \pm 0.07$ (NC) |
| EPSulfoxide | $0.34 \pm 0.09$ | $0.25 \pm 0.04$ (NC) |
| DBT Sulfone | $0.27 \pm 0.08$ | $0.34 \pm 0.04$ (NC) |
| EPSulfone | $0.62 \pm 0.20$ | $0.55 \pm 0.11$ (C) |

$K_m$ and $V_{max}$ were determined by double-reciprocal evaluation of enzyme activity data measured under standard conditions of assay (described in the text) and optimal ratios of organic solvent (dimethylformamide) to aqueous buffer.

Sulfatase was purchased from Sigma Chemical Company (S-9751), Type H-2 from Helix pomatia.

Data are presented as the mean $\pm$ standard deviation of four experiments.
C = apparent competitive inhibition
NC = apparent noncompetitive inhibition

substrates (i.e., noncompetitive inhibition was evident with some of the compounds). Also, it is likely that the modulation of these enzymes by the organic sulfur compounds may be significantly affected by the enzymatic action on the "inhibitor" itself. This would certainly account for the apparently mixed nature of the effects on kinetic parameters.

In order to further evaluate enzyme inhibition by DBT, EPS, and oxidized products, the reversibility of the binding of the compounds to the enzymes of interest was measured. These experiments were done by simply varying the preincubation time for enzyme and test compound (before triggering the reaction by addition of standard substrate) recognizing that an increase in preincubation time will generally produce an apparently lower $I_{50}$ for irreversible ligands. The results are shown in Figure 4.1. Statistical analysis of the data by t-test demonstrated that all of the interactions studied were reversible.

## 4.3    Horseradish Peroxidase Assays

### 4.3.1    Activity Against Standard Substrate in Organic Media

The studies with the hydrogen peroxide-horseradish peroxidase enzymatic system were first performed in hydrated dimethylformamide and hydrated dioxane with standard substrate. As shown in Table 4.3, enzyme activity was very well maintained in a hydrated dimethylformamide matrix and was partially maintained in the dioxane.

Assays were performed either at room temperature or at 30°C. Except as noted, enzymes were dissolved in buffer or water at 20 mg/ml concentration. Hydrogen peroxide (200 nM) was added to the enzyme solution in peroxidase assays and the concentrated enzyme solution was then added to the organic media. Vigorous agitation was utilized in assays. Aliquots were taken at various intervals and filtered to remove enzyme.

### 4.3.2    Activity Against DBT in Aqueous Organic Media

**UV Screening of Assays in 0.5% Aqueous Media, DBT [0.29mM]**
Change in UV spectrum was initially used as a monitor of horseradish peroxidase activity against DBT, as it had been shown that scans of DBT, DBT sulfoxide, DBT sulfone, and biphenol showed certain differences (see Section 5.1).

Dimethylformamide, dioxane, and a range of solvents of lower hydrophilicity (heptane, isopropyl ether, hexane, ethyl acetate, toluene, and benzene) were selected to evaluate horseradish peroxidase activity. Both the amount of DBT added and the amount of enzyme used were varied. In general, the DBT was added directly to the solvent. Horseradish peroxidase (20 mg/ml) plus 200 nM $H_2O_2$ was dissolved in double distilled water (pH 5.5). The trace concentration of $H_2O_2$ was selected as that which is just below the threshold for producing spectrophotometric interference. 100 - 250 $\mu$l of enzyme-cofactor mixture was added to each 20 ml solvent-substrate sample. Each sample was incubated with continuous agitation to overcome diffusional energy reaction barriers. Each sample was analyzed by UV spectroscopy at T = 1 hour, 5 days, and 7 days. While

65
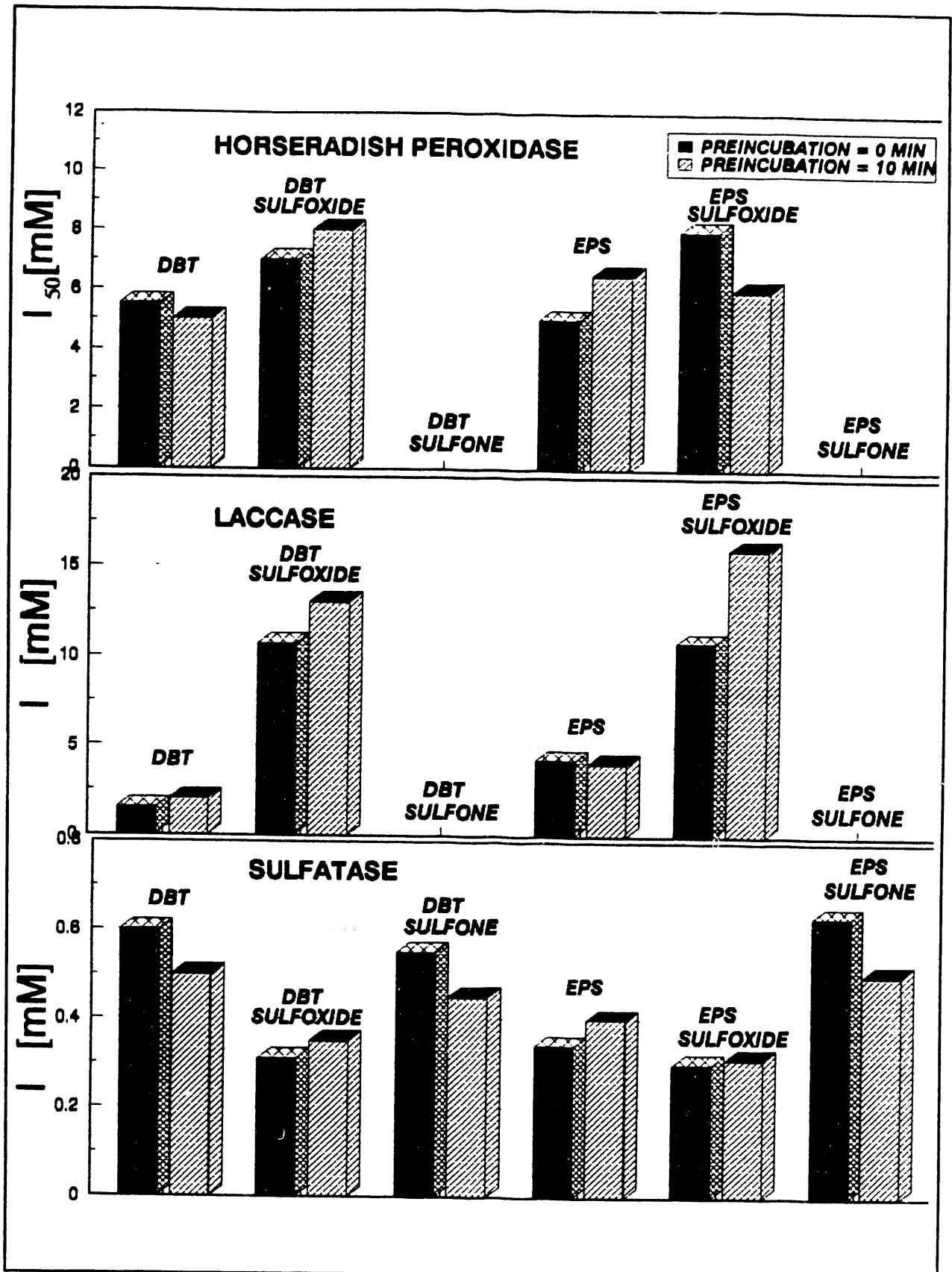
FIGURE 4.1: REVERSIBILITY OF ENZYME INHIBITIONS IN $I_{50}$ EXPERIMENTS

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## TABLE 4.3: RETENTION OF HORSERADISH PEROXIDASE ACTIVITY IN 5% AQUEOUS DIOXANE OR DIMETHYLFORMAMIDE

| Assay System | Enzyme Activity (% Control) |
|---|---|
| Standard Buffer | 100% |
| 5% Buffer + 95% Dioxane | 10 to 20% |
| 5% Buffer + 95% Dimethylformamide | 100% |

Buffer = 2.5 mM Tris, pH 6.2.
Standard Buffer = Antipyrine/phenol substrate in peroxide/acetate buffer reagent system. This horseradish peroxidase assay procedure monitors the development over time of color at 510 nm with antipyrine in phenol as the substrate, $H_2O_2$ as co-factor. The reaction is linear for at least 20 minutes under the conditions of assay. Enzyme activity in solvent plus buffer was calculated as delta O.D./minute and expressed as % control.

changes in spectra were observed (data not shown), it was difficult to identify trends, especially since solvent evaporation caused loss of control of reactant concentrations.

In any event, it was evident that UV spectrophotometric analyses could only provide qualitative information in regard to chemical alterations of the organic sulfur "model" compounds.

### GC and HPLC Monitoring of Assays in Several 0.5% Aqueous Solvents [DBT, 0.2 mM] and [DBT, 20mM] with Laccase Added

Attention was then given to gas chromatographic techniques to obtain more quantitative analyses and identification of the reaction products. One set of the above experiments was repeated to obtain analysis by gas chromatography. The DBT concentration was 0.29 mM. An aliquot of each sample was filtered and analyzed by GC at 7 days. The GC results are shown in Table 4.4. It can be seen that several new compounds appeared at this time, particularly in the heptane, hexane, and ethyl acetate media. It was observed that the two most apolar solvents, heptane and hexane, seemed to provide good media for the enzymatic reaction of horseradish peroxidase and DBT with only trace amounts of $H_2O_2$ added. In experiments with ethyl acetate, a considerably more polar solvent, a second substantial peak with a very long retention time on the GC was observed.

On Day 8, to the above solutions, enough new DBT was added to make them 20 mM. The toluene and benzene systems were discarded. The others (heptane, hexane, isopropyl ether, and ethyl acetate) were kept under agitating conditions until Day 12 when laccase was added to the media. These dual enzyme samples were shaken for 2 additional days prior to GC analysis. Results (day 14) are summarized in Table 4.4. As the proportions of observed materials were altered substantially for all of the samples, it appeared that laccase had exhibited substantial activity. Subsequent experiments (data not shown) did not confirm this observation.

### 1% Aqueous Acetonitrile, Ethyl Acetate, and Isopropyl Ether [DBT 3.0 mM]

The assays of horseradish peroxidase against DBT in three hydrated solvents (acetonitrile, ethyl acetate, and isopropyl ether) were repeated with DBT at 3.0 mM. The protocols were as follows: DBT stocks with 3.0 mM DBT were prepared. Aliquots of 9.5 ml solvent/DBT were mixed with 0.1 ml enzyme stock (which was 1 mg/ml). The vials were sealed and kept shaking vigorously. Samples were removed and filtered at 1 hour and 24 hours. Some brown color was observed in all the horseradish peroxidase vials. There was one vial for each solvent type. The samples were analyzed by HPLC.

The results of this set of experiments are shown in Figure 4.2 A&B. Figure 4.2-A represents the concentrations of DBT sulfoxide, DBT sulfone, and o,o'-biphenol in the reaction mixture at 1 hour and 24 hours. Figure 4.2-B shows the DBT concentrations at 1 and 24 hours. It can be seen that the DBT concentrations were all above 3 mM at T = 1 hr, indicating some solvent evaporation prior to analysis. This difficulty was most

## TABLE 4.4: GC ANALYSIS OF HORSERADISH PEROXIDASE ASSAYS IN 5% AQUEOUS MEDIA VS DBT

| Solvent | Time (Days) | [DBT] (mM) | Percents of Total Area at Various Retention Times (min) | | | |
|---|---|---|---|---|---|---|
| | | | 11.6 min | 11.7 to 17.6 min | 17.9 to 20 min | 20.1 to 24 min |
| Initial Assay | | | | | | |
| Heptane | 7 | 0.29 | 82.8 | 12.5 | 4.7 | 0.0 |
| Isopropyl Ether | 7 | 0.29 | 95.3 | 2.1 | 1.6 | 0.0 |
| Hexane | 7 | 0.29 | 85.8 | 14.2 | 0.0 | 0.0 |
| Ethyl Acetate | 7 | 0.29 | 68.4 | 14.1 | 5.0 | 12.5 |
| Benzene | 7 | 0.29 | 90.7 | 4.5 | 2.5 | 2.3 |
| Toluene | 7 | 0.29 | 97.4 | 2.6 | 0.0 | 0.0 |
| Laccase Added on Day 12 | | | | | | |
| Heptane | 14 | 20 | 99.1 | 0.4 | 0.5 | 0.1 |
| Isopropyl ether | 14 | 20 | 99.8 | 0.2 | 0.0 | 0.0 |
| Hexane | 14 | 20 | 100.0 | 0.0 | 0.0 | 0.0 |
| Ethyl acetate | 14 | 20 | 99.9 | 0.0 | 0.1 | 0.0 |

Standards run in dioxane show retention times as follows:

| | |
|---|---|
| Biphenol | - 10.42 min.; |
| DBT | - 11.6 min.; |
| DBT Sulfoxide/DBT Sulfone | - 17.7 min. |
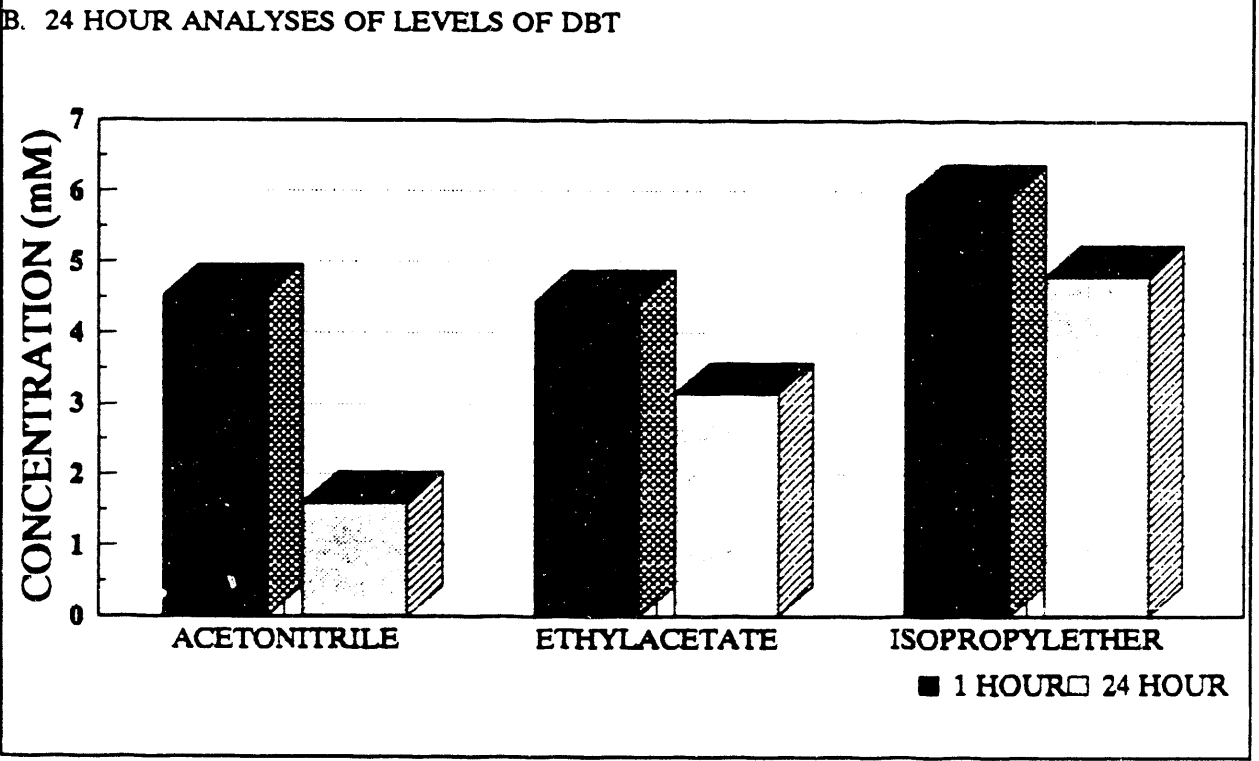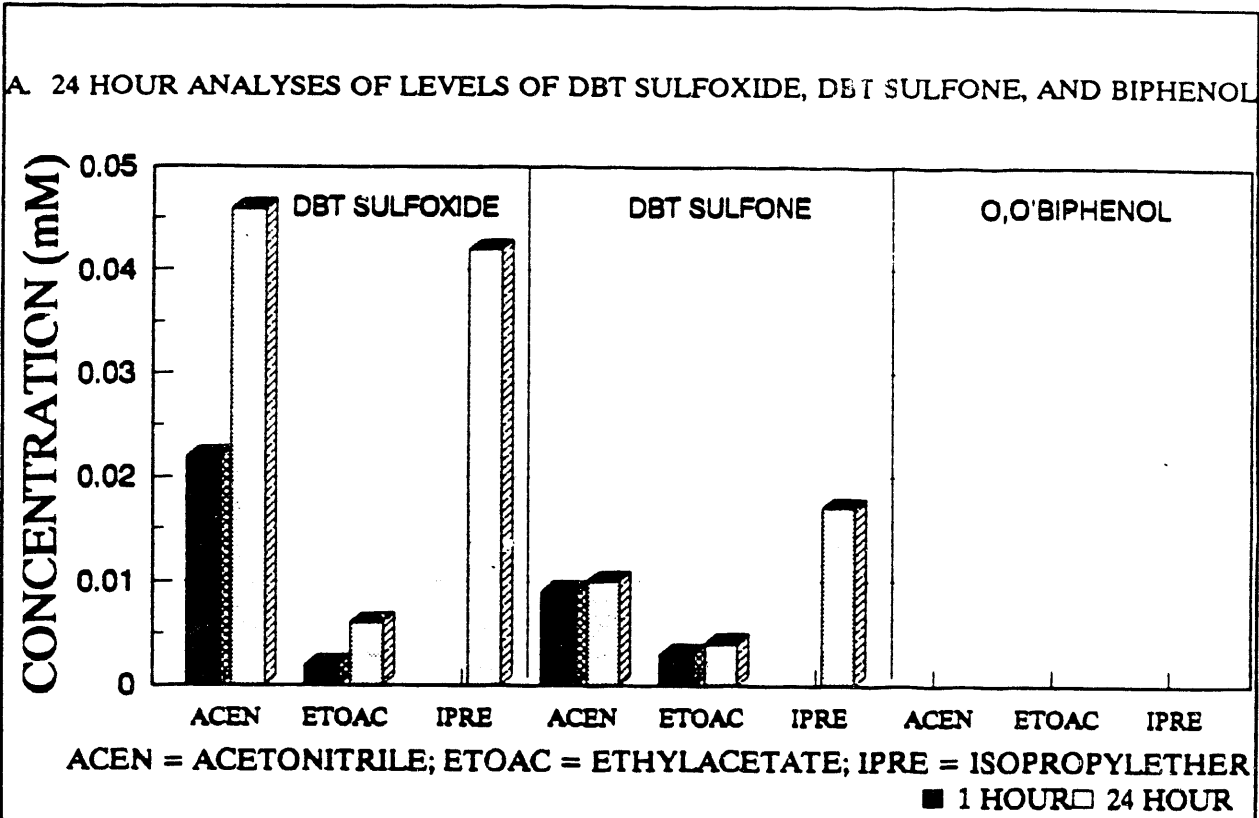
FIGURE 4.2: ASSAYS OF HORSERADISH PEROXIDASE IN ACETONITRILE, ETHYLACETATE, AND ISOPROPYL ETHER VS DBT

severe in the isopropyl ether. Because of the small sample numbers, no significance can be attached to the observed decreases. Peaks occuring at the retention times of sulfoxide and sulfone appear in all samples at 24 hours in low concentrations.

### 1% Aqueous Acetonitrile and 50% Aqueous Acetonitrile, Dimethylformamide, and Ethyl Acetate [0.1mM DBT]

A stock solution of 0.1 mM DBT in acetonitrile was prepared and 9.9 mls was added to each of 2 glass scintillation vials. To one vial was added 100 $\mu$l of 1 mg/ml horseradish peroxidase in distilled water. As a control, 100 $\mu$l of distilled water was added to the second vial. The two vials were placed on a rotating shaker at room temperature.

At 1, 24, and 48 hours, 500 $\mu$l was removed from each sample and filtered through a 0.22 $\mu$ Nylon 66 syringe filter unit. After seven days (168 hours) a final aliquot was taken from each sample. These aliquots were screened by GC and HPLC for the presence of DBT oxidation products and the results can be found in Table 4.5-A (first experiment). The concentrations of DBT observed, particularly at the later time points, were lower than the actual initial DBT level. This difference was presumably due to losses during sample handling, although some conversion to unknown products may have occurred. The decrease in concentration of DBT and concomitant increase in DBT sulfoxide/DBT sulfone at 168 hours indicated that some sulfur oxidation had occurred.

A repeat of this experiment was started using more samples in order to confirm the preliminary findings. A new stock solution of 0.1 mM DBT in acetonitrile was prepared. A sample of this stock solution was filtered through a 0.22 $\mu$ filter unit and retained as a time = 0 control.

To each of six screw cap tubes, a total of 4.95 ml of the 0.1 mM DBT stock was added. To each of three glass scintillation vials, 9.9 ml of this stock was added. Fifty (50) $\mu$l of 1 mg/ml horseradish peroxidase in distilled water was added to three screw cap tubes containing DBT. 100 $\mu$l of the horseradish peroxidase solution was added to two of the scintillation vials. Finally, 50 $\mu$l of distilled water was added to the last set of three screw cap tubes and 100 $\mu$l distilled water was added to the last scintillation vial. The samples were placed on a shaker at ~24°C for rapid mixing.

500 $\mu$l aliquots were removed at 1, 24, and 48 hours and filtered through the 0.22 $\mu$ filter units. Larger aliquots of ~3 mls were removed and filtered at time $\approx$ 168 hours. In order to have the products of interest be as concentrated as possible for detection, the 168 hour aliquots were evaporated to dryness prior to analysis. Each aliquot was placed 100 $\mu$l at a time in a test tube in a heating block at 60°C. A gentle stream of $N_2$ was blown over the liquid to help speed the evaporation of solvent to dryness. This was repeated until a total of 500 $\mu$l of each aliquot had been reduced to dryness. The dried sample was then redissolved in 50 $\mu$l of acetonitrile. The 168 hour concentrated aliquots were then analyzed by GC and HPLC and some were analyzed by GC/MS. Table 4.5-B

## TABLE 4.5-A: HPLC AND GC ANALYSIS OF HORSERADISH PEROXIDASE ACTIVITY VS DBT IN ACETONITRILE

| Time | Method | o,o'-Biphenol (mM) | DBT (mM) | DBT* Sulf- oxide (mM) | DBT* Sulfone (mM) | DBT** oxide/one (mM) | o-Hydroxy Biphenyl (mM) |
|------|--------|------|------|------|------|------|------|
| **First Experiment** | | | | | | | |
| 1 | GC | ND | | | | | |
|   | HPLC | 0.0 | 0.076 | 0.0 | 0.0 | -- | 0.0 |
| 24 | GC | ND | | | | | |
|   | HPLC | 0.0 | 0.087 | 0.0 | 0.0 | -- | 0.0 |
| 48 | GC | 0.002 | 0.08 | -- | -- | 0.0 | 0.0 |
|   | HPLC | 0.0 | 0.029 | 0.012 | 0.0 | -- | 0.0 |
| 168 | GC | 0.0 | 0.16 | -- | -- | 0.44 | 0.0 |
|   | HPLC | 0.0 | 0.022 | 0.06 | 0.035 | -- | 0.0 |
| **First Experiment Control** | | | | | | | |
| 1 | GC | ND | | | | | |
|   | HPLC | 0.0 | 0.068 | 0.0 | 0.0 | -- | 0.0 |
| 24 | GC | ND | | | | | |
|   | HPLC | 0.0 | 0.89 | 0.0 | 0.0 | -- | 0.0 |
| 48 | GC | 0.0 | 0.0 | -- | -- | 0.0 | 0.0 |
|   | HPLC | 0.0 | 0.073 | 0.0 | 0.0 | -- | 0.0 |
| 168 | GC | 0.0 | 0.017 | -- | -- | 0.0 | 0.0 |
|   | HPLC | 0.0 | 0.049 | 0.0 | 0.0 | -- | 0.0 |

* = HPLC only; ** = GC only
ND = sample not analyzed

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## TABLE 4.5-B: HPLC AND GC ANALYSIS OF HORSERADISH PEROXIDASE ACTIVITY VS 1% mM DBT IN AQUEOUS ACETONITRILE

| Time | Method | o,o'- Biphenol (mM) | DBT (mM) | DBT* Sulf- oxide (mM) | DBT* Sulfone (mM) | DBT** oxide/one (mM) | o- Hydroxy Biphenyl (mM) |
|------|--------|------|------|------|------|------|------|
| Second Experiment | | | | | | | |
| 168 | GC | 0.0 | 0.05 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.052 | 0.0 | 0.0 | -- | 0.0 |
| 168 | GC | 0.0 | 0.06 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.048 | 0.0 | trace | -- | 0.0 |
| 168 | GC | 0.0 | 0.02 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.038 | 0.0 | 0.0 | -- | 0.0 |
| 168 | GC | 0.0 | 0.02 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.04 | 0.0 | 0.0 | -- | 0.0 |
| 168 | GC | 0.0 | 0.02 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.039 | 0.0 | trace | -- | 0.0 |
| Second Experiment Control | | | | | | | |
| 168 | GC | 0.0 | 0.02 | -- | -- | 0.0 | 0.0 |
|     | HPLC | | 0.037 | trace | trace | -- | 0.0 |
| 168 | GC | 0.0 | 0.017 | -- | -- | 0.0 | 0.0 |
|     | HPLC | | 0.049 | 0.0 | 0.0 | -- | 0.0 |

* = HPLC only;  ** = GC only

gives a summary of the results of the GC and HPLC analysis of the second experiment. Traces of DBT sulfone were seen in some samples, however, similar observations were made in controls. Thus, no oxidation of DBT at sulfur could be claimed.

Subsequently, some concern was raised that the horseradish peroxidase used in the second experiment described above might not have been as active as previously thought. To determine that his was not the cause of the irreproducibility of the results, a new lot of enzyme was obta.ned for the following round of experiments. Stock solutions of DBT at 0.2 mM were prepared using the following solvents: acetonitrile, dimethylformamide, and ethyl acetate. A stock of 0.1 mM DBT in acetonitrile was also used.

As a third repeat of the horseradish peroxidase assay in 1% acetonitrile, 4.95 ml of 0.1 mM DBT in acetonitrile was added to each of four screw cap test tubes. To two of the tubes, 50 $\mu$l of horseradish peroxidase (1 mg/ml in distilled water) solution was added while 50 $\mu$l of distilled water was added to the second pair of tubes.

For each of the 0.2 mM DBT stock solutions in the three solvents, 2.5 ml was added to each of four screw cap test tubes. For each set, 2,450 $\mu$l distilled water and 50$\mu$l of enzyme (10 mg/ml) in distilled water were added to two tubes while 2,500 $\mu$l distilled water was added to the second pair of tubes in each set. All four sets of tubes were shaken to mix and a 0.5 ml aliquot was removed and filtered through a 0.22 $\mu$ syringe filter unit for a time = 0 sample.

All sixteen tubes were placed in a shaker housed in a constant temperature room with the temperature at 24°C. Depending on the sample set, an intermediate time aliquot (1 ml) was removed from each tube at time = 2 or 3 days. In the case of the 50% ethyl acetate samples, the tubes were allowed to stand and the aqueous and organic layers were allowed to separate before 0.5 mls were removed from each layer.

Since experiments one and two of this series had been performed without use of the co-factor, $H_2O_2$, no $H_2O_2$ had been added to the samples in these third experiments. The rationale for not using peroxide is discussed in Section 4.7. In order to get the most information, it was decided to modify experiment three on day 2 or 3 by adding enough $H_2O_2$ to each of the tubes to have the final concentration of 0.05 mM in each sample tube. Thus, after the withdrawal of the intermediate time aliquots in experiment 3, 17.3 $\mu$l of 0.0101 M $H_2O_2$ solution (31% $H_2O_2$ diluted 1,000 times) was added to the remaining 3.5 ml of sample in each tube. The tubes were returned to the shaker. Aliquots were taken on day 5 or 6 and analyzed from experiment 3.

The results (data not shown) indicated no enzyme activity. Sulfur oxidation products were found sporadically in samples, especially in those to which peroxide had been added and in those which were 50% aqueous.

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Examination of H₂O₂/DBT Reactivity

For these experiments, 20 mM DBT solutions in isopropyl ether were prepared. Four concentrations of $H_2O_2$ were tested: u.3, 0.15, 0.06, and 0.03 percent. The analyses were made after three days. The GC results, shown in Table 4.6, indicate that at these concentrations some oxidation does occur. It should be noted that the amount utilized in the experiments is much less than 0.03 percent.

TABLE 4.6: GC ANALYSIS: CONCENTRATION EFFECT OF $H_2O_2$ ON DBT IN ISOPROPYL ETHER

PERCENTS OF AREAS UNDER CURVES AT VARIOUS
RETENTION TIMES

| Conc. H2O2 (%) | RT 10.42* (%) | RT 11.6** (%) | RT 11.7 to 17.6 (%) | RT 17.7 to 17.9 *** (%) | RT 17.9 to 20 (%) | RT 20.1 to 24 (%) |
|---|---|---|---|---|---|---|
| 0.3 | 0.0 | 97.1 | 0.8 | 0.7 | 41.3 | 0.0 |
| 0.15 | 0.0 | 93.5 | 0.3 | 0.0 | 6.1 | 0.0 |
| 0.06 | 0.0 | 99.4 | 0.0 | 0.0 | 0.6 | 0.0 |
| 0.03 | 0.0 | 95.6 | 0.3 | 0.0 | 4.1 | 0.0 |

* Diphenol
** DBT
*** D3T sulfoxide/DBT sulfone

## 4.4 Laccase Assays

### 4.4.1 Standard Substrate (Syringaldazine) in Buffer

The standard assay method for laccase was obtained from Sigma. The method is as follows.

1.    Dissolve laccase in water (concentration not given); solutions of about 2 mg/ml laccase and also 10X and 100X dilutions were used.

2.    Prepare 0.1 M (potassium) phosphate buffer at pH 6.5.

3.    Dissolve 7 mg of syringaldazine in 100 ml of methanol ($1.94 \times 10^{-4}$ M).

4.          3 mls of buffer are warmed to 30°C in a test tube, 1 ml of enzyme solution (kept at room temperature) is added, and the tube is covered and inverted several times to mix.

5.          0.5 ml of the syringaldazine solution (prewarmed to 30°C) is added and the solution is mixed again by tube inversion.

6.          At 5 minutes, a sample is taken and the absorbance is read at 530 nm vs. a solution of buffer and enzyme (the enzyme does not have absorbance at this wavelength). Using this method, one activity unit is that amount of enzyme which gives an increase in absorbance at 530 nm of 0.001/minute.

The enzyme received from Sigma had activity as stated on the package label. The assay was to be modified for assays in organic solvents and it was possible that the assay product would not have absorbance above 500 nm in the solvents tested. Thus it was important to look also at the decrease in the absorbance of syringaldazine during the reaction.

To assess the correlation of decrease in absorbance at the lambda maximum of syringaldazine (352 nm in buffer) with the increase in absorption at 530 nm, the assay was repeated, making measurements at 352 and 530 nm every 10 seconds for 10 minutes. The assay was linear over the ten minute period. The slope of the appearance of product (A530) ($1.2 \times 10^{-3}$ absorbance units/minute) was a little more than twice as large as the slope of the disappearance of substrate ($-5.1 \times 10^{-4}$ absorbance units/minute), probably due to the differences in extinction coefficients for substrate and product (data not shown). The ability to correlate the decrease of UV absorbance with activity was important because the assay product does not have the high absorbance in the visible range in the organic media which are to be utilized.

### 4.4.2    Standard Substrate (Syringaldazine) in 5% Aqueous Dimethylformamide

Syringaldazine dissolves in both hydrated dioxane and hydrated dimethylformamide. The solutions are stable at elevated temperatures (30°C) as seen by unchanging spectra with maximum absorbance at 360 nm in the dimethylformamide and 356 nm in the dioxane. The solutions are transparent above 500 nm.

The enzyme, when placed directly (dry method) in hydrated dimethylformamide or dioxane, does not dissolve completely. When enzyme is first suspended in buffer or water (wet method) and this solution is then added to the organic solvent, a clear or nearly clear solution is obtained. The amount of activity in these suspensions or solutions is not necessarily a function of clarity, however, the suspensions have been found to contain active enzyme. Several combinations of media and methods of preparation of laccase reagent have been employed.

The preliminary assay method utilized laccase in hydrated organic solvents. For example: an aliquot of 12.5 mg of laccase was added dry to 5 ml of 5% aqueous dimethylformamide. There was undissolved material in the container and a portion of it was filtered through a 0.45 $\mu$ filter. An aliquot of 0.1 ml of enzyme solution was mixed with 2.4 ml aqueous dimethylformamide in a cuvette, then 0.5 ml of syringaldazine in the aqueous dimethylformamide was added. After mixing, the cuvette was placed in the UV/VIS spectrophotometer and kept at 30°C with a circulating water bath. Scans were made from 220 to 600 nm every 10 minutes. It was found that no change in absorbance occurred above 500nm, however, the absorbance at 360 nm did decrease with time and the shape of the scan at the lower wavelengths changed over a two hour period.

The test was run twice; the enzyme solution was kept at room temperature for several hours in between runs. In both cases, there was an apparent reaction (judging from decrease at 360 nm) but the rates of decrease were vastly different, the second test rate was about 1/100th of the first. It appeared that the enzyme activity was decreased about 100 fold in the course of the day. The results of the two tests are shown in Figures 4.3A and B. It is interesting to note that in this assay the apparent rate of the reduction of lambda maximum of substrate was -9.9 x $10^3$ units/minute, whereas in the buffered system the rate of reduction was -5.1 x $10^4$ units/minute. Possibly differences in extinction coefficient in solvent account for the different apparent rates.

The standard assay procedure given in Section 4.4.1 was modified by substituting hydrated dioxane or hydrated dimethylformamide for all other solvents (e.g., 3 ml media, 1 ml enzyme in media, 0.5 ml substrate in media). A 0.001 M phosphate buffer at pH 6.5 was prepared and found to be miscible with the solvents. The following assay conditions were used:

a. Buffer/Dioxane: The stock syringaldazine was 1.8 x $10^4$ M in 5 percent buffer/dioxane.

   1. Wet method of enzyme addition: The stock laccase solution was 0.042 mg/ml in 5 percent dioxane/buffer.

   2. Dry method of enzyme addition: The stock laccase solution as 0.02 mg/ml in 5 percent dioxane/buffer.

b. Buffer/dimethylformamide: The stock syringaldazine solution was 2.1 x $10^4$ M in 5 percent buffer/dimethylformamide.

   1. Wet method of enzyme addition: The stock laccase solution was 0.042 mg/ml in 5 percent buffer/dimethylformamide.

   2. Dry method of enzyme addition: The stock laccase solution was 0.02 mg/ml in 5 percent buffer/dimethylformamide.

A. First Reaction

SLOPE = -0.0099

SYRINGALDAZINE ALONE          LACCASE PLUS
                              SYRINGALDAZINE

B. Second Reaction

SLOPE = -0.000073

LACCASE AND
SYRINGALDAZINE

FIGURE 4.3-A&B: UV ANALYSIS OF ASSAYS OF LACCASE IN BUFFERED
DIMETHYLFORMAMIDE VS SYRINGALDAZINE

The results of these experiments are shown in graph form in Figures 4.4 A-D. The results seem to indicate activity against syringaldazine in both dimethylformamide and dioxane.

### 4.4.3 Stability of Laccase in Ethyl Acetate and Acetonitrile

A coal process utilizing enzymes in organic media would require high enzyme stability. The assays planned with model compounds might also be carried out for several days as the reaction conditions were not optimized. Thus the lifetime of activity of laccase in solvent was of interest.

To examine the lifetime of laccase in the hydrated organic solvents, the following experimental procedure was followed:

1.      laccase stock solution was prepared as described previously; syringaldazine solutions in ethyl acetate and acetonitrile;

2.      aliquots of the enzyme stock solution were added to tubes of ethyl acetate and acetonitrile;

3.      one aliquot of each of the enzyme solutions in hydrated organic solvent was mixed with syringaldazine solution (in the matching solvent) and changes in absorbance in the range 200 to 800 nm were measured;

4.      the unused enzyme solutions in hydrated organic solvents were stored at room temperature and at one day and at five days, aliquots were removed and assays with syringaldazine were repeated.

The results, changes in absorbance at selected wavelengths where changes were significant, are shown in Figure 4.5, which plot the absorbances at each time point and show the least squares fit for the first 30-60 minutes of the assay. It can be seen that the activity of laccase vs. syringaldazine (as indicated by the slopes of the lines) was not significantly changed over the five day period.

### 4.4.4 I thylphenylsulfide (EPS) as Substrate

### Assay in 5% hydrated Organic Media - UV Analysis

EPS is soluble in dimethylformamide/buffer and dioxane/buffer. EPS was scanned in 5 percent aqueous dimethylformamide and 5 percent aqueous dioxane and the absorbance maxima were: 258 nm in aqueous dioxane and 272 nm in aqueous dimethylformamide. Unlike the syringaldazine assay, where the product has absorbance at a distinct wavelength in the visible range, changes were observed only in the UV range.
Laccase assays were run with EPS as shown below.

A) UV ASSAY OF LACCASE SUSPENDED IN 5% BUFFERED DIOXANE

220nm 356nm

B) UV ANALYSIS OF ASSAY OF LACCASE DISSOLVED IN 5% BUFFERED DIOXANE VS SYRINGALDAZINE

220nm 244
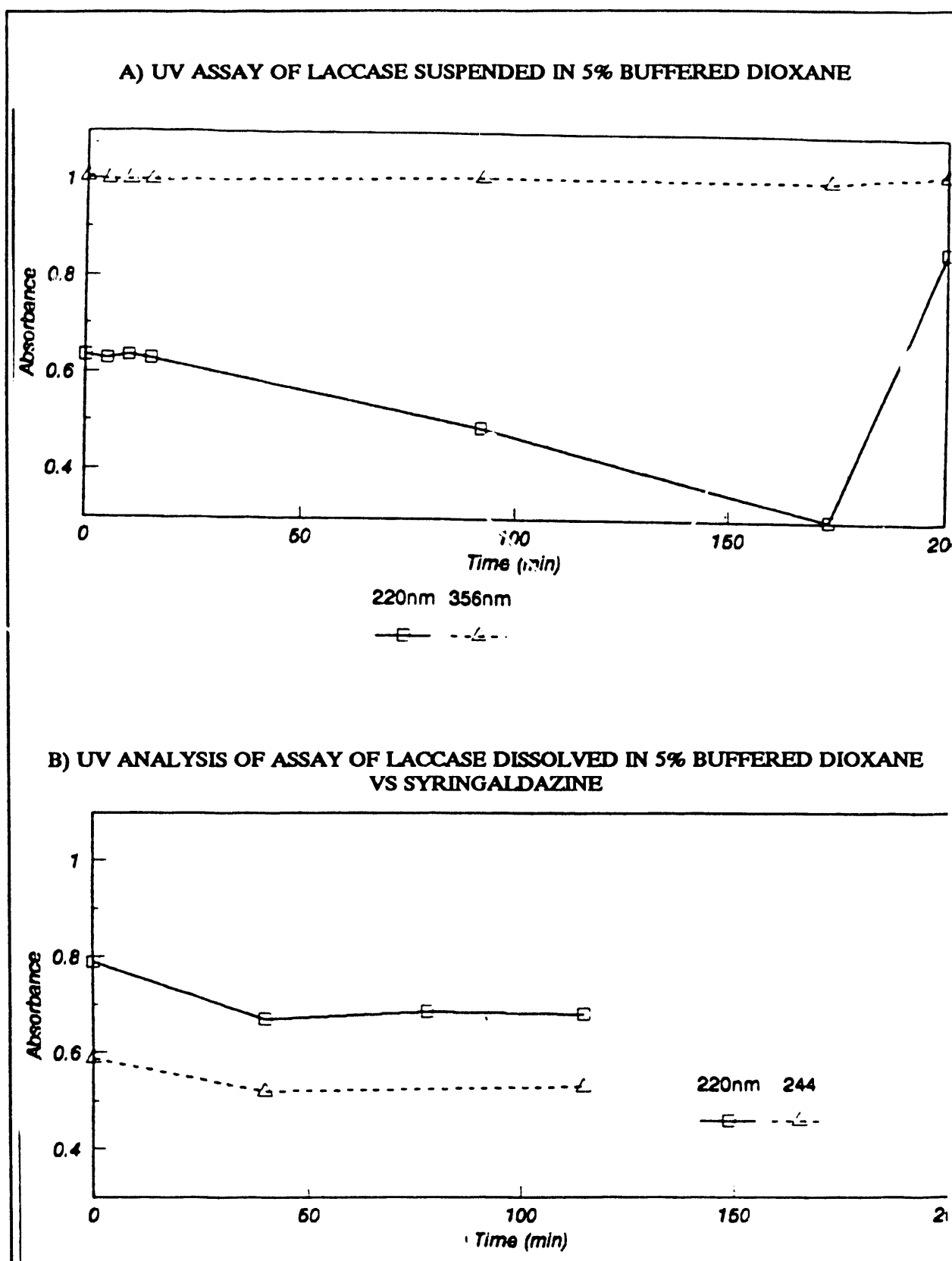
FIGURE 4.4-A&B: UV ANALYSIS OF LACCASE ASSAYS IN DIOXANE VS SYRINGALDAZINE

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C) UV ASSAY OF LACCASE DISSOLVED IN 5% BUFFERED DIMETHYLFORMAMIDE VS SYRINGALDAZINE
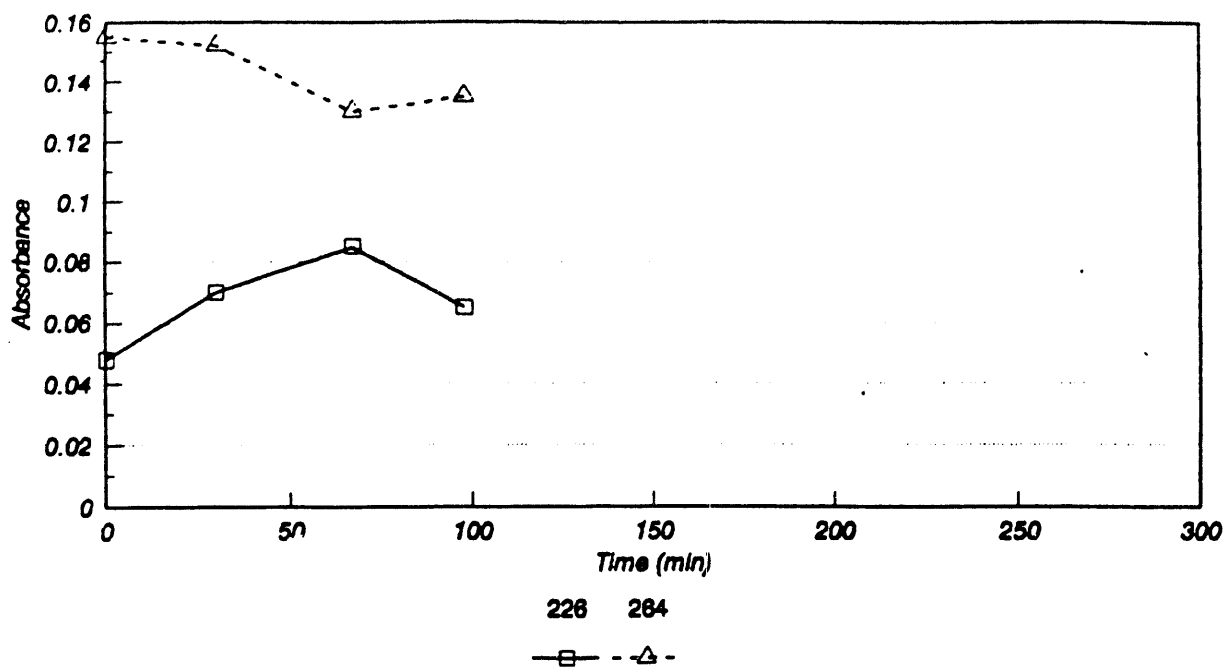
D) UV ASSAY OF LACCASE SUSPENDED IN 5% BUFFERED DIMETHYLFORMAMIDE VS SYRINGALDAZINE
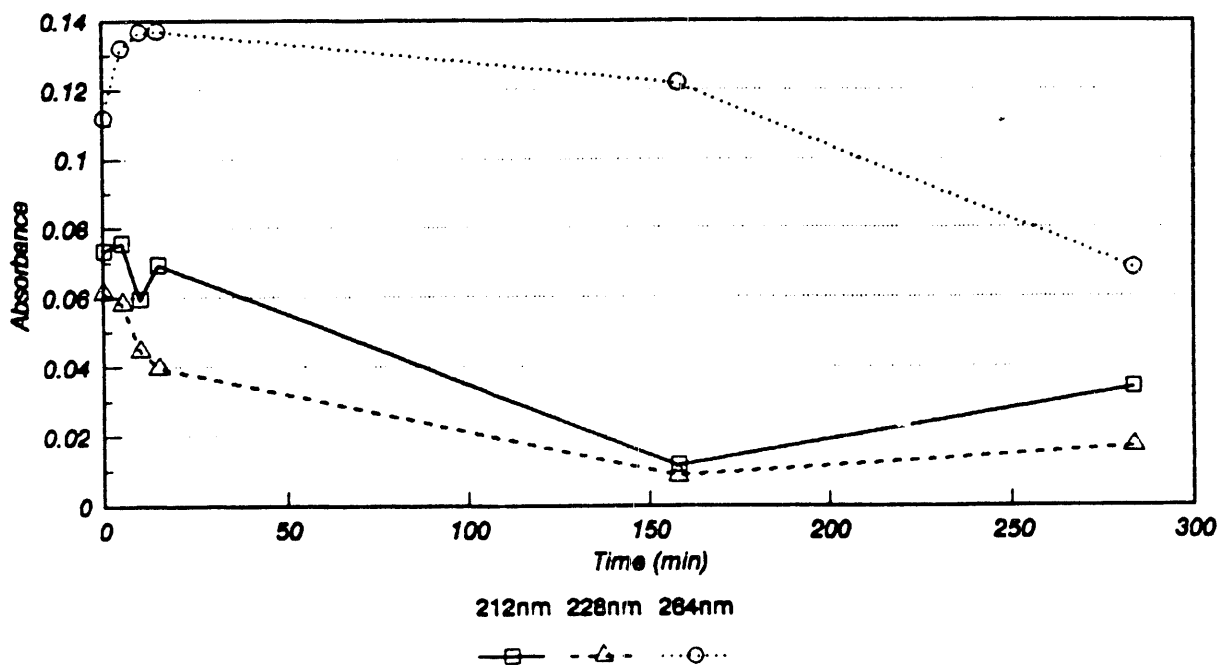
FIGURE 4.4-C&D: UV ANALYSIS OF LACCASE ASSAYS IN DIMETHYLFORMAMIDE VS SYRINGALDAZINE

A) RETENTION OF LACCASE ACTIVITY IN ACETONITRILE
VS SYRINGALDAZINE

DAY 0                DAY 1                DAY 5
SLOPE -0.000179  SLOPE -0.000133  SLOPE -0.000116

B) RETENTION OF LACCASE ACTIVITY IN ETHYLACETATE
VS SYRINGALDAZINE

DAY 0                DAY 1                DAY 5
SLOPE -0.000085  SLOPE -0.000049  SLOPE -0.000082
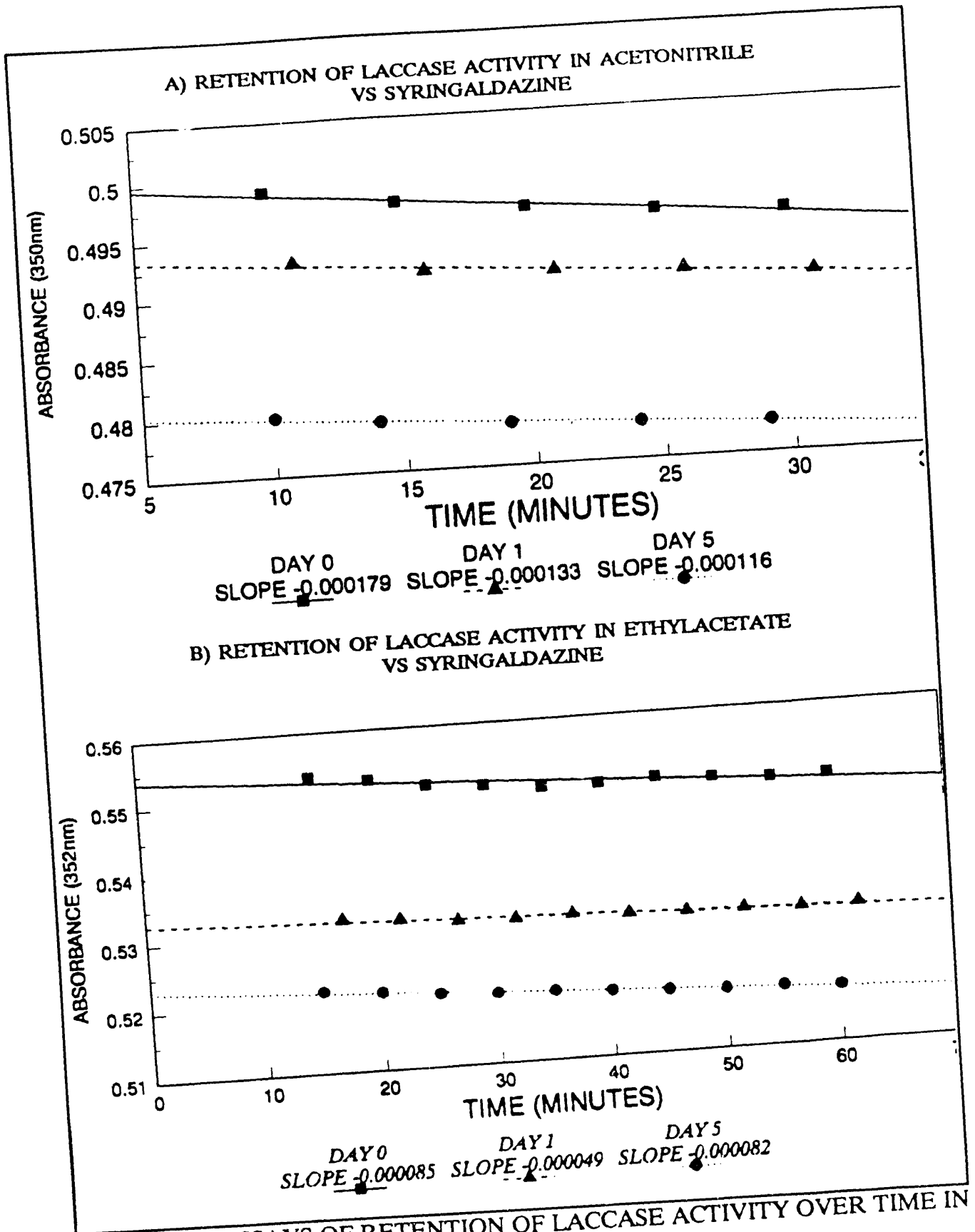
FIGURE 4.5: ASSAYS OF RETENTION OF LACCASE ACTIVITY OVER TIME IN
TWO MEDIA

The standard laccase assay procedure given in Section 4.4.1 was modified by substituting hydrated dioxane or hydrated dimethylformamide for all other solvents (e.g., 3 ml media, 1 ml enzyme in media, 0.5 ml substrate in media). A phosphate buffer at pH 6.5 was prepared and found to be miscible with the solvents.

a.      Buffer/Dioxane:   The stock EPS solution was $1.38 \times 10^3$ M in 5 percent buffer/dioxane.

        1. <u>Wet method of enzyme addition</u>: The stock laccase solution was 0.042 mg/ml in 5 percent buffer/dioxane.

        2. <u>Dry method of enzyme addition</u>: The stock laccase solution was 0.02 mg/ml in 5 percent buffer/dioxane.

b.      Buffer/dimethylformamide: The stock EPS solution was $1.48 \times 10^3$ M in 5 percent buffer/dimethylformamide.

        1. <u>Wet method of enzyme addition</u>: The stock laccase solution was 0.042 mg/ml in 5 percent buffer/dimethylformamide.

        2. <u>Dry method of enzyme addition</u>: The stock laccase solution was 0.04 mg/ml in 5 percent buffer/dimethylformamide.
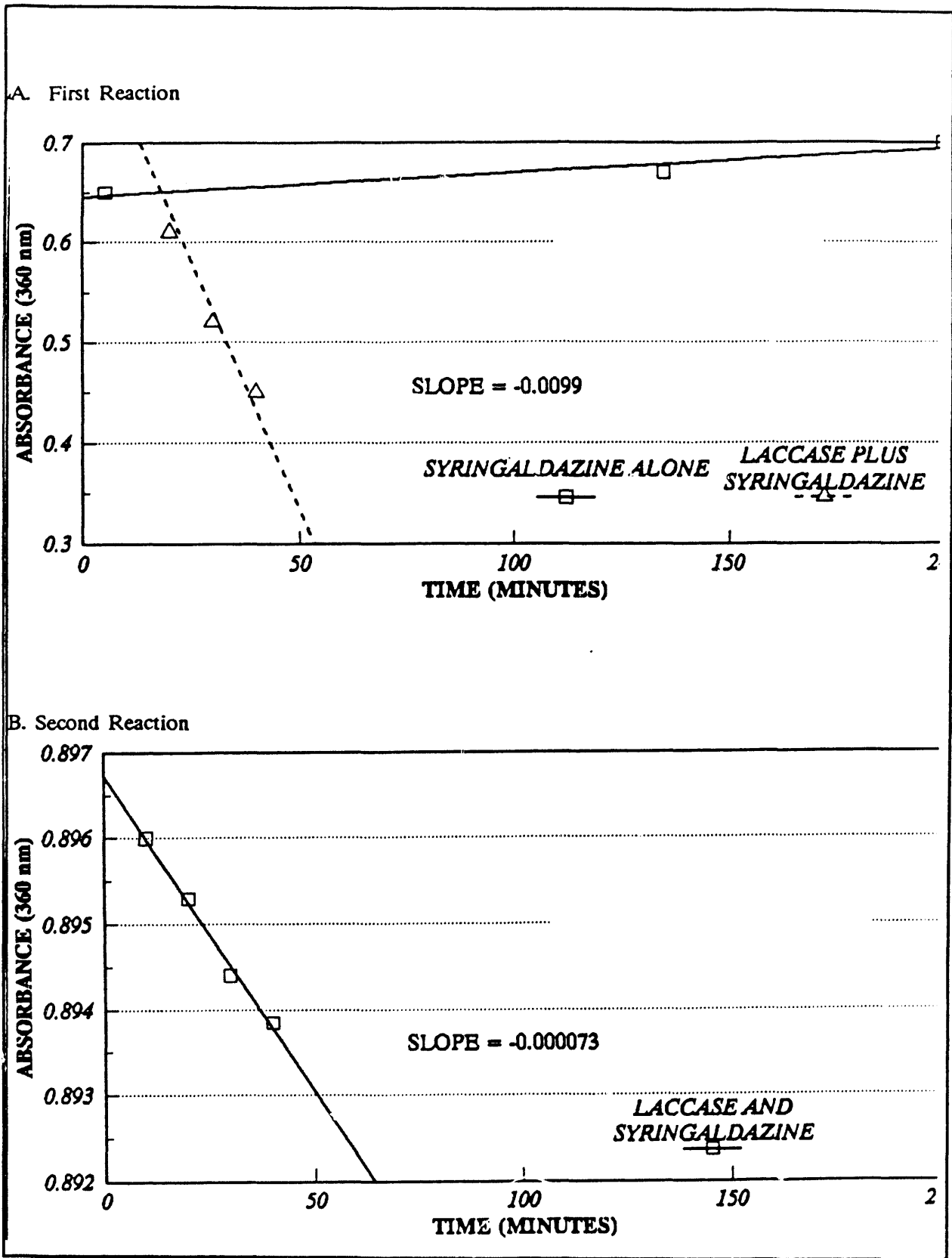
The results from the <u>dry</u> method of enzyme addition are shown in Figures 4.6 A-B. Changes in absorbance were observed; because no work was performed with expected products, no further conclusions can be made.

### TLC Analysis of EPS Reactions in 1% Aqueous Ethyl Acetate or Acetonitrile

Consideration was given to monitoring EPS/laccase reactions by TLC. Assays were set-up using 0.2 mM ethyl phenyl sulfide (EPS) as the substrate in either ethyl acetate or acetonitrile with 1% buffered water. However, it was found that the detection limits of EPS and its "4S" components differ greatly from that of DBT and its components. The lower limit of detection on the TLC plate was a 5 $\mu$l spot of a 20 mM solution of EPS. If a 1% conversion of EPS (initial concentration = 0.2 mM) occurred, there would be 0.002 mM of the component(s). In order to see this small amount, 50 ml would have to be spotted. This was obviously beyond the limits of the experiment, this investigation was discontinued.

### 4.4.5    DBT as Substrate in Various Hydrated Organic Media

    5% Phosphate Buffer/95% Dimethylformamide or Dioxane [DBT 0.2 - 0.3mM; Laccase .004 mg/ml]

DBT is soluble in dimethylformamide/buffer and dioxane/buffer. The standard laccase assay procedure given in Section 4.4.1 was modified by substituting hydrated dioxane or hydrated dimethylformamide for all other solvents (e.g., 3 ml media, 1 ml enzyme in

**A) UV ANALYSIS OF LACCASE ACTIVITY
SUSPENDED IN 5% BUFFERED DIOXANE VS EPS**
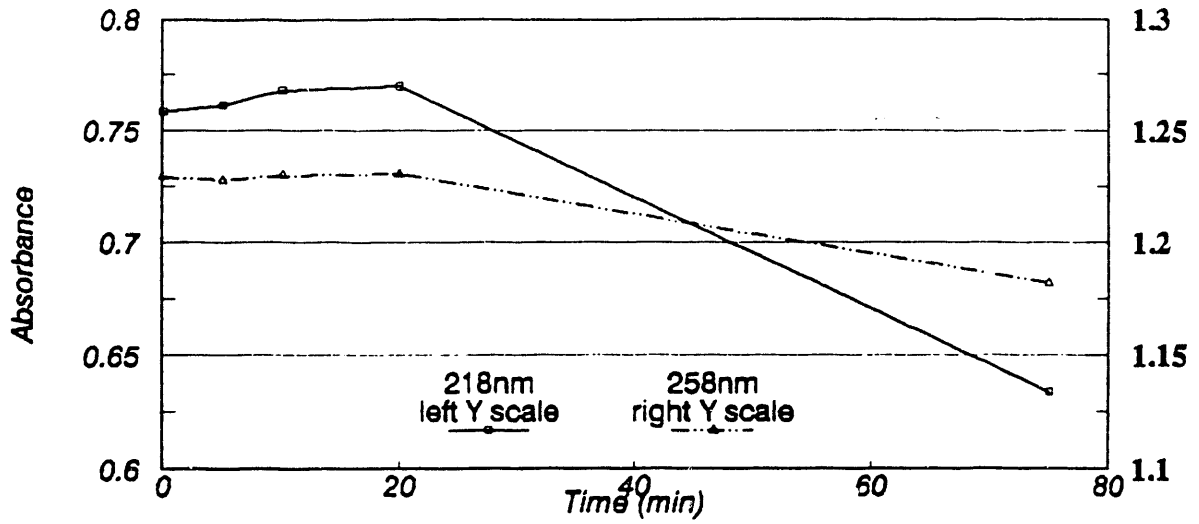
218nm
left Y scale

258nm
right Y scale

**B) UV ANALYSIS OF LACCASE ACTIVITY
SUSPENDED IN 5% BUFFERED DIMETHYLFORMAMIDE VS EPS**

206nm
left Y scale

238nm
left Y scale

242nm
left Y scale

254nm
left Y scale

268nm
right Y scale
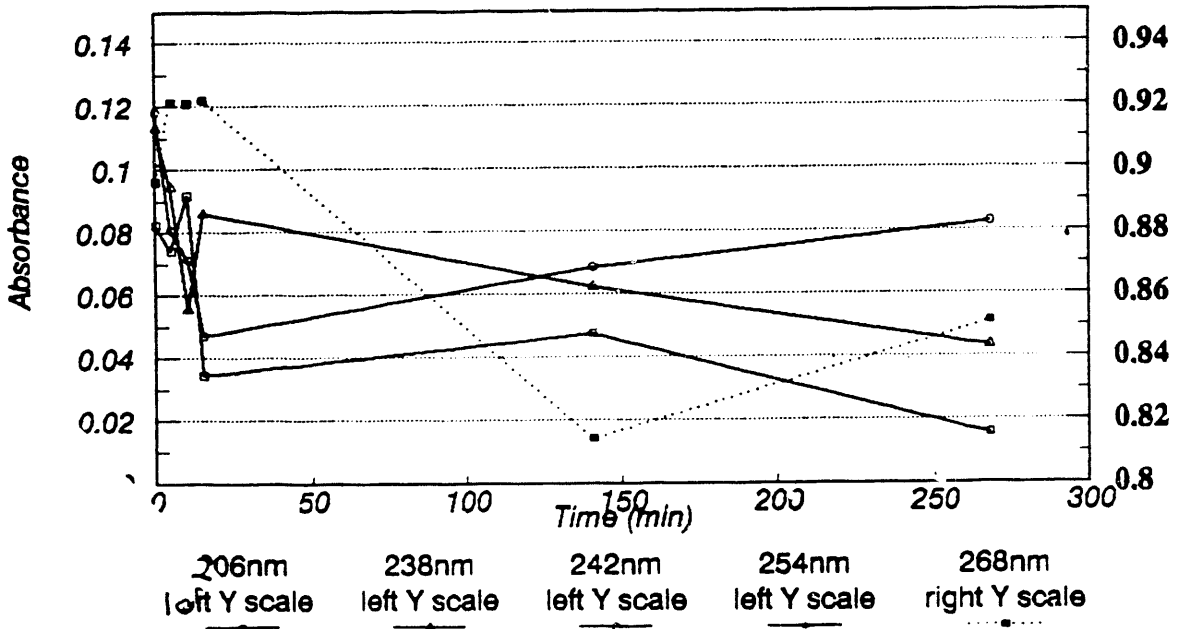
FIGURE 4.6-A&B: UV ANALYSIS OF ASSAYS OF LACCASE VS EPS

media, 0.5 ml substrate in media). A phosphate buffer at pH 6.5 was prepared and found to be miscible with the solvents. The following assay conditions were used.

a.    Buffer/Dioxane: The stock DBT solution was 2.78 x $10^{-4}$ M in 5 percent buffer/dioxane.

1. Wet method of enzyme addition: The stock solution of enzyme was 0.042 mg/ml in 5 percent buffer/dioxane.

2. Dry method of enzyme addition: The stock solution of enzyme was 0.02 mg/ml in 5 percent buffer/dioxane.

b.    Buffer/dimethylformamide: The stock solution of DBT was 2.17 x $10^{-4}$ M in 5 percent buffer/dimethylformamide.

1. Wet method of enzyme addition: The stock solution of laccase was 0.042 mg/ml solution of laccase in 5 percent buffer/dimethylformamide.

2. Dry method of enzyme addition: The stock solution of laccase was 0.02 mg/ml in 5 percent buffer/dimethylformamide.

The results for the dry addition method are shown in Figures 4.7 A-B. Plainly some changes in DBT were occurring in these systems.

Unlike the syringaldazine assays, where the product has absorbance at a distinct wavelength in the visible range, DBT and the DBT reaction products with laccase have overlapping spectra in the UV. The interpretation of results, therefore, is more difficult. Although it is clear that activity was seen, the rates of reaction could not be determined easily. Gas chromatography was used in subsequent assays to monitor the reaction progress.

## 5% Phosphate Buffer/95% Dimethylformamide or Dioxane [DBT 20mM; Laccase 0.13 mg/ml]

The buffer was 0.001 M phosphate (pH 6.5); the solvents were dimethylformamide and dioxane. The first set of assays was done in 5% buffer/95% solvent (v/v) with a final DBT concentration of 20 mM. For the dry method assays, dry laccase - 5 mg in 10 ml of buffer/dimethylformamide media (0.5 mg/ml) and 4.8 mg in 10 ml of buffer/dioxane media (0.48 mg/ml) - was added. The assays were done without agitation at 30°C. For the wet method, 19.9 mg laccase was added to 8 ml of buffer but it did not all dissolve and the solution was filtered. The stock concentration was thus less than 2.49 mg/ml. Of the stock, 0.5 ml was added to 9.5 ml of 21 mM buffer/dimethylformamide and buffer/dioxane media. The final enzyme concentration was less than 0.13 mg/ml and the final DBT concentration was 20 mM.

The changes in DBT concentration over time were measured daily for two days by GC, and the results are summarized below.

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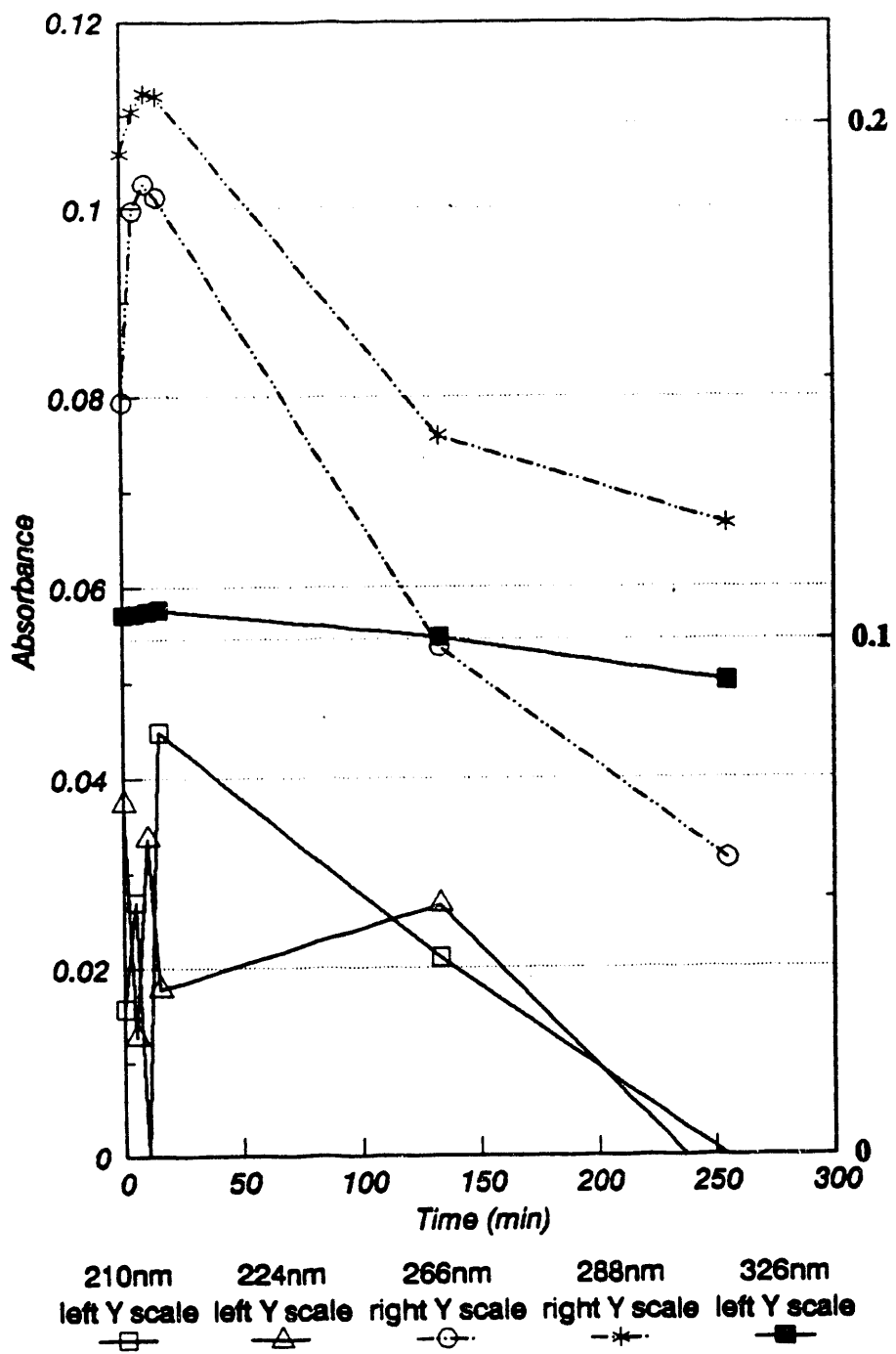
FIGURE 4.7-A: UV ANALYSIS OF LACCASE ASSAYS SUSPENDED IN 5% BUFFERED DIMETHYLFORMAMIDE VS DBT
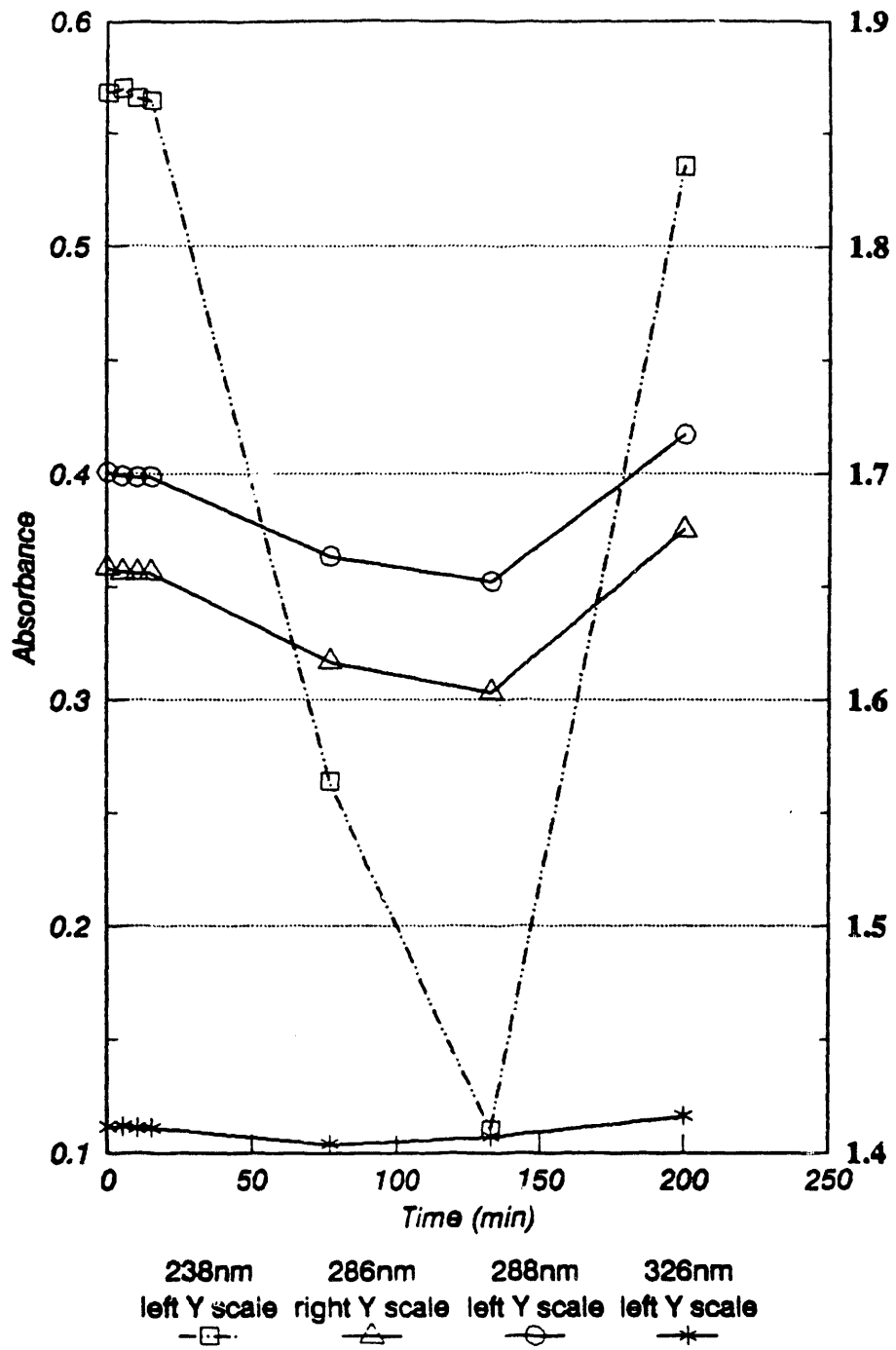
FIGURE 4.7-B: UV ANALYSIS OF LACCASE ASSAYS SUSPENDED IN 5% BUFFERED DIOXANE VS DBT

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• <u>Dry</u> Method (Buffer/dimethylformamide): Down 17.2% at 24 hours; up at 48 hours (evaporation had occurred).

.• <u>Wet</u> Method (Buffer/dimethylformamide): Down 4.55% at 24 hours; up at 48 hours (evaporation had occurred).

• <u>Dry</u> Method (Buffer/Dioxane): Down 13.9% at 24 hours; up at 48 hours (evaporation had occurred).

• <u>Wet</u> Method (Buffer/Dioxane): Up 11.2% at 24 hours; up at 48 hours (evaporation had occurred).

### 1% Phosphate Buffer/99% Dimethylformamide or Dioxane [ DBT 20mM; Laccase 0.5 mg/ml]

The buffer was 0.001 M phosphate (pH 6.5); the solvents were dimethylformamide and dioxane. This set of assays was done in 1% buffer/99% solvent (v/v) with a final DBT concentration of 20 mM. Only the <u>dry method</u> of assay was used and dry laccase - 5 mg in 10 ml of buffer/dimethylformamide media (0.5 mg/ml) and 5 mg in 10 ml of buffer/dioxane media (0.5 mg/ml) - was added. The assays were done without agitation at 30°C. The final DBT concentration was 20 mM. The results (not shown) showed no change in DBT concentration over two days.

### 5% Acetate Buffer/95% Dimethylformamide or Dioxane [DBT 20mM; 0.5 mg/ml Enzyme]

The buffer was 0.01 M acetate (pH 5); the solvents were dimethylformamide and dioxane. This set of assays was done in 5% buffer/95% solvent (v/v) with a final DBT concentration of 20 mM. The <u>dry method</u> of assay was used and dry laccase - about 5 mg in 10 ml of media (0.5 mg/ml) - was added. Additional assays at the same concentration by the "wet" method were also carried out. The assays were done without agitation at 30°C. The final DBT concentration was 20 mM.

The samples were analyzed after two days by GC. The results are shown in Table 4.7. In the dimethylformamide assays, the changes from day 0 to day 1 are not significant. In the dioxane, the percentage of unknowns did increase in one day.

### 5% Acetate Buffer/95% Dimethylformamide or Dioxane [20 mM DBT; 0.2 mg/ml Laccase)

In these experiments, the conditions were: organic solvent (95%), 0.01 M sodium acetate (5%), laccase 0.05 mg/ml, and DBT 20 mM. The total volume per tube was 10 ml. The temperature was 30°C. Two organic solvents were used: dimethylformamide and dioxane. Four sets of experiments were run, each in duplicate: dimethylformamide-enzyme added dry; dimethylformamide-enzyme added in buffer solution; dioxane-enzyme added dry;

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TABLE 4.7: GC ANALYSIS OF LACCASE ASSAYS VS DBT IN DIMETHYLFORMAMIDE OR DIOXANE

| Method of Enzyme Addition/ Solvent | Time (days) | Percents of Total Area at Various Retention Times (min) | | | | |
|---|---|---|---|---|---|---|
| | | 11.6* | 11.7 to 17.6 | 17.7 to 17.9** | 17.9 to 20 | 20.1 to 24 |
| Dry Addition | | | | | | |
| Dimethyl-formamide | 0 | 98.2 | 1.7 | 0.0 | 0.0 | 0.0 |
| | 0 | 99.9 | 0.0 | 0.0 | 0.1 | 0.0 |
| Dimethyl-formamide | 1 | 99.6 | 0.1 | 0.0 | 0.1 | 0.0 |
| | 1 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Dioxane | 0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 0 | 99.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| Dioxane | 1 | 99.7 | 0.2 | 0.0 | 0.0 | 0.0 |
| | 1 | 99.3 | 0.5 | 0.1 | 0.2 | 0.0 |
| Wet Addition | | | | | | |
| Dimethyl-formamide | 0 | 98.2 | 1.7 | 0.0 | 0.0 | 0.0 |
| | 0 | 99.7 | 2.0 | 0.0 | 0.2 | 0.1 |
| Dimethyl-formamide | 1 | 99.6 | 0.3 | 0.0 | 0.0 | 0.0 |
| | 1 | 99.2 | 0.1 | 0.0 | 0.3 | 0.4 |
| Dioxane | 0 | 99.6 | 0.1 | 0.0 | 0.0 | 0.0 |
| | 0 | 99.8 | 0.1 | 0.0 | 0.1 | 0.0 |
| Dioxane | 1 | 99.5 | 0.3 | 0.0 | 0.1 | 0.0 |
| | 1 | 99.6 | 0.4 | 0.0 | 0.0 | 0.0 |

* DBT
** DBT sulfoxide/DBT sulfone

and dioxane-enzyme added in buffer solution. Samples were taken at t = 0 and t = 24 hours and analyzed by GC. As can be seen in the summaries of average areas under curves or peaks at various retention times, shown in Table 4.8 A-B. the only set of conditions which produced any drop in DBT was the dimethylformamide with wet enzyme addition.

### 0.5% Aqueous/Ten Different Organic Solvents [20mM DBT; 0.1 mg/ml Enzyme]

Additional solvents tested without water or buffer added were: tetrahydrofuran, toluene, heptane, hexane, isopropyl ether, ethyl acetate, dioxane, acetonitrile, dimethylformamide, and benzene. The laccase (100 $\mu$l of 20 mg/ml) was added to 20 ml solvent with 20 mM DBT. For the first two systems, a sample was taken at time zero and another at fourteen days. For the latter eight systems, aliquots were taken at two days. The aliquots were filtered and analyzed by GC. The results are shown in Table 4.9. It can be seen that some new compounds appeared. Both DBT sulfoxide and DBT sulfone were observed in several samples, although some of these compounds were also seen in the controls. Unknowns were observed in the heptane and dimethylformamide assays.

### 0.5% and 0.1% PIPES and Acetate Buffers in Four Organic Solvents [2mM DBT]

The activity of laccase against DBT was tested in four organic solvents: acetonitrile; ethyl acetate; dioxane; and dimethylformamide. Laccase was tested at a final concentration of 0.2 mg/ml in two buffers: 1 mM PIPES, pH 7.05; and 1 mM acetate buffer, pH 5.5. The two buffer concentrations used were 0.5% and 1% in organic solvent. The DBT concentration was 2 mM. Controls without laccase and without DBT were included for each solvent. The total volume per tube was 5 ml. The tubes were tightly capped, laid on their side, and placed in a shaker at 28°C. This allowed for adequate mixing and aeration. After three days, 50 $\mu$l from each tube was applied onto TLC plates (silica gel; Merck No. 5628) and run with chloroform:acetone [80:20] as eluent. The plates were examined by UV light. If a 1% conversion of DBT to its oxidation products had occurred, the application of 50 $\mu$l of sample would have detected it. No "4S" oxidation products were observed with any of the solvents at either pH and buffer concentration. It was, therefore, apparent that laccase activity against DBT, if any, caused less than 1% conversion under the conditions tested.

### 1% Phosphate Buffer/99% Acetonitrile or Ethyl Acetate [2.0 mM DBT; 0.2 mg/ml Laccase]

Distilled water was brought up to pH 7 with dibasic phosphate. Laccase (19.4 mg) was added to 1 ml of the buffer. Six screw cap test tubes were prepared as follows: three tubes (1-3) each received 9 ml of acetonitrile and 1 ml of 21 mM DBT in dimethylformamide; and three tubes (4-6) each received 9 ml of ethyl acetate and 1 ml of 21 mM DBT in dimethylformamide.

A magnetic stir bar was added to each tube along with 100 $\mu$l of the enzyme solution. Final concentration of laccase was 0.2 mg/ml. Some precipitate was observed. Samples were taken for GC at t = 0, 24 hrs, 2 days, 3 days, 7 days, 8 days, and 16 days.

## TABLE 4.8-A: GC ANALYSIS OF LACCASE ASSAYS IN ACETATE/DIMETHYLFORMAMIDE VS DBT

| Retention Time (minutes) | A. Enzyme Predissolved in Acetate Buffer % of Total Peak Area | | B. Enzyme Added Dry % of Total Peak Area | |
|---|---|---|---|---|
| | T=0 (n=1) | T=24hr (n=2) | T=0 (n=2) | T=24hr (n=2) |
| 5.8-7.0 | 0.00 | 1.59 | 0.00 | 0.00 |
| 8.0 - 9.0 | 0.00 | 0.00 | 0.00 | 0.00 |
| 10.0-10.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 11.0-11.9* | 98.20 | 97.83 | 99.09 | 99.78 |
| 12.0-12.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13.0-13.9 | 0.01 | 0.00 | 0.00 | 0.01 |
| 14.0-14.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 15.0-15.9 | 0.36 | 0.15 | 0.00 | 0.00 |
| 16.0-16.5 | 0.48 | 0.04 | 0.00 | 0.02 |
| 16.5-16.9 | 0.00 | 0.00 | 0.85 | 0.03 |
| 17.0-17.5 | 0.82 | 0.00 | 0.00 | 0.00 |
| 17.5-17.9** | 0.00 | 0.02 | 0.01 | 0.00 |
| 18.0-18.5 | 0.00 | 0.00 | 0.00 | 0.05 |
| 18.5-18.9 | 0.10 | 0.00 | 0.00 | 0.00 |
| 19.0-19.9 | 0.00 | 0.15 | 0.03 | 0.00 |
| 20.0-20.9 | 0.02 | 0.00 | 0.01 | 0.02 |
| 21.0-21.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 22.0-22.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 23.0-23.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 24.0-24.9 | 0.00 | 0.22 | 0.00 | 0.08 |
| 25.0-25.9 | 0.00 | 0.00 | 0.00 | 0.00 |

\* DBT
\*\* DBT Sulfoxide/DbT Sulfone

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## TABLE 4.8-B: GC ANALYSIS OF LACCASE ASSAYS IN 5% ACETATE/DIOXANE VS DBT

| | A. Enzyme Predissolved in Acetate Buffer | | B. Enzyme Added Dry | |
|---|---|---|---|---|
| Retention Time | % of Total Peak Area | | % of Total Peak Area | |
| (minutes) | T=0 (n=1) | T=24hr (n=2) | T=0 (n=2) | T=24hr (n=2) |
| 5.8-7.0 | 0.00 | 0.00 | 0.00 | 0.00 |
| 8.0 - 9.0 | 0.00 | 0.00 | 0.00 | 0.00 |
| 10.0-10.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 11.0-11.9* | 99.97 | 99.48 | 99.81 | 99.58 |
| 12.0-12.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13.0-13.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 14.0-14.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 15.0-15.9 | 0.00 | 0.04 | 0.01 | 0.00 |
| 16.0-16.5 | 0.00 | 0.11 | 0.12 | 0.07 |
| 16.5-16.9 | 0.00 | 0.03 | 0.00 | 0.00 |
| 17.0-17.5 | 0.00 | 0.17 | 0.00 | 0.29 |
| 17.5-17.9** | 0.01 | 0.11 | 0.00 | 0.00 |
| 18.0-18.5 | 0.00 | 0.00 | 0.00 | 0.06 |
| 18.5-18.9 | 0.02 | 0.05 | 0.05 | 0.00 |
| 19.0-19.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 20.0-20.9 | 0.00 | 0.01 | 0.00 | 0.00 |
| 21.0-21.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 22.0-22.9 | 0.00 | 0.01 | 0.01 | 0.00 |
| 23.0-23.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 24.0-24.9 | 0.00 | 0.00 | 0.00 | 0.00 |
| 25.0-25.9 | 0.00 | 0.00 | 0.00 | 0.00 |

* DBT
** DBT Sulfoxide/DBT Sulfone

# TABLE 4.9: GC ANALYSIS OF LACCASE ASSAYS VS DBT

| Solvent | Time | Percents of Total Area at Various Retention Times (min) | | | | |
|---|---|---|---|---|---|---|
| | | 11.6* | 11.7 to 17.6 | 17.7 to 17.9** | 17.9 to 20 | 20.1 to 24 |
| DBT STANDARDS | | | | | | |
| Acetate/Dimethyl-formamide | | 99.6 | 0.3 | 0.1 | 0.1 | 0.0 |
| Aqueous Toluene | | 99.5 | 0.3 | 0.0 | 0.2 | 0.0 |
| Acetate/Dimethyl-formamide | | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Acetate/Dioxane | | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SAMPLES | | | | | | |
| Toluene | 0 | 99.8 | 0.2 | 0.0 | 0.0 | 0.0 |
| | 14 | 99.2 | 0.8 | 0.0 | 0.0 | 0.0 |
| Tetrahydrofuran | 0 | 99.9 | 0.1 | 0.0 | 0.0 | 0.0 |
| | 14 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Heptane | 2 | 97.7 | 0.7 | 0.0 | 0.9 | 0.7 |
| Hexane | 2 | 99.9 | 0.1 | 0.0 | 0.0 | 0.0 |
| Dimethyl-formamide | 2 | 98.7 | 1.2 | 0.0 | 0.1 | 0.0 |
| Dioxane | 2 | 99.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| Acetonitrile | 2 | 99.7 | 0.2 | 0.0 | 0.0 | 0.0 |
| Benzene | 2 | 99.8 | 0.2 | 0.0 | 0.0 | 0.0 |
| Isopropyl ether | 2 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Ethyl acetate | 2 | 99.9 | 0.1 | 0.0 | 0.0 | 0.0 |

* DBT
** DBT sulfoxide/DBT sulfone

For each GC run, the areas of peaks or curves were converted to percents of total area (excluding solvent). All analyses (at least two for each sample) were averaged. The results, shown in Figure 4.8 A&B, indicate that there was reactivity against the DBT; however, it is not yet clear what the products were, as they did not necessarily fall only where known products were expected. After two days, the percentage of total area attributed to observed products began to decrease. By the 16th day, only DBT was seen in any of the samples. To simplify examination of these experiments, the areas of all peaks emerging "pre-DBT" on the GC were summed, as were the areas of all peaks emerging "post-DBT". For the acetonitrile assay, through Day 2 there was a drop in %DBT explained by a rise in both "pre-DBT" and "post-DBT" peaks. Subsequently, the % content of DBT returned to the original value. The ethyl acetate samples showed a similar but less dramatic pattern of change in %DBT. The difference was almost entirely due to "post-DBT" material. It was thought that this pattern could be due to conversion of intermediates from compounds separated on the GC to those that were not seen, either because they did not come off the column during the period of observation (e.g., too early or too late) or they had precipitated in solution and were removed by filtration before GC analysis.

On Day 17, experiments were made to see if the products had precipitated. For Samples 1-3, 2 ml were taken from each, and 1 ml of ethylacetate and 1 ml of 0.1 M NaHCO$_3$ were added to each. No phase separation occurred, but the solution was clear. A sample was filtered and run on the GC. Only DBT was seen, confirming the results reported in the paragraph above. Additionally, 1 ml of the reaction mixture was mixed with 1 ml of 1% acetic acid and a filtered aliquot was run on the GC. Again, nothing but DBT was found. For Samples 4-6, 2 ml was taken from each, and 1 ml of ethyl acetate and 1 ml of .1 M NaHCO$_3$ were added to each. Phase separation occurred and the solutions were clear. The organic layers were removed by pipette. Samples were filtered and run on the GC. In the aqueous phase, a peak at the position assigned to the sulfone/sulfoxide accounted for 11.3% of the area, with DBT being the remainder. In the organic phase, most of the material seen was DBT, although a trace amount of material appeared at later retention times.

The series of acetonitrile and ethyl acetate assays just described was repeated and each tube was initially cloudy with flocculent material. It seemed that the amount of this material increased with time. It was likely that the precipitate was enzyme, however there was some concern that some that reaction products might be filtered out during the filtration prior to analysis. Thus, at each measurement time, two aliquots were taken and while one was simply filtered as before, the other was mixed with an equal volume of distilled water. In both the ethyl acetate and the acetonitrile aliquots, at each time immediate clearing occurred in the diluted aliquot. The ethyl acetate samples phase separated and the upper phase was removed to a separate container prior to filtering (two filtered samples were thus obtained for each of the diluted ethyl acetate aliquots).

Each sample was analyzed by GC with two or more injections. In looking at the data from the undiluted samples, no significant change in DBT concentration was observed and no significant appearance of new compounds was seen in filtered assay aliquots taken at Day 1, and at Days 15 or 16. In the aliquots which were diluted or extracted with water, many new peaks were observed in the GC analyses, but these often appeared in the enzyme-
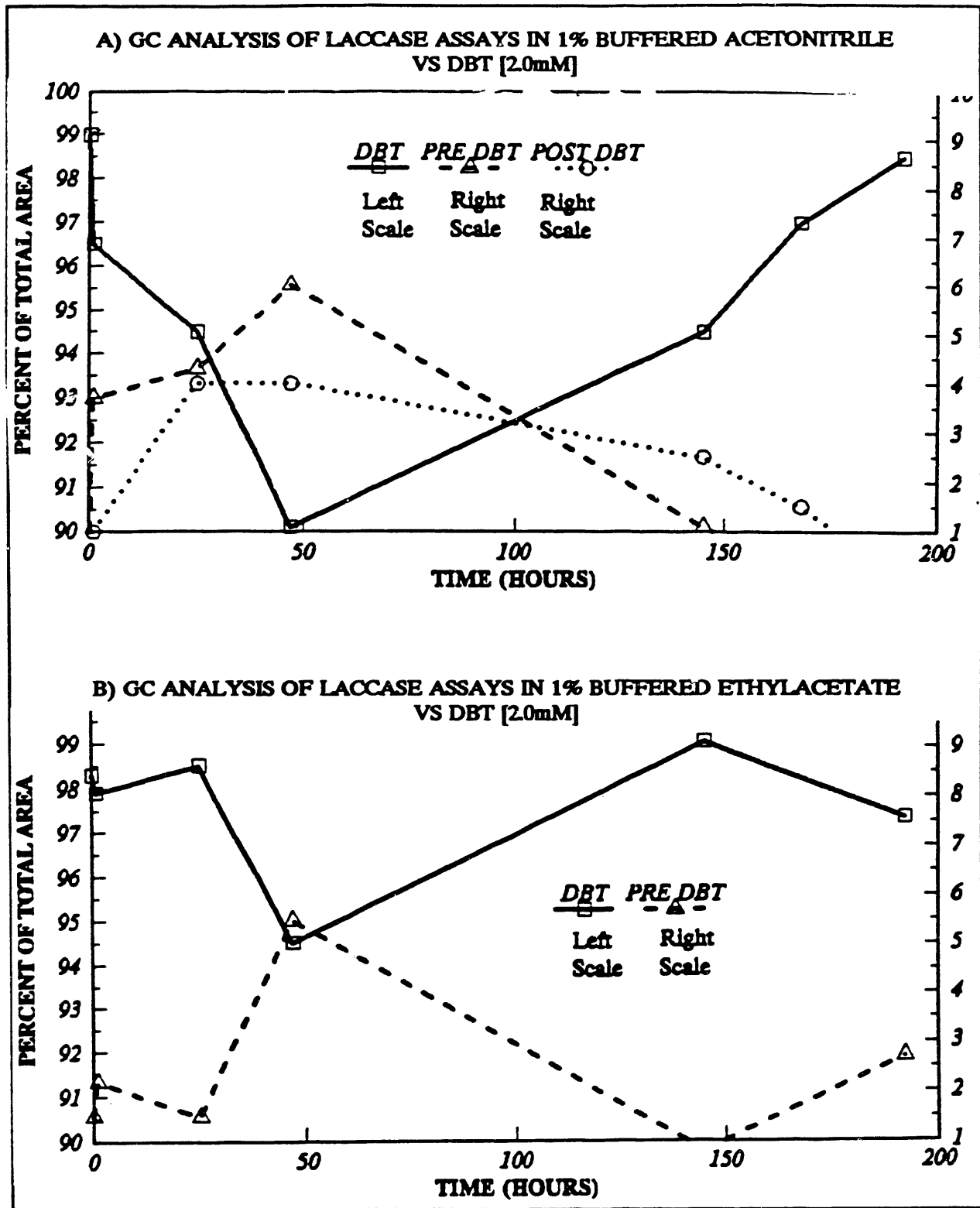
FIGURE 4.8: GC ANALYSIS OF LACCASE ASSAYS IN 1% BUFFERED MEDIA VS 2.0 mM DBT

only controls as well. The results were erratic and not reproducible. One sample was also run on the GC-MS and it did not show the same peaks. It was confirmed that the enzyme used had the normal level of activity vs. syringaldazine.

These assays of laccase vs. DBT in hydrated acetonitrile and hydrated ethyl acetate were repeated. Previously, a GC-mass spectroscopy analysis of the 24 hour aliquot from the laccase/DBT assay in acetonitrile had shown the DBT peak and a small peak which ran earlier than o,o'-biphenol. This second peak was identified from the Library as 1,1-thiobisbenzene. It was suggested that this peak was actually o,o'-biphenol.

To evaluate this hypothesis, the comparable sample from the repeat assay was analyzed by GC and a very small o,o'-biphenol peak was observed. Another 24 hour aliquot from this repeat experiment (laccase/DBT in acetonitrile) was analyzed by GC-MS and neither o,o'-biphenol nor the early peak seen in the previously reported sample was observed. These results are conflicting, and because there had been carry-over problems with the GC at that time, the GC-MS data seemed more reliable.

### 1% Aqueous Organic Acetonitrile, Ethyl Acetate, Isopropyl Ether [3.0 mM DBT]

The assays of laccase against DBT in selected hydrated solvents were repeated with DBT at 3.0 mM. The protocols were as follows: DBT stocks with 3.0 mM DBT were prepared. Aliquots of 9.5 ml solvent/DBT were mixed with 0.1 ml enzyme stock (which was 1 mg/ml). The vials were sealed and kept shaking vigorously. Samples were removed and filtered at 1 hour and 24 hours. The samples were analyzed by HPLC.

The results of this set of experiments are shown in Figures 4.9 A-B. The two figures represent the concentrations of DBT, DBT sulfoxide, DBT sulfone, and o,o'-biphenol in the reaction mixture at 1 hour and 24 hours for the laccase assays. It can be seen that the DBT change is not significant in acetonitrile and ethyl acetate. The rise in concentration in isopropyl ether may be due to solvent evaporation. Sulfoxide and sulfone appeared at 24 hours, but in low concentrations. The largest amount of identified products were in the acetonitrile samples.

### 1% Aqueous Acetonitrile [0.1 mM DBT]

A stock solution of 0.1 mM DBT in acetonitrile was prepared and 9.9 mls was added to each of 2 glass scintillation vials. To one vial 100 $\mu$l of 1 mg/ml laccase in distilled water was added. As a control, 100 $\mu$l of distilled water was added to the second vial. The three vials were placed on a rotating shaker at room temperature.

At 1, 24, and 48 hours, 500 $\mu$l was removed from each sample and filtered through a 0.22 $\mu$ Nylon 66 syringe filter unit. After seven days (168 hr) a final aliquot was taken from each sample. These aliquots were screened by GC and HPLC for the presence of DBT oxidation products. The results of this analysis can be found in Table 4.10-A. Some biphenol, o-OH-biphenyl, DBT sulfoxide and DBT sulfone were observed.

A) 24 HOUR LEVELS OF DBT SULFOXIDE, DBT SULFONE, AND DIPHENOL

ACEN = ACETONITRILE; ETOAC = ETHYLACETATE; IPRE = ISOPROPYLETHER

■ 1 HOUR □ 24 HOUR
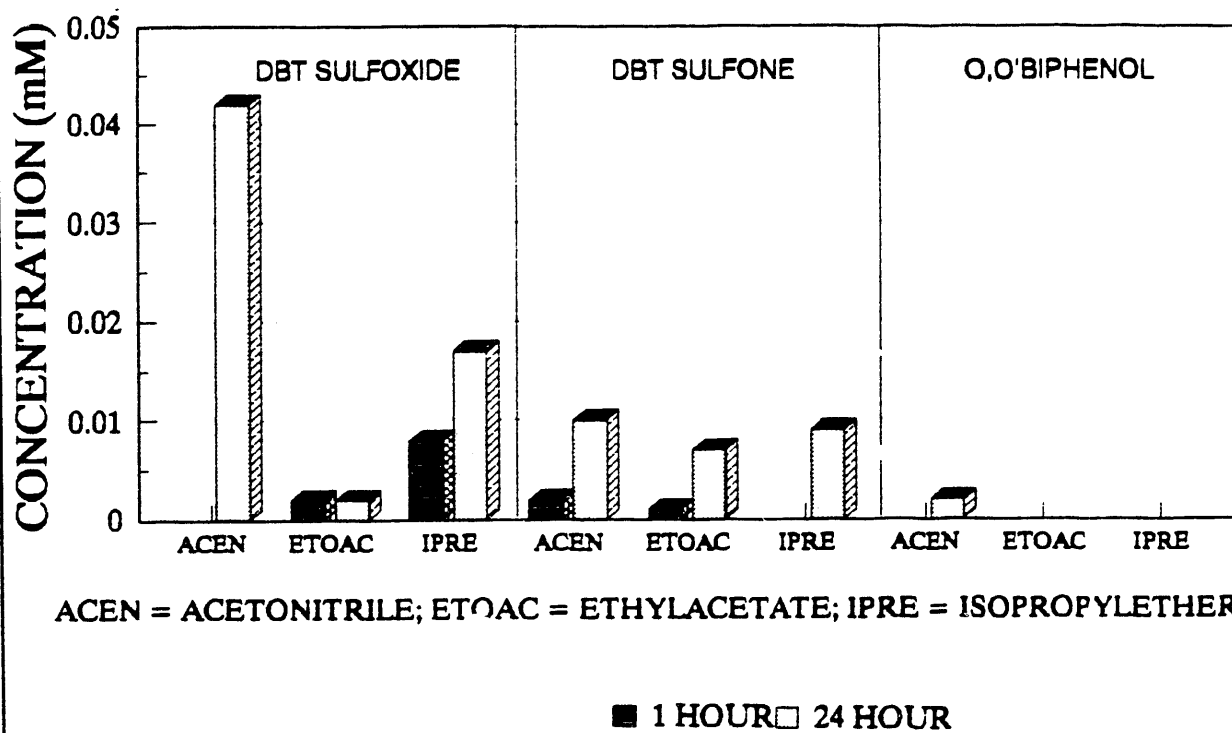
B) 24 HOUR LEVELS OF DBT

■ 1 HOUR □ 24 HOUR
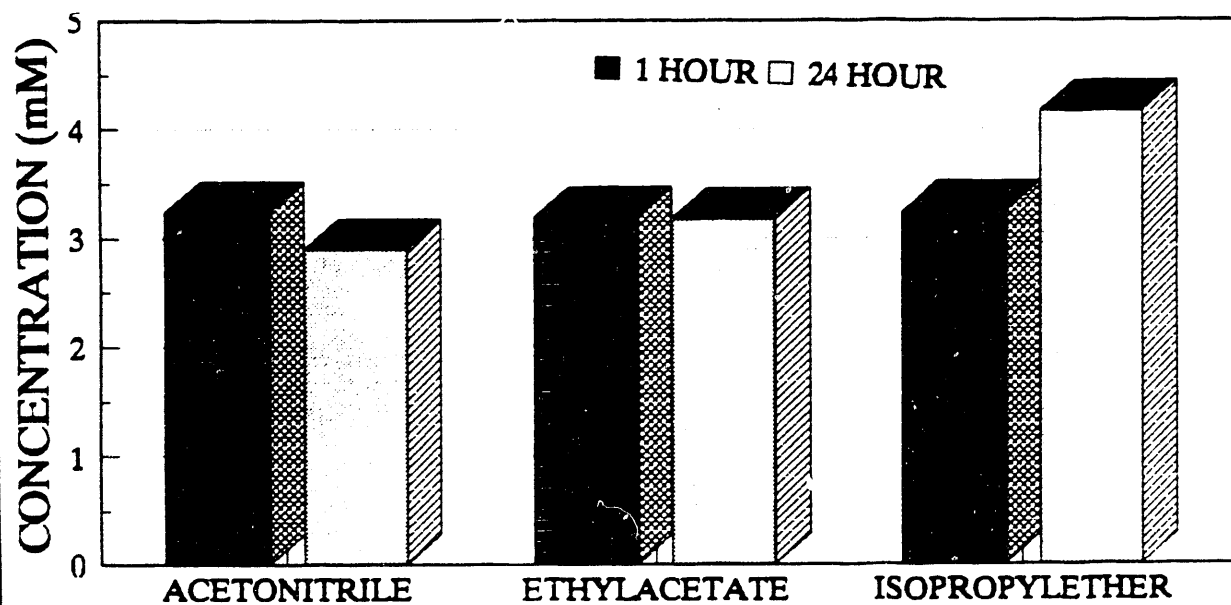
FIGURE 4.9-A&B: HPLC ANALYSIS OF LACCASE ASSAYED IN ACETONITRILE, ETHYL ACETATE, OR ISOPROPYL ETHER VS DBT [3.0 mM]

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Since there had been indications of the presence of DBT oxidation products in the above assays (first experiment), the work was repeated in quadruplicate. A new stock solution of 0.1 mM DBT in acetonitrile was prepared. A sample of this stock solution was filtered through a 0.22 $\mu$ filter unit and retained as a time = 0 control.

To each of six screw cap tubes, a total of 4.95 ml of the 0.1 mM DBT stock was added. To three of the screw cap tubes containing DBT, 50 $\mu$l of 1 mg/ml laccase in distilled water was added. Distilled water, 50 $\mu$l, was added to the last set of three screw cap tubes. The samples were placed on a shaker at room temperature for rapid mixing.

500 $\mu$l aliquots were removed at 1, 24, and 48 hours and filtered through the 0.2 2$\mu$ filter units. Larger aliquots of ~3 mls were removed and filtered at time $\approx$ 168 hours. In order to have the products of interest be as concentrated as possible for detection, the 168 hour aliquots were evaporated to dryness prior to analysis. Each aliquot was placed 100 $\mu$l at a time in a test tube in a heating block at 60°C. A gentle stream of $N_2$ was blown over the liquid to help speed the evaporation of solvent to dryness. This was repeated until a total of 500 $\mu$l of each aliquot had been reduced to dryness. The dried sample was then redissolved in 50 $\mu$l of acetonitrile. These concentrated aliquots were then analyzed by GC and HPLC and some were analyzed by GC/MS. Table 4.10-B summarizes the results of the GC and HPLC analysis. The significant decrease in the concentration of DBT from the initial value of 0.1 mM suggests that DBT was lost, probably in the drying/evaporation process. Traces of DBT sulfoxide and DBT sulfone were seen, however these were present in some controls as well.

### 1% Aqueous Acetonitrile Laccase from USDA [0.2mM DBT]

Assays versus 0.2 mM DBT in 99% acetonitrile were performed with laccase obtained from the Forest Products Laboratory of the U.S. Department of Agriculture in order to determine whether the lot of laccase used in previous assays was possibly the reason we had not clearly seen laccase activity against DBT. This new lot of laccase was provided as a solution of 5 mgs laccase/ml in 0.1 M phosphate buffer, pH 6. This solution had been stored in frozen aliquots and was freshly thawed just prior to use. The final concentration of the samples was ~0.2 mM DBT and 0.05 mgs/ml laccase.

| Sample Set | 0.2 mM DBT in CH³CN (ml) | Laccase (5 mg/ml) in 0.1 M Phosphate Buffer pH6 (ml) | CH₃CN (ml) | Distilled H₂O |
|---|---|---|---|---|
| 1 | 4.95 | 0.05 | 0.0 | 0.0 |
| 2 | 0.0 | 0.05 | 4.95 | 0.0 |
| 3 | 4.95 | 0.0 | 0.0 | 0.05 |

## TABLE 4.10-A: HPLC AND GC ANALYSIS OF LACCASE ASSAYS IN 1% AQUEOUS ACETONITRILE VS DBT [0.1 mM]

| Time | Method | o,o'-Biphenol (mM) | DBT (mM) | DBT* Sulf-oxide (mM) | DBT* Sulfone (mM) | DBT** oxide/one (mM) | o-Hydroxy Biphenyl (mM) |
|---|---|---|---|---|---|---|---|
| First Experiment | | | | | | | |
| 1 | GC<br>HPLC | ND<br>0.0 | 0.058 | 0.0 | 0.0 | -- | 0.0 |
| 24 | GC<br>HPLC | ND<br>0.0 | 0.093 | 0.0 | 0.0 | -- | 0.0 |
| 48 | GC<br>HPLC | ND<br>0.0 | 0.07 | 0.0 | 0.0 | -- | 0.0 |
| 168 | GC<br>HPLC | 0.01<br>0.0 | 0.05<br>0.046 | --<br>0.071 | --<br>0.0 | 0.02<br>-- | 0.01<br>0.0 |
| First Experiment Control | | | | | | | |
| 1 | GC<br>HPLC | ND<br>0.0 | 0.068 | 0.0 | 0.0 | -- | 0.0 |
| 24 | GC<br>HPLC | ND<br>0.0 | 0.89 | 0.0 | 0.0 | -- | 0.0 |
| 48 | GC<br>HPLC | ND<br>0.0 | 0.073 | 0.0 | 0.0 | 0.0<br>-- | 0.0<br>0.0 |
| 168 | GC<br>HPLC | 0.0<br>0.0 | 0.017<br>0.049 | --<br>0.0 | --<br>0.0 | 0.0<br>-- | 0.0<br>0.0 |

* = HPLC only;  ** = GC only
ND = sample not analyzed

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## TABLE 4.10-B: HPLC AND GC ANALYSIS OF LACCASE ASSAYS IN 1% AQUEOUS ACETONITRILE VS [0.1 mM] DBT

| Time | Method | o,o'-Biphenol (mM) | DBT (mM) | DBT* Sulfoxide (mM) | DBT* Sulfone (mM) | DBT** oxide/one (mM) | o-Hydroxy Biphenyl (mM) |
|------|--------|-------|-------|-------|-------|-------|-------|
| Second Experiment | | | | | | | |
| 168 | GC | 0.0 | 0.04 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.055 | 0.0 | trace | -- | 0.0 |
| 168 | GC | 0.0 | 0.007 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.027 | 0.0 | trace | -- | 0.0 |
| Second Experiment Control | | | | | | | |
| 168 | GC | 0.0 | 0.02 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.037 | trace | trace | -- | 0.0 |
| 168 | GC | 0.0 | 0.017 | -- | -- | 0.0 | 0.0 |
|     | HPLC | 0.0 | 0.049 | 0.0 | 0.0 | -- | 0.0 |

* = HPLC only; ** = GC only

Results for separate reactors are shown..

The amounts of materials indicated above were added to three screw cap test tubes for each sample set and mixed by inverting the tube several times. A 1 ml time 0 aliquot was withdrawn and filtered through a 0.22 μ Nylon 66 filter unit (Rainin No. 38-159) and refrigerated until analysis. The tubes were then placed on a shaker in a controlled temperature room (25°C). Aliquots were removed and filtered at 1 hour and at 24 hours and were also refrigerated until analysis.

The time = 24 hour samples were analyzed by gas chromatography. Aliquots (100 μl) of samples were transferred to silanized "Reacti Vials" and evaporated at room temperature to complete dryness. The dried samples were derivatized for 15 minutes at 65°C in freshly opened Tri-Sil/BSA Formula D (Pierce No. 49010) prior to analysis. The results of the GC analysis are summarized in Table 4.11.

As can be seen, the only products detected (o-hydroxybiphenyl and DBT sulfone/sulfoxide) in the samples were also present in similar concentration in the controls. Whether this lack of effect on DBT at these conditions is due to the fact that a precipitate (buffer salts or laccase) formed immediately upon addition of the laccase solution to the samples and controls or rather the laccase truly has no effect on DBT is still open to question.

A second puzzling result was that only ~50 to 65% of the amount of DBT that should have been present in the samples and controls was detected. The DBT peaks detected were relatively consistent injection to injection and from sample to sample. It seemed that DBT was lost during the drying and derivatization process.

TABLE 4.11: GC ANALYSIS OF ASSAYS WITH USDA LACCASE IN 1% AQUEOUS ACETONITRILE VS 0.2 mM DBT

| Sample Description | o-Hydroxy Biphenyl (mM) | DBT (mM) | o,o'- Biphenol (mM) | DBT Sulfone/ Sulfoxide (mM) |
|---|---|---|---|---|
| Laccase + DBT in 99% Acetonitrile | 0.002 0.002 0.002 | 0.01 0.12 0.13 | 0.0 0.0 0.0 | 0.0002 0.0001 0.0 |
| Laccase in 99% Acetonitrile | 0.001 0.001 | 0.0 0.0 | 0.0 0.0 | 0.0002 0.0 |
| DBT in 99% Acetonitrile | 0.001 0.001 | 0.11 0.11 | 0.0 0.0 | 0.0001 0.0002 |

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## 4.4.6    DBT Sulfone as Substrate

Assay samples contained 0.2 mM DBT sulfone as the substrate, laccase as the enzyme (0.2 mg/ml) and either acetonitrile or ethyl acetate with 1% water (buffered to pH = 7) as the solvent. Blank samples (i.e, DBT sulfone with no laccase and laccase without DBT sulfone) were also prepared. The samples were constantly stirred at room temperature for one week. Aliquots were removed at 0 hours, 24 hours, and 7 days. Upon removal, aliquots were filtered through a 0.2 $\mu$m pore size syringe filter unit.

In an attempt to avoid the extensive time requirements for gas chromatography analysis, thin-layer chromatography was chosen as an alternate method. Plastic-backed silica gel 60 TLC plates (Merck No. 5735) with an indicator that fluoresces at 254 nm were used. By exposing the developed plate to UV light, a positive detection of a compound would be indicated by the lack of fluorescence. The solvent system used was chloroform:acetone (80:20). Samples were spotted on the plates with either 1 $\mu$l Microcaps[R] or with a 100 $\mu$l syringe. In the case of multiple drops of liquid per sample, the TLC plate was dried under a blower (room temperature) between applications of sample.

In the assay sample mentioned above, the purpose of the experiment was to determine whether laccase could convert DBT sulfone to o,o'-biphenol or, for that matter, to any other compound. It was determined that 5 $\mu$l of 0.2 mM (initial concentration of DBT sulfone in the assay) "4S" standards could be detected in the described TLC system. If a 1% conversion of DBT sulfone to o,o'-biphenol is assumed, in order for the o,o'-biphenol to be detectable, 500 $\mu$l of sample wold have to be spotted. (500 $\mu$l of the o,o'-biphenol standard could be detected by the TLC system.) However, no o,o'-biphenol was detected in the assay samples ( t = 24 hours and 7 days) in ethyl acetate and acetonitrile even when such large volumes were spotted (data not shown). It is clear that the activity, if any, of laccase against DBT sulfone is negligible in the solvent systems tried.

## 4.5    Discussion of Laccase, Horseradish Peroxidase, and Sulfatase Studies

## 4.5.1    Enzyme Kinetic Studies

One of the more important aspects of this program was the elucidation of the level of interaction of model coal and their oxidation products with the commercial enzymes. Two model compounds, dibenzothiophene [DBT] and ethylphenylsulfide [EPS] and their sulfur oxidation products and o,o'-biphenol were treated as potential inhibitors of the three enzymes in their reactions with standard substrates.

These assays were performed in mixtures which facilitated solvation of all components: enzyme, standard substrate, and model compound. Thus they were not directly comparable with the other assays performed subsequently nor were they necessarily representative of ideal coal processing conditions.

In the measurement of Km (affinity) and Vmax (activity) for each inhibitor-enzyme pair, one major pitfall must be noted, namely, it is not known that each enzyme

follows Michaelis-Menton kinetics. Enzymes such as horseradish peroxidase which require a co-factor may not be adequately evaluated by simple double-reciprocal plots. Very little is know about the mechanisms of laccase activities.

It was clear that none of the three enzymes were readily inhibited by the o,o'-biphenol, the end-product of DBT desulfurization. This is an optimistic indication for the feasibility of the anticipated industrial process. Furthermore, the sulfones are seen to react poorly with the horseradish peroxidase, however they do react with sulfatase. It is thus suggested that a sulfatase or related enzyme will be required in addition to enzymes such as laccase and horseradish peroxidase in a full desulfurization process. It is also significant to note that the $I_{50}$ for each compound and its respective sulfoxide is very similar (differences are only two- or three-fold), so it is suggested that these enzymes may carry out at least two steps in the desulfurization process, namely oxidation to the sulfoxide and the corresponding sulfone.

### 4.5.2    Horseradish Peroxidase and Laccase Assays

A second aspect of the work was to evaluate the feasibility of using enzymes in organic solvents for industrial processing. For this reason, the activity and stability of the selected enzymes in organic solvents was first evaluated. Horseradish peroxidase and laccase assays against standard substrates in hydrated organic solvents were carried out, and activities slightly less than or comparable to that seen in aqueous solution were seen.

The studies with the hydrogen peroxide-horseradish peroxidase enzymatic system were first performed in hydrated dimethylformamide and hydrated dioxane with standard substrate. Enzyme activity was very well maintained in a hydrated dimethylformamide matrix and was partially maintained in the dioxane. As shown in the works of Klibanov and others previously discussed, this result was expected. Less work has been published on the use of laccase in such systems, so the demonstration of maintenance of activity of laccase (against standard substrate) for 5 days of storage in organic solvent at room temperature was of importance. These results would indicate that the lifetimes of enzymatic activity will be in accord with the needs of a bioprocessing venue.

The next aspect of the work was to evaluate the activity of the enzymes vs model coal compounds. In the section above, indications that the model compounds did bind to the enzymes were discussed. Laccase (two types) and horseradish peroxidase were assayed vs DBT under a broad variety of conditions. Aqueous organic media tested were: DMF, acetonitrile, ethyl acetate, isopropyl ether, dioxane, heptane, hexane, benzene, and toluene. Hydration levels ranged from 0.5% to 50% aqueous. Hydration was accomplished with either water or buffer (acetate, phosphate, or PIPES). Concentrations of DBT ranged from 20 mM to 0.1 mM. Methods of analysis included: UV, HPLC, GC, GC-MS, and TLC.

Utilizing the UV spectrophotometric analyses, it was expected that it would be possible to determine reaction conditions that facilitate degradation of DBT by the enzymes. Indeed both horseradish peroxidase and laccase induced changes in the DBT UV spectrum. These were more dramatic in the case of horseradish peroxidase. A survey of a wide range of organic solvents demonstrated that more hydrophilic solvents such as isopropyl ether and ethyl acetate provide a more favorable environment for horseradish peroxidase reactivity

Also, controls were run to demonstrate the enzymatic nature of the effects on DBT (i.e., the marked loss of peaks in 300+ nm range were not seen with hydrogen peroxide alone).

In some horseradish peroxidase assays vs. DBT, evidence of DBT sulfoxide and DBT sulfone production without peroxide was seen at seven days. Additionally, DBTsulfoxide and o-hydroxybiphenyl were seen in 168 hour laccase experiment. One factor later observed, however, was that in these tests scintillation vials were used as reactors and after seven days some of the cap liners had loosened. It is speculated that the changes in the reaction mixture were caused by this liner loosening rather than by enzyme activity, since the results could not be repeated when reactors with Teflon liners were used.

The need for using hydrogen peroxide in the horseradish peroxidase assays was debated. This co-factor is commonly utilized with the enzyme and its role depends on the type of reaction catalyzed. Klibanov[27] and Dordick[28] have discussed both reactions which do require hydrogen peroxide (for example in the polymerization of phenols) and those which use, instead of peroxide, a fumarate as an oxygen donor. It is not clear that a sulfur oxidation reaction would require peroxide, but another oxidizing factor other than molecular oxygen might be required.

While the results of the assays of laccase in 50% aqueous organic media were not shown, it was clear that this media itself was excellent for oxidation of DBT sulfur. Shaking with air in these conditions caused considerable conversion and might be considered in a non-biological coal treatment process. It was shown in addition that peroxide alone reacts with DBT at sulfur.

In the many reactions analyzed, the occasional appearance of sulfur oxidation products was observed. Overall, these did not become significant by comparison with controls and it appeared that while DBT was a substrate for the enzymes, oxidation was not occurring at sulfur. It was possible that ring oxidation products were subsequently substrate for sulfur oxidation, but no such products were identified and no significant reduction in DBT was observed.

The rationale for the narrowing of the assay methods was that though several buffers were tested, it became apparent that the buffer was becoming very concentrated in the solvents and that this at the very least did not favor DBT solvation. Several sulfur oxidation products were seen in an impractical solvent, isopropyl ether (which probably had to do with peroxide and with solvent evaporation) and this solvent was dropped. The solvent which consistently gave the greatest conversion seemed to be acetonitrile, so this was pursued to the greatest extent. It had seemed that a water immiscible solvent would be best for processing, but none of these seemed to promote activity vs DBT.

1% Aqueous acetonitrile [0.1 mM DBT] had appeared to be the most reactive system for laccase assays, based on a small number of test samples. When the assays were repeated, however, there was no evidence of DBT sulfur oxidation products. The reactivity observed by UV, and by the appearance of unknowns in GC and HPLC analyses suggests that laccase may act to a limited extent on the carbon ring portions of DBT.

Laccase from USDA was also tested vs DBT [0.2mM] in experiments otherwise identical to earlier work with enzyme from Sigma (1% aqueous acetonitrile). In these assays, an amount of biphenol greater than that seen without enzyme was observed, however the difference was not statistically significant and no significant concentration of intermediates was seen. It appeared that under the conditions tested the USDA laccase was not able to catalyze sulfur oxidation on DBT.

Limited experiments with DBT sulfone as substrate for laccase were carried out. In acetonitrile no reactivity was found. This is not surprising, considering that the $I_{50}$ found for DBT sulfone with laccase was over 20 mM, indicating very little binding interaction with this enzyme.

No tests of the activity of either laccase or horseradish peroxidase vs DBT sulfoxide were carried out. One reason for this was that the commercially available DBT sulfoxide is greatly contaminated with DBT sulfone. Even when the product was freshly purified in our laboratory, it appeared that rapid oxidation occurred as soon as it was placed in solution.

## 5.0     METHODS OF ANALYSIS

### 5.1     UV Spectrophotometric Analysis

One method of characterizing DBT and its sulfur oxidation products is UV/VIS spectrophotometry. The spectral properties of DBT and the intermediates and product of the 4S pathway (i.e., DBT sulfoxide, sulfone, sulfonate, sulfate, and o,o'-biphenol) each exhibit a characteristic UV absorption spectrum. These are shown in Figure 5.1. This method is fast, somewhat sensitive (0.2 mM DBT), and is certainly an indication of change in media composition. It is useful especially in noting the decrease in DBT concentration and the presence of colored open-ring products. Because the various oxidation products have overlapping spectra, the results are difficult to interpret with much detail.

### 5.2     Gas Chromatography as a Means of Monitoring Model Compound Level and Reaction Products

Another method of sample analysis is gas chromatography. One of the reasons for utilizing gas chromatography is to observe more clearly the change in substrate concentration without interference from new species which are formed in the reaction mixture, as is the case with UV spectroscopy. It was possible to quantitate DBT (in the range of 0.2 to 20 mM) and DBT sulfur oxidation products in the reaction media. Below 0.2 mM it is difficult to detect these compounds accurately. Thus, samples were concentrated before analysis. One shortcoming was that buffer and/or media components from microbial sources seemed to alter retention times of the intermediate eluting compounds. Although standards for DBT ring-opening products were not available, it appeared that the GC method did not show such products.

A Varian 3700 gas chromatograph using FID detection was initially fitted with a 3% SP2250, 100/120 Supelcoport column. The carrier gas was $N_2$ at 30 ml/min. The injector and detector were set at 240°C. The temperature program was started at 150°C and held for 5 minutes; the rise was 10 ° C to 260 ° C; the temperature was then held for 5 minutes. The attenuation of the instrument was set at 1 and the range was $10^{11}$ amps. A Varian 4270 integrator was used to record and report the gas chromatograph detector output. The "turn-around-time" for each sample in this method was thirty minutes, so the number of measurements made per day was limited.

In the chromatogram shown in Figure 5.2, 1 μl of a mixture of the four compounds (DBT, DBT sulfoxide, DBT sulfone, and o,o'-biphenol) at concentrations of 0.6 mM was injected.

106
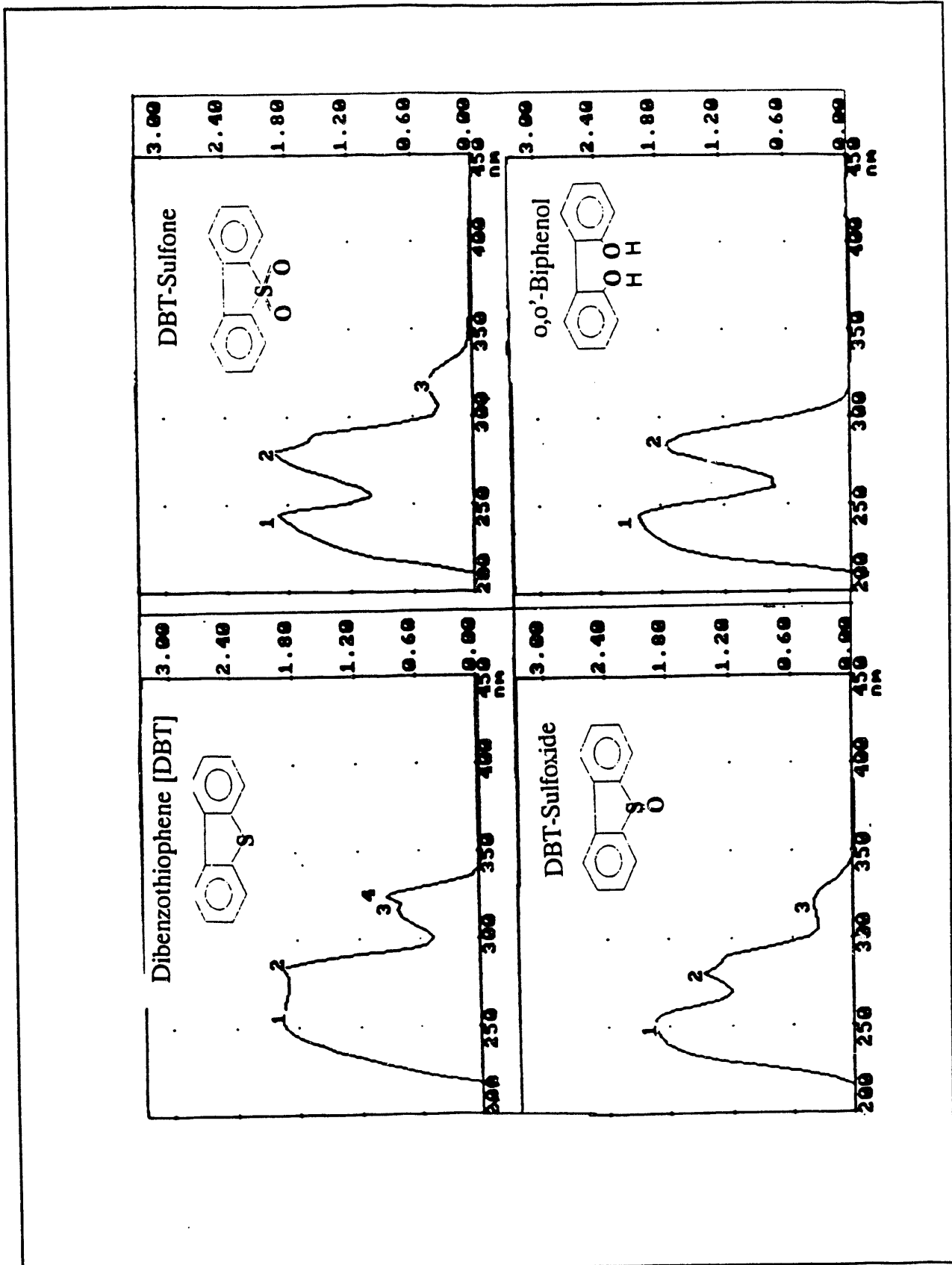
FIGURE 5.1: UV SPECTRA OF DBT AND RELATED COMPOUNDS

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711 0

p-Nitrophenol

A. 88

5. 84 32

o-Hydroxy Biphenyl

6. 59

7. 29

DBT

8. 59

8. 97

o,o'-Biphenol

11. 98

12. 54

DBT-Sulfoxide/Sulfone
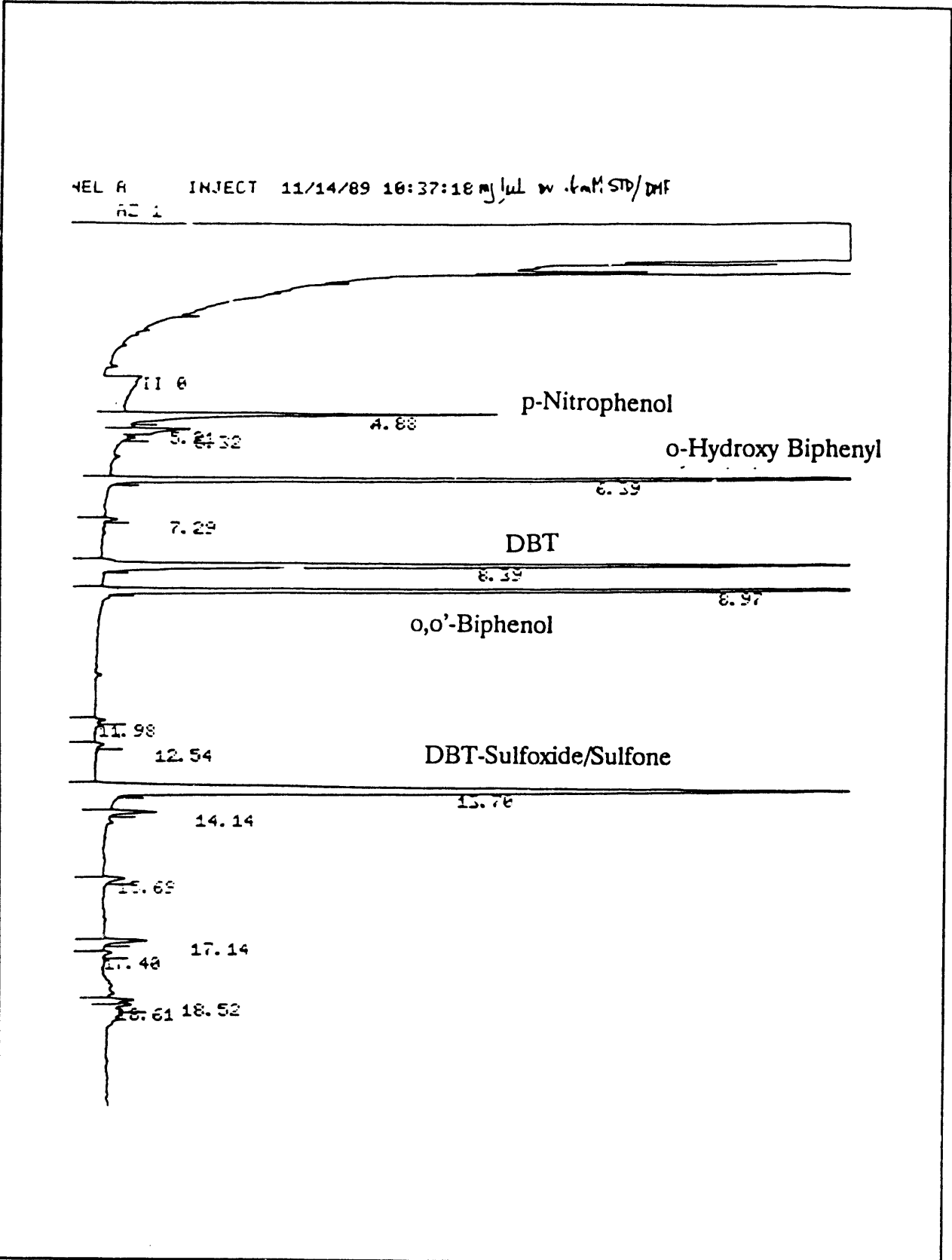
13. 78

14. 14

15. 69

17. 14

17. 48

18. 61  18. 52

FIGURE 5.2: GC STANDARD (1.0μl OF A SOLUTION WHICH WAS 1.2 mM IN DBT AND ITS SULFUR OXIDATION PRODUCTS)

Two changes were subsequently made in the GC procedure during the program. One change was to add the use of a derivatizing reagent. This eliminated some of the difficulties with reproducibility which had been experienced. In addition, the packed column was replaced with a capillary column. The column chosen was a SPB(TM)-1 from Supelco, which is similar to that commonly used on GC-MS equipment. The column has the following characteristics: dimethylpolysiloxane phase; bonded phase; nonpolar; 15 m length, 0.2 mm I.D., and 0.2 $\mu$ film thickness; and described as useful in the separation of solvents, petroleum products, and many other industrial chemicals including acidic, basic, and other active compounds.

When using capillary GC it is sometimes necessary to use split/splitless injection to obtain good resolution of early peaks without interference from solvent peaks. The conditions were as follows: the injector and detector were set at 260°C. The temperature program was started at 120°C and held for 2 minutes, and then increased at 6°C/minute to a final temperature of 240°C. At injection the splitter was turned off and then turned on at 0.75 minutes. This program ran for 30 minutes.

Preparation of samples for GC analysis included filtration and concentration. Assay samples were often dried and resuspended to concentrate the contents. The prepared concentrates were derivatized with TMS-BSA in dimethylformamide; by mixing the reagent TriSil-BSA 50:50 with wet samples or adding the reagent neat to dry samples. These mixtures were heated at 65°C for 15 minutes prior to injection.

## 5.3    GC-MS

The GC program was transferred to the GC-MS. Although the retention times were slightly longer and the sensitivity seemed to be less (on the GC chromatogram) the program was satisfactory. The system utilized with our standards and assay samples was as follows: a Hewlett Packard 5890A gas chromatograph was used with a 5988a mass spectrometer column - Hewlett Packard HP1 cross-linked methylsilicone gum (12m x 0.22 mm, 0.33 $\mu$ film thickness);

The source temperature was 200°C; the analyzer temperature was 280°C and the Initial Column Temperature [$T_i$] was 35°C; the Final Column Temperature [$T_f$] was 300°C. The program was to hold at $T_i$ for 10 minutes, follow a Temperature Rise Rate of 7°C/minute to the final temperature, and then hold at $T_f$ for 5 minutes.

The instrument is a versatile computer driven machine and just learning the "lingo" of the computer menus is not trivial. The manufacturer provided a large file of reference spectra (a library) for use in compound identification. When a sample is run, the software is designed to choose representative scans from the major peaks and to choose the compounds from the library which best match the same scans. Scans from a sample may not exactly resemble the scans from the library, because the sample collection conditions are not necessarily the same as those used in the library data base. For this reason, the matching parameters are not too rigorous and many potential matches are made. If no spectrum in the library resembles the sample spectrum, no identification is made. A very useful feature of the software is that the operator can interactively review the individual

column peaks and select other scans in each peak for analysis. In this way, if the column peak contains more than one compound, multiple identifications can be made.

The compounds in the reference library included DBT, DBT sulfoxide, and o,o'-biphenol. The DBT sulfone was not present and was added by the operator from an in-house standard. Prior to this, when a sample containing DBT sulfone was processed, the identification given from the library was thioanthrene, which has the correct molecular weight, but the wrong molecular formula and structure.

Two examples of GC-MS runs are described and illustrative here as examples of the utility of the method. The first example is a "4S" standard chromatogram (solvent was acetonitrile, contents were equimolar amounts of o,o'-biphenol, DBT, DBT sulfoxide, and DBT sulfone). In this standard run, five major peaks were found above the set threshold. The fifth peak was less than two percent of the total area and was an unidentified contaminant in the standard. The column scan is shown in Figure 5.3 and copies of the computer printouts of the program identifications of the four peaks are included in Appendix B. The first peak was clearly and correctly identified as the o,o'-biphenol. The second peak was correctly identified as the DBT. The third and fourth peaks (expected to be the DBT sulfoxide and DBT sulfone) ran very close and were only partially resolved, as on the DynaGen GC program; and the distribution of areas was not equal. Peak number three was apparently difficult to identify from the sample scan, but the correct compound, DBT sulfoxide, was one of the tentative identifications made from the computer library. Peak number four was not correctly identified by the computer, as the DBT sulfone was not in the compound library, however, the correct mass ion of 216 was found in the scan.

The second GC-MS analysis (Figure 5.4) shown was from an actual laccase assay. Two column peaks were of interest, although because we set low threshold limits for this run seventeen peaks were selected. Peaks 5 and 10 were examined in detail. The major peak, consistent with our expectations, was identified as DBT. The much smaller earlier peak eluted near the retention time of o,o'-biphenol, however, it was identified from the library as a reduced form of DBT in which the bond directly between the two benzyl rings has been reduced (benzene, 1,1'-thiobis).

Since the compounds of interest were occurring in our samples at very low concentrations, the single ion mass spectra mode [SIMS] was employed in subsequent analyses. From the standards, information on relative abundances of ions was collected and only certain mass/ion ratios were collected. With the use of a broad latitude on acceptance, the identity of "4S" compounds in the samples could be confirmed. An example of the column chromatograph and output for a standard run are included in Appendix C. This method is very sensitive and it can given positive identifications of

110
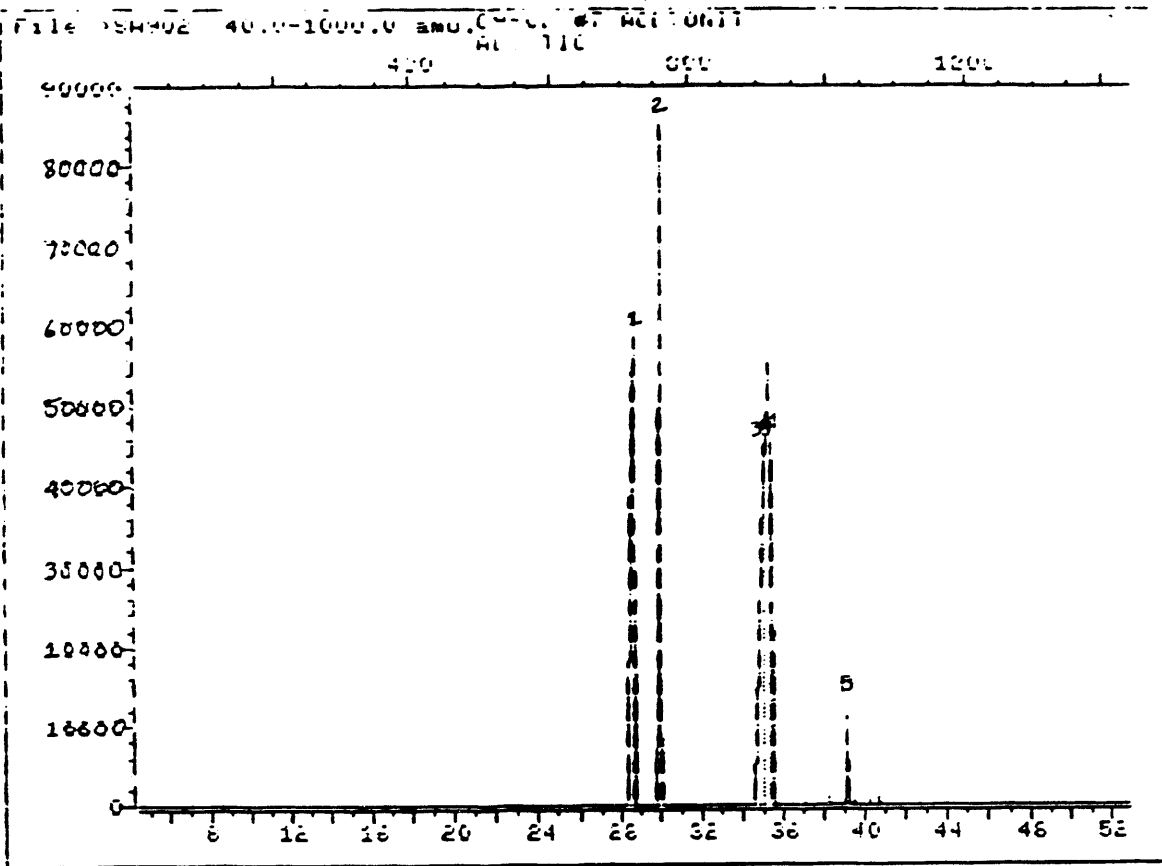
FIGURE 5.3: GC-MS CHROMATOGRAPH OF LACCASE ASSAY SAMPLE

111
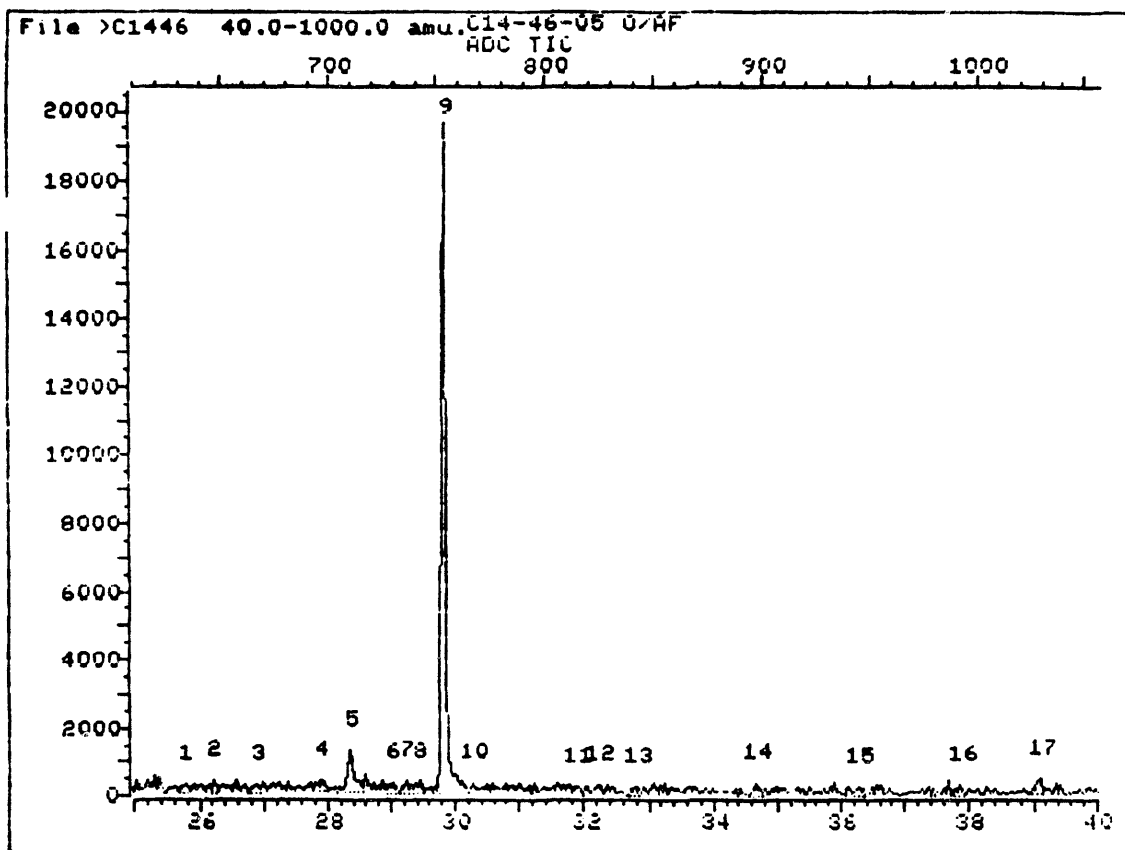
FIGURE 5.4: GC-MS CHROMATOGRAPH OF DBT AND DBT SULFUR OXIDATION PRODUCTS

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compounds. The short coming is that the sulfur containing compounds change oxidation state on the hot column, possibly even before they separate, and their quantitation is thus very difficult.

## 5.4 HPLC Analysis

HPLC was performed on a Waters system with a Waters C18 Resolve 5 micron spherical column. The first HPLC work was performed with a gradient program (method A) as follows:

- Injection volume    : 2 $\mu$l

- Eluent A    : water

- Eluent B    : acetonitrile:water (70:30)

- Gradient program    :

| Time (min) | Flow Rate (ml/min) | %A | %B | Curve |
|---|---|---|---|---|
| Initial | 2.0 | 60 | 40 | * |
| 10 | 2.0 | 30 | 70 | 6 |
| 15 | 2.0 | 0 | 100 | 6 |
| 20 | 2.0 | 60 | 40 | 6 |

- HPLC system:    Waters model 510 pump system (2);  Waters U6K injector; Waters Lambda-max model 481LC spectrophotometer;  (lambda = 254 nm; AUFS = 0.01; response time = 0.5A);  Waters automated gradient controller; and   Spectra Physics SP 4270 integrator.

For the second method (B), the mobile phase, was: tetrahydrofuran:acetonitrile: water [23:18:59]; flow rate, 1.5 ml/minute; injection volume, 2 microliters; detection at 242 nm using a Waters Lambda Max 281 LC spectrophotometer.

The method was modified again (Method C) so as to reduce the program time by about 10 minutes. The modification was in the mobile phase composition. This has been changed to tetrahydrofuran:acetonitrile:water (23:20:57). DBT, the peak that elutes last, now has a retention time of ~19 minutes (as opposed to ~30 minutes). A standard chromatogram is shown in Figure 5.5. DBT sulfoxide and DBT sulfone remain separated in this method.

113
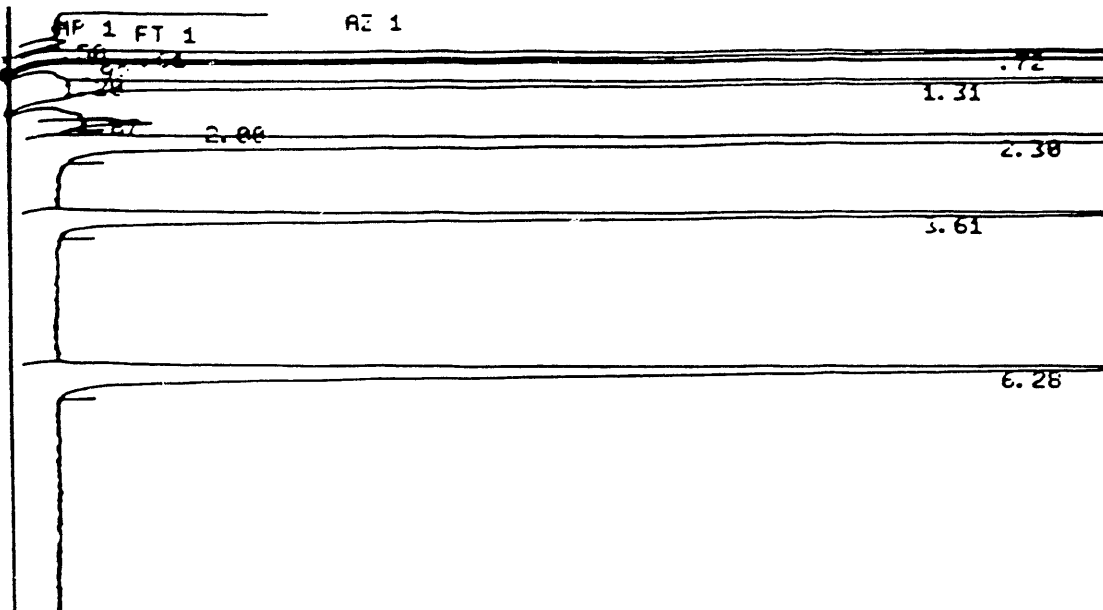
Retention times:  DBT-sulfoxide - 1.31, DBT-sulfone - 2.3, o,o'-biphenol - 3.61,
o-hydroxy biphenyl - 6.28, DBT - 19.28
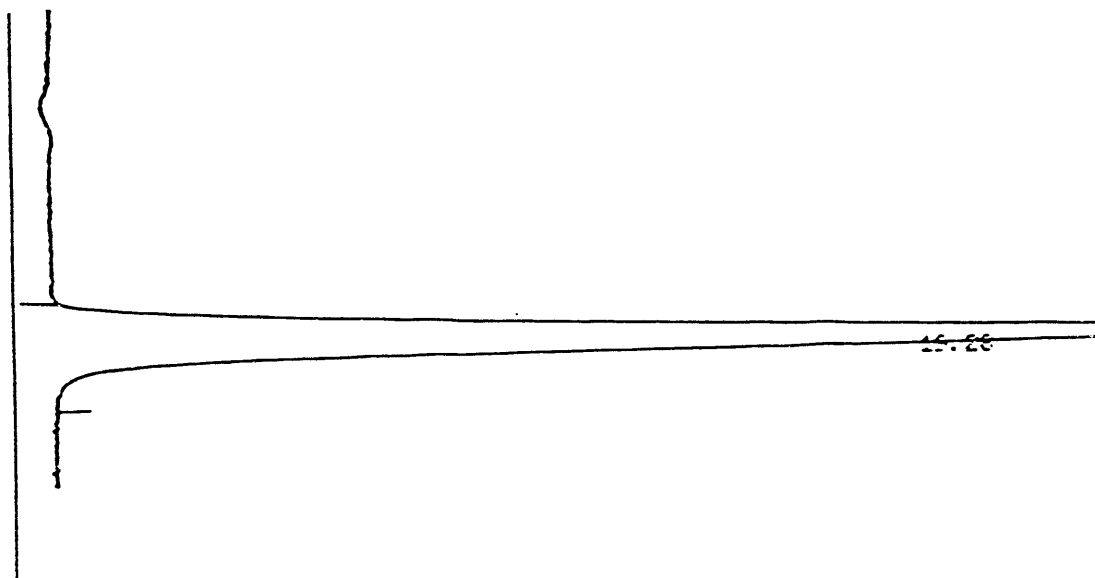
FIGURE 5.5: HPLC STANDARD 14.0 µl OF A 0.1 mM SOLUTION OF DBT AND DBT SULFUR
OXIDATION PRODUCTS

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## 5.5    Studies of Materials Loss During Sample Concentration

### 5.5.1    Drying Samples in Glass Tubes

As mentioned previously, it was suspected that the concentration process was resulting in a loss of some material, possibly due to adherence to glass. In order to confirm the suspected loss of phenols due to drying, a standard solution of o-hydroxybiphenyl and one of a mixed "4S" standard containing DBT sulfoxide, DBT sulfone, o,o'-biphenol, o-hydroxybiphenyl, and DBT was prepared in 90% acetonitrile, 10% Tris buffer. A small volume of each of these standards was dried in a heat block at 40°C and analyzed either: (1) immediately after drying; (2) on keeping for 1 hour at 40°C after drying; and (3) on keeping for 2 hours at 40°C after drying.

The results are presented in Fig. 5.6. It is clear that a substantial loss of DBT and o-hydroxybiphenyl occurs during such a procedure. This loss may be due to adherence of these materials to the glass vessel rather than to volatilization. One potential method of eliminating this problem is to coat the glassware with DriCote (Fisher No. D140-50). An initial experiment was performed to determine any differences in levels of recovered materials from samples evaporated in silane coated glassware. Equal volumes, 300 μl, of 0.1 mM "4S" standards were placed in two silane coated vials. One sample was allowed to evaporate until the volume had been reduced to 13.5 μl. The second sample was allowed to evaporate to complete dryness and then was resuspended to a volume of 13.5 μl. Both samples were analyzed by HPLC. Figure 5.7 shows the results. In this case, the quantities of phenolic compounds were better conserved, however, losses of DBT sulfoxide and DBT sulfone were larger than in unsilanized vials.

### 5.5.2    Difficulties in GC Analysis of Extracts from GB-1 ICF Assays Derivatized in Silanized Vials

Another difficulty with this method was subsequently noted. When a trial extraction of neat GB-1 ICF was performed to determine whether there were materials present in the GB-1 ICF that possibly may interfere in the analysis of the assay samples, previously unnoticed late eluting peaks were discovered. While these peaks occurred at retention times other than at our peaks of interest, it was decided to try to modify the GC program in order to prevent these materials from adhering to either the column or injection liner. It was feared that if these compounds did not elute until a subsequent sample was run, the analysis could have been impaired. Raising the final column temperature and extending the length of time at this final temperature did not prevent these materials from being retained in the system. When subsequent blank runs (with no sample injected) showed peaks eluting, we knew that just the modifications of the GC program were not eliminating the problem.

It was suspected that a possible source of these materials was the silane treated vials used for concentration and derivatization of the extracts. Silane-treated and untreated vials to which no sample had been added were derivatized with TriSilBSA/Formula D under identical conditions and analyzed on the GC. The silane treated vials showed these extraneous peaks while the untreated vials showed none. A varied silanization procedure was attempted by rinsing the coated vials with methanol rather than water prior to drying. Standard amounts of model compounds were analyzed in these methanol rinsed silanized vials and showed no extraneous peaks. However, compared with standards from water rinsed silanized vials, the amounts of phenolic compounds detected were dramatically reduced.

The extraneous peaks eluted even after one or two subsequent blank runs with the GC temperature program lengthened to a rather impractical analysis time for each sample. This, combined with the fact that some model compounds could not be adequately analyzed if non-silane treated vials were used for derivatization, eliminated the use of gas chromatography as a practical method of analysis and strongly indicated that HPLC analysis would be a preferred method when the detection of very low concentrations of products is sought.

## 5.5.3    Using SepPacs^R for Concentration of Phenols

Disposable SepPak^R cartridges (Water Associates) were tested as a means of separating and concentrating all the reaction products or the phenols selectively. The C18 and CN SepPaks tried were not effective in binding either the phenols or the DBT sulfone preferentially and pretreatment of the sample by making it acidic did not improve the procedure. It appears that because the initial samples were in acetonitrile, they did not readily partition onto the SepPak.

## 5.5.4    Extraction of Phenols from Assay Samples

When GB-1 ICF was incubated with 0.05 mM sulfone for 4 days at 25°C, the formation of o,o'-biphenol and o-hydroxybiphenyl was detected. In order to confirm o-hydroxybiphenyl, a scheme was developed to separate the phenols from the ICF-sulfone mixture. This was based on the principle that the phenols will have greater solubility in basic solutions. The procedure was initially done with a standard mix containing o-hydroxybiphenyl, o,o'-biphenol, and sulfone in 90% acetonitrile and 10% Tris buffer. To 2.5 ml of this solution was added 2.5 ml of 0.1 N NaOH and 2.5 ml methylene chloride.

This was mixed thoroughly and the layers were allowed to separate. The top basic layer was removed. To the methylene chloride layer was added 2 ml of base and after vortexing and separation of the layers, the methylene chloride layer was extracted twice more with base. All the base layers were pooled, acidified by the addition of concentrated HCl, and extracted twice in 2 ml of methylene chloride. At each extraction, the layers were analyzed by HPLC to make sure that no more phenols were left behind in the methylene chloride/base layers. The methylene chloride layers were pooled, dried to concentrate the phenols, and resuspended in a small volume of acetonitrile. This was then analyzed by

116
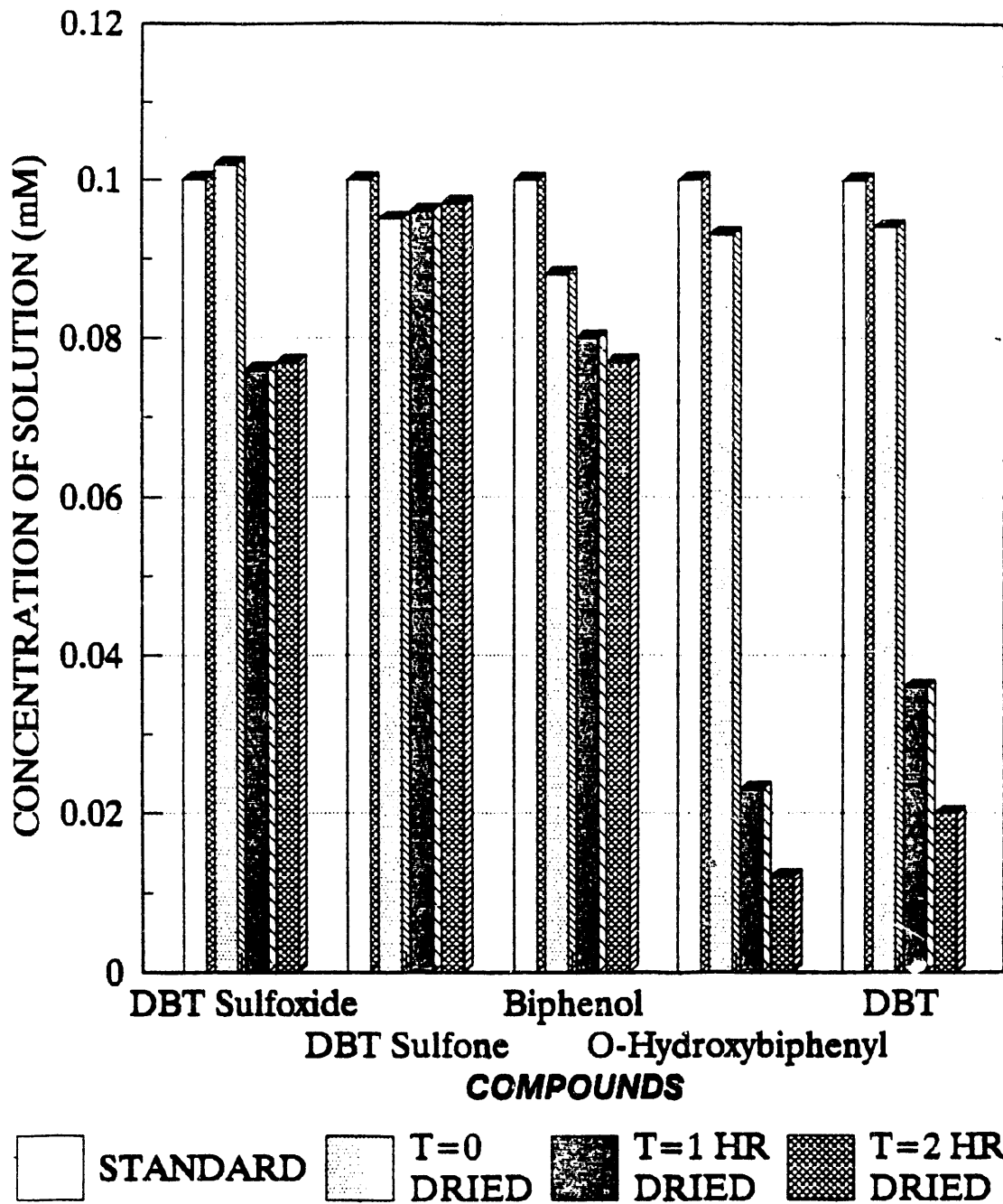
FIGURE 5.6: LOSSES OF DBT AND DBT OXIDATION PRODUCTS DURING DRYING IN GLASS TUBES
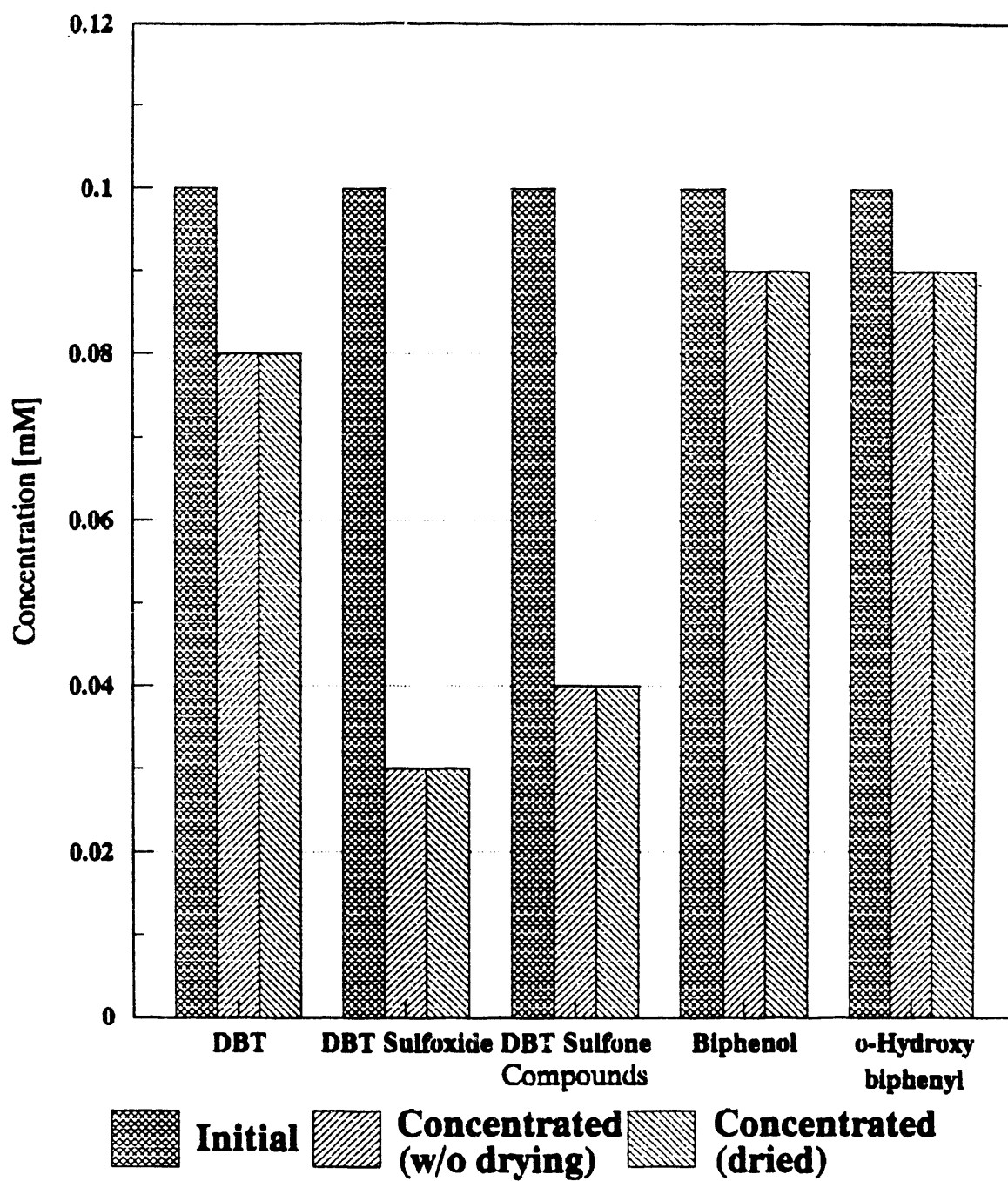
FIGURE 5.7: RESULTS OF USING SILANIZED VIALS FOR SAMPLE DRYING

HPLC. By this procedure, about 50% of the o,o'-biphenol and only 10 to 20% of the o-hydroxybiphenyl could be recovered. It became apparent that the loss was occurring during the concentration/drying step. The concentration of the phenols by evaporation of methylene chloride was a required step since the amount of phenol in the samples was not high enough to be detectable without concentration.

# 6.0 CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Conclusions

### • Organism from Novel Source Uses DBT

One organism (GB-1) from a novel source, the soil of a hydrothermal vent in the Guyamas basin of the Gulf of California, was isolated and shown to utilize the model compound DBT as a sole carbon and sulfur source. DBT sulfur oxidation intermediates were observed in the growth media while prior core indicated that microbes that produced red "Kodama"[22] products did not perform sulfur oxidation. It was shown that GB-1 carried out both processes.

### • An Enzyme Consortium Active Against DBT Was Isolated from GB-1

The organism has been fractionated and the activity against model compounds has been retained, largely in the intracellular fraction.

### • Activity of Enzyme Fractions Was Retained in Organic Solvent

Unlike the parent organism, the fractionated enzyme materials were functional in organic solvents. This suggests that coal processing could be carried out in an organic media. This is important because the coal surface is more readilly wetted by some organic solvents, and it is expected that this this would lower the energy barrier of enzyme access to the coal matrix, thus facilitating the coal processing.

### • Commercial Enzymes Tested Did Not Show Activity Against the Sulfur in DBT

It had been hoped that known enzymes with oxidative capabilities would be active against organic sulfur when utilized on hydrated organic media. After extensive testing, it was found that the enzymes did have binding affinity for the sulfur containing compounds, and they did show some activity against the model compound DBT. This activity did not appear, however, to be related to sulfur oxidation.

### • During Long Test Periods in Oxygenated Conditions, Spontaneous DBT Sulfur Oxidation Occurred in Organic Media

It is know that oxygen present in air oxidizes sulfur in coal over time. It was shown during the program that shaking the model compounds in aerated aqueous organic solvent, especially at higher amounts of hydration (50%), produced oxidation without biological catalyst. It is reasonable to expect that an organic media for coal processing may also facilitate sulfur removal.

## 6.2     Recommendations

This program has led to very interesting developments which could be carried much further. Some noteworthy recommendations are discussed below.

## CONTINUE ISOLATION AND CONCENTRATION OF GB-1 ENZYMES

Ultimately in a biodesulfurization process a consortium of organisms, recombinant organisms, or enzyme "cocktails" (measured mixtures) will be needed for complete and economical production. For this reason a diverse series of biocatalysts sources must be maintained. As stated above, the organism GB-1, isolated from a unique source, may be a crucial source of enzymes for coal desulfurization. It is very important to continue the work of isolating and characterizing these enzymes and co-factors (if any). The intracellular fraction should be further fractionated by methods such as were discussed in Section 3. Metals such as molybdenum may be needed in order to produce the enzymes efficiently. Changes in solvation must be further examined, including both organic and aqueous systems.

## PERFORM SIMILAR FRACTIONATIONS ON OTHER ORGANISMS

Many other organisms have shown activity against model compounds and coal. It is important to fractionate these organisms and examine such fractions for activity against the substrates which the whole organisms have utilized. It is possible that some previously tested organisms may have been poisoned by model compounds, whereas they may contain or produce enzymes which are fully capable of attacking organic sulfur. While the task may seem daunting, patterns of enzyme activities which would emerge from in vitro studies could actually simplify the choice of processes and the choice of genes for gene engineering if desired by showing commonalities of specific functions among organisms.

## DEVELOP STANDARD ASSAYS FOR MODEL COMPOUNDS AND COAL

Recommendations 1 and 2 would be accomplished much more readily if some standard assay methods could be utilized by all laboratories concerned. Assays for both coal and model compounds could be standardized by choosing A) standard times of measurement; b) standard methods of measuring activity (i.e., the way of monitoring the increase of products and decrease of starting material); c) if the substrate is coal, one type of demineralized coal should be used and some method of materials balance which fits all scenarios should be devised, i.e., all mass in media, gas produced (if microbes are used), biomass, and coal mass should be accounted for.

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# 7.0    MATERIALS SOURCES

While the sources of supplies are frequently given in the text, the following list of supply sources is given for simplicity:

The commercial enzymes used in our experiments were laccase (No. L-5510) and horseradish peroxidase (No. P-8125) which were both obtained from Sigma Chemical Company. Sulfatase was also purchased from Sigma (no.S-9751). Additionally, some laccase from a different source was obtained from Dr. C. Walsh who received it from Dr. Kirk at the USDA.

Dimethylformamide was from Burdick and Jackson (No. 076). Acetonitrile (No. A998-4) and Ethyl Acetate (No. E-195) were both HPLC grade and were obtained from Fisher Scientific.

Model compounds were obtained from various sources: DBT (Aldrich), DBT sulfoxide (K&K Biochemicals), DBT sulfone (Lancaster Biochemicals), EPS (Aldrich), ethylphenylsulfoxide (Lancaster Biochemicals), ethylphenylsulfone (Lancaster Biochemicals), and o,o'-biphenol (Aldrich).

The syringe filter units used for aliquots removed from the assay samples were 0.2 $\mu$ pore size, 3 mm diameter Nylon 66 membranes in a polypropylene housing. These disposable syringe filter units were purchased from Rainin Instrument Company (No. 38-159). Larger diameter (13 mm) and pore size (0.45 $\mu$) syringe filter units (Rainin No. 38-154) were also used to pre-filter aliquots from microbial enzyme samples that contained suspended material.

The samples for GC were derivatized with Tri-Sil/BSA; Formula D (in dimethylformamide) from Pierce Chemical Company (No. 49010).

## 8.0 REFERENCES

1. Kargi, F., "Microbial Desulfurization of Coal" in Advances in Biotechnologica Processes 3 (Alan R. Liss, Inc., New York, 1984).

2. Meyers, R., Coal Desulfurization (Marcel Dekker, Inc., New York, 1977).

3. Dugan, P.R., in "Biotechnology for the Mining, Metal Refining, and Fossil Fuel Processing Industries," Biotech. and Bioeng. Symposium 16 (1986), pp 185-203, Interscience.

4. Ensley, B.D., in "Microbial Degradation of Organic Compounds" (1984), Dekker, New York, pp 309-317.

5. Monticello, D.T. and Finnerty, W.R., in "Ann. Rev. Microbiol. 39" (1985), pp 371-389.

6. Biological Coal Beneficiation - Literature Review, Final Report, November, 1986, for EPRI, prepared by Battelle Pacific Northwest Laboratories.

7. "Proceedings of Bioprocessing of Fossil Fuels Workshop." (1989) DOE and Idaho National Engineering Laboratory Bioprocessing Center.

8. "Proceedings of First International Symposium on the Biological Processing of Coal." (1990) In press. Electric Power Research Institute.

9. Zaks, A. and Klibanov, A.M., Proc. Natl. Acad. Sci. USA, 82, 3192 (1985).

10. U.S. Patent No. 4,562,156 (to Isbister et al. in 1985).

11. Cohen, M.S. and Gabriele, P.D., Appl. Environ. Microbiol. 44, 23 (1982).

12. Jannasch, H.W., Wirsen, C.O., Molyneau, S.J., and Langworthy, T.A., Applied and Environ. Microbiol. 54(5):1203-1209 (1988).

13. Zaks, A. and Klibanov, A.M. (1988) J. Biol. Chem. 263:8017-8021.

14. Zaks, A. and Kilbanov, A.M.. J. Am. Chem. Soc. 108 (1986):2767-2768.

15. Scott, C.D. and Faison, B.D. in "Proceedings of Workshop on Biological Treatment of Coal" (1987).

16. Bazylinski, D.A., C.O. Wirsen, and H.W. Jannasch. (1989). Appl. Environ. Microbiol. 55:324-326.

17. Report to Houston Lighting and Power.

18. Kodama, K., Nakatani, S., Umehara, K., Shimizu, K., Minoda, Y., and Yamada, K. (1970) Ag. Biol. Chem. 34:1320-1324.

19. Bazylinski, D.A. (1989) Op. Cit.

20. Wolin, E.A., M.J. Wolin, and R.S. Wolfe. (1963) J. Biol. Chem. 238:2882-2886.

21. Buck, J.D. (1982) Appl. Environ. Microbiol. 44:992-993.

22. Kodama, K. (1970) Op. Cit.

23. Monticello, D.J., D. Bakker, and W.R. Finnerty. (1985) Appl. Environ. Microbiol. 49:756-760.

24. Laborde, A.L. and D.T. Gibson. (1977) Appl. Environ. Microbiol. 34:783-790.

25. Wyza, R. 1989. ARC Tech. Personal Communication.

26. Fersht, A., "Enzyme Structure and Mechanism." (1977) W.H. Freeman and Co., Ltd., San Francisco.

27. Klibanov, A.M., Tu, T-M, Scott, K.P. Science (1983) 221:259-261.

28. Dordick, J.S., Klibanov, A.M., Marletta, M.A. Biochemistry (1986) 25:2946-2951.

# APPENDIX A

**Final Report from Woods Hole Oceanographic Institution**

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## "ISOLATION OF MARINE DIBENZOTHIOPHENE- AND ETHYLPHENYLSULFIDE-TRANSFORMING MICROORGANISMS"

## L Sources of Inocula

Sources of inocula for the isolation of marine dibenzothiophene (DBT) and ethylphenylsulfide (EPS) came from a number of different sources including sediment and water from: 1) Eel Pond, Woods Hole, MA; 2) the Black Sea; and 3) the Guaymas Basin. Enrichments using material from the first two sites were negative for these types of organisms. Because of this and other reasons (see below), we concentrated our efforts on samples from the Guaymas Basin.

The Guaymas Basin is one of a series of deep semi-closed basins in the Gulf of California (Byrne and Emery, 1960) which is tectonically active (Curray et al., 1979). It is separated by a 20 km transform fault into two rift valleys, the Northern and Southern Troughs (Simoneit and Lonsdale, 1982). Hydrothermal vents are numerous in the Southern Trough which is the area we sampled with the use of the DSV *ALVIN* during February 1987. Exuding hydrothermal fluids discharge through chimneys at between 270°-325°C and through sediment at about 50°C (Lonsdale et al., 1980; Lonsdale and Becker, 1985; personal observations).

The Guaymas Basin is unique in that rapidly accumulating hemipelagic sediment which amasses at a rate of 1.0-1.2 m per 1000 yr (Curray et al., 1979) is pyrolyzed under high temperature conditions to petroleum-like products (Simoneit and Lonsdale, 1982; Simoneit, 1984, 1985). These products consist of gasoline-range aliphatic and aromatic hydrocarbons and predominantly residual polar asphaltic material (Simoneit and Lonsdale, 1982). The sediments also contain large amounts of sulfur (Bazylinski et al., 1988) some of which is found in organic compounds particularly in the form of cyclic polysulfides (Kawka and Simoneit, 1987).

Geochemical analyses of extracted petroleums from sediments of the Southern Trough of the Guaymas Basin and microbiological studies of the area suggest that microbial degradation of hydrocarbons is a significant process at the site (Simoneit, 1985; Bazylinski et al., 1988, 1989). Because of the presence of organic sulfur compounds and hydrocarbon-utilizing bacteria as well, we thought this area to be a likely source of material from which to isolate DBT- and/or EPS-utilizing microorganisms.

## II. Experimental Procedures

Approximately 1-2 g of sediment and water were used as inocula and added to flasks containing the following basal artificial seawater medium (g $L^{-1}$ unless otherwise noted): NaCl, 23.477; $MgCl_2 \cdot 6H_2O$, 4.981; $Na_2SO_4$, 3.917; $CaCl_2 \cdot 2H_2O$, 1.102; KCl, 0.664; $NaHCO_3$, 0.192; KBr, 0.096; $H_3BO_3$, 0.026; $SrCl_2 \cdot 6H_2O$, 0.024; $NH_4Cl$, 0.5; and 5 ml of Wolfe's mineral elixir (Wolin et al., 1963) modified by the addition of 0.4 g $L^{-1}$ of $Na_2MoO_4 \cdot 2H_2O$ rather than 0.01 g $L^{-1}$. The medium was autoclaved after which 2.7 ml $L^{-1}$ of sterile potassium phosphate buffer (750 mM, pH 7.2) and 5 ml $L^{-1}$ of a filter sterilized vitamin solution (Bazylinski et al., 1989) were added. The pH of the medium was adjusted to 7.2 with sterile 0.1 M NaOH or HCl. Sterile DBT or EPS was added to the medium at a concentration of 0.1% (w/v) as the sole carbon source. DBT was dissolved in acetone or ether and filter-sterilized and EPS was filter-sterilized directly (solvent resistant FP Vericel; porosity, 0.2 μm; Gelman Sciences, Inc., Ann Arbor, MI). The acetone or ether was evaporated under a stream of sterile nitrogen gas.

Previous results with hydrocarbon-utilizing bacteria isolated from oily Guaymas Basin sediments showed that most if not all of these bacteria are mesophilic (growth optima >20°C and <45°C) (Bazylinski et al., 1989). For this reason, all enrichment cultures were incubated at 25°C.

Enrichment cultures were checked daily for: 1) turbidity, 2) appearance of colored compounds, 3) change in the appearance of DBT and EPS, and 4) fluorescence under short wave ultraviolet light (indicating the presence of certain intermediates in the desulfurization of DBT - J. Marquise, personal communication). Cultures were considered positive if any of the above criteria were met and were then used for subsequent steps in isolation of DBT- and EPS-utilizing microorganisms.

Material from positive enrichment cultures were streaked onto similar medium solidified with 1.5% Purified Agar (Difco). Some agar plates also contained various amounts (0.01-0.05%, w/v) of Yeast Extract (Difco). DBT was dissolved in acetone and ether (2%, w/v) and

sprayed onto the surface of the agar plates using a nebulizer. Approximately 1.0-1.5 ml was sprayed onto each agar plate. Once separated colonies were apparent, material from these colonies were streaked onto new plates. This was repeated at least three times to ensure the use of a pure culture.

## III. Results

*EPS:* - All enrichment cultures containing EPS using inocula from all sampled sites were negative for turbidity (growth). In addition, there was never any change in the appearance of the EPS and microscopic examination of the culture showed no increase in bacterial numbers.

*DBT:* - All enrichment cultures containing DBT with inocula from Eel Pond and the Black Sea were negative based on the previously mentioned criteria.

Although most enrichment cultures inoculated with Guaymas Basin sediment were also negative, one culture containing sediment from an oily core obtained on *ALVIN* dive number 1967 was positive. After two weeks of incubation, the culture fluid was red and some of the DBT disappeared. The culture was slightly turbid and microscopic examination of the culture indicated large numbers of a motile, rod-shaped bacterium. There was no fluorescence under short wave ultraviolet light. A sample of red culture fluid was filtered and analyzed spectrophotometrically. The pigment had a visible absorbance maximum at about 392 nm.

The enrichment procedure was repeated using more sediment from the same source and material from the original positive enrichment was streaked onto DBT agar plates as described earlier. As in the first enrichment culture, culture fluid from the second also became reddish and slightly turbid after two weeks. Microscopic examination of the culture showed organisms of similar morphology to those observed in the first enrichment culture. This result suggests that these organisms are distributed throughout the sediment of the sample. Red areas became apparent on the DBT-containing agar plates (with and without yeast extract) in about 11-12 days. However, single colonies grew much more quickly when yeast extract was included. Several colonies were restreaked onto DBT agar plates containing 0.05% (w/v) yeast extract. In two weeks colonies formed with reddish zones surrounding them. This red pigment eventually diffused throughout the medium indicating that the pigment was water soluble. In addition, sprayed water-insoluble DBT particles disappeared around the colonies. This and the fact that no reddish color appeared on control agar plates lacking DBT indicates that the red material results from a bacterial transformation of DBT. This restreaking was repeated once more.

Once returned to liquid culture, the strain also produced red pigment from DBT which occasionally precipitated out of the growth medium late in the growth cycle. Several strains were purified and kept. These were designated GB1DBTa, GB1DBTb, GB1DBTc, and GB1DBTd. The organism was rod-shaped, motile, and stained variably with the Gram stain. The KOH test for the determination of the Gram reaction (Buck, 1982) was also equivocable.

Smaller colonies which did have clear zones on DBT agar plates and did not produce any pigment were also restreaked to obtain a pure culture. Two strains were obtained and designated GB2DBTa and GB2DBTb. The organism was vibrioid-to-helical in morphology, motile, and Gram-negative. There was no indication that this organism could transform DBT in any way but more specific tests should be done to determine this.

## IV. Final Considerations

This study has resulted in the isolation of at least one organism (strain GB1DBT) which can transform DBT to a red water soluble compound(s). It is unclear whether this organism can remove sulfur during this transformation. In addition, the red compound has not been identified. It is interesting that a strain of *Pseudomonas putida* has been reported to convert DBT to DBT sulfone via DBT-5-oxide and to 3-hydroxy-2-formyl benzothiophene via a previously described pathway (Kodama et al., 1970, 1972) while also producing an unidentified red-colored product (Mormile and Atlas, 1989). Further studies may show that strain GB1DBT and the strain of *P. putida* utilize similar pathways of DBT oxidation.

## V. References Cited

Bazylinski, D. A., J. W. Farrington and H. W. Jannasch. 1988. Hydrocarbons in surface sediments from a Guaymas Basin hydrothermal vent site. Org. Geochem. 12: 547-558.

Bazylinski, D. A., C. O. Wirsen and H. W. Jannasch. 1989. Microbial utilization of naturally occurring hydrocarbons at the Guaymas Basin hydrothermal vent site. Appl. Environ. Microbiol. 55. In press.

Byrne, J. V. and K. O. Emery. 1960. Sediments in the Gulf of California. Geol. Soc. Am. Bull. 71: 983-1010.

Buck, J. D. 1982. Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. Appl. Environ. Microbiol. 44: 992-993.

Curray, J. R., D. G. Moore, J. E. Aguayo, M.-P. Aubry, G. Einsele, D. J. Fornari, J. Gieskes, J. C. Guerrero, M. Kastner, K. Kelts, Y. Matoba, A. Molina-Cruz, J. Niemitz, J. Rueda, A. D. Saunders, B. R. T. Simoneit, and V. Vacquier. 1979. Leg 64 seeks evidence on the development of basins. Geotimes 24: 18-20.

Kawka, O. E. and B. R. T. Simoneit. 1987. Survey of hydrothermally-generated petroleums from the Guaymas Basin spreading center. Org. Geochem. 11: 311-328.

Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. III. Isolation and identification of products from dibenzothiophene. Agric. Biol. Chem. **34:** 1320-1324.

Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda and K. Yamada. 1973. Identification of products from dibenthothiophene and its proposed oxidation pathway. Agric. Biol. Chem. **37:** 45-50.

Lonsdale, P. F., J. L. Bischoff, V. M. Burns, M. Kastner and R. E. Sweeney. 1980. A high-temperature thermal deposit on the seabed at a Gulf of California spreading center. Earth Planet. Sci. Lett. **49:** 8-20.

Lonsdale, P. F. and K. Becker. 1985. Hydrothermal plumes, hot springs, and conductive heat flow in the Southern Trough of Guaymas Basin. Earth Planet. Sci. Lett. **73:** 211-225.

Mormile, M. R. and R. M. Atlas. 1989. Biotransformation of dibenzothiophene and dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. **35:** 603-605.

Simoneit, B. R. T. 1984. Hydrothermal effects on organic matter-high vs. low temperature components. Org. Geochem. **6:** 857-864.

Simoneit, B. R. T. 1985. Hydrothermal petroleum: composition and utility as a biogenic carbon source. Biol. Bull. Soc. Wash. **6:** 49-56.

Simoneit, B. R. T. and P. F. Lonsdale. 1982. Hydrothermal petroleum in mineralized mounds at the seabed of Guaymas Basin. Nature (London) **295:** 198-202.

Wolin, E. A., M. J. Wolin and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:** 2882-2886.

# APPENDIX B

## Examples of GC-MS Analysis

(1)   A standard containing o,o'-biphenol, DBT, DBT sulfoxide and DBT sulfone

(2)   A laccase assay sample
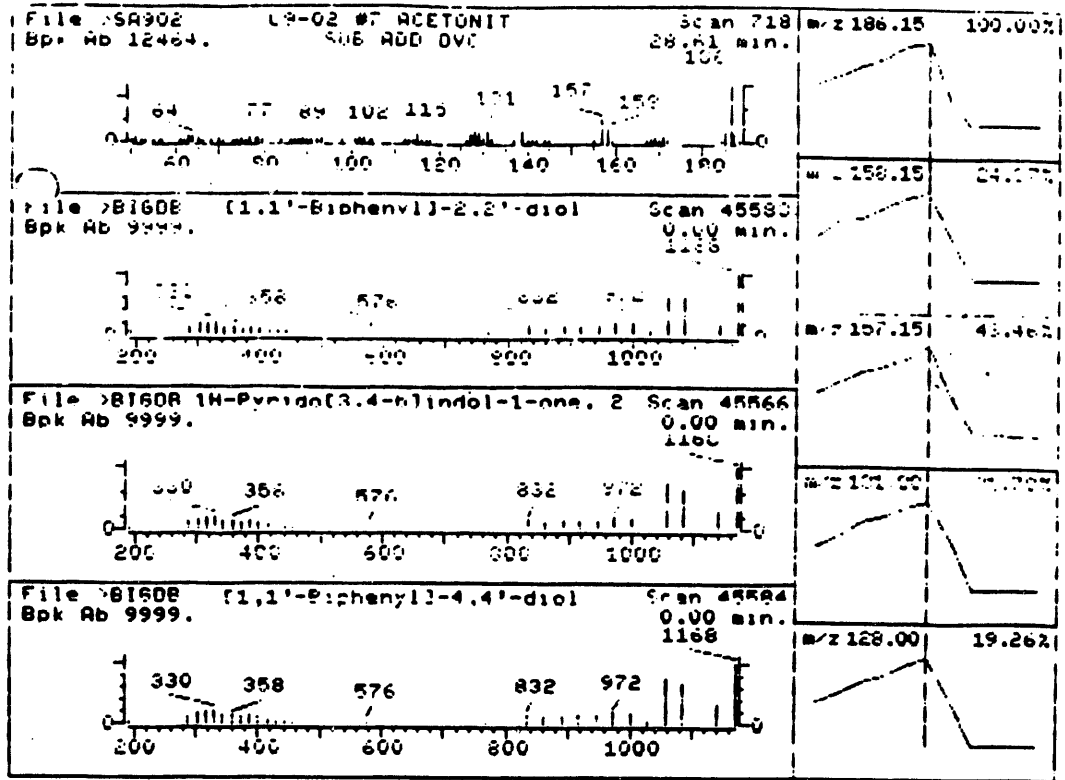
1. [1,1'-Biphenyl]-2,2'-diol
2. 1H-Pyrido[3,4-b]indol-1-one, 2,3,4,9-tetrahydro-
3. [1,1'-Biphenyl]-4,4'-diol
4. Phenanthrene, octahydro-
5. 1,4-Naphthalenedione, 2,3-dimethyl-

Sample file: >SAY02      Spectrum #:        759
Search speed: 1      fitting option: 5      No. of ion ranges searched:    49

| | Prob. | CAS # | CON # | Root | R | DR | #FLG | TILT | % | CON | C_I | F_I |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 90* | 132650 | 45303 | "BIGDB | 78 | 5 | 1 | 0 | 95 | 3 | | |
| 2. | 90* | 132650 | 45302 | "BIGDB | 62 | 17 | 0 | 0 | 85 | 5 | | |
| 3. | 95* | 132650 | 45301 | "BIGDB | 67 | 14 | 0 | 0 | 70 | 5 | | |
| 4. | 86* | 132650 | 45061 | "BIGDB | 70 | 43 | 2 | 0 | 80 | 1 | 65 | 5 |
| 5. | 86* | 132650 | 45304 | "BIGDB | 66 | 40 | 2 | -1 | 89 | 3 | 60 | 3 |
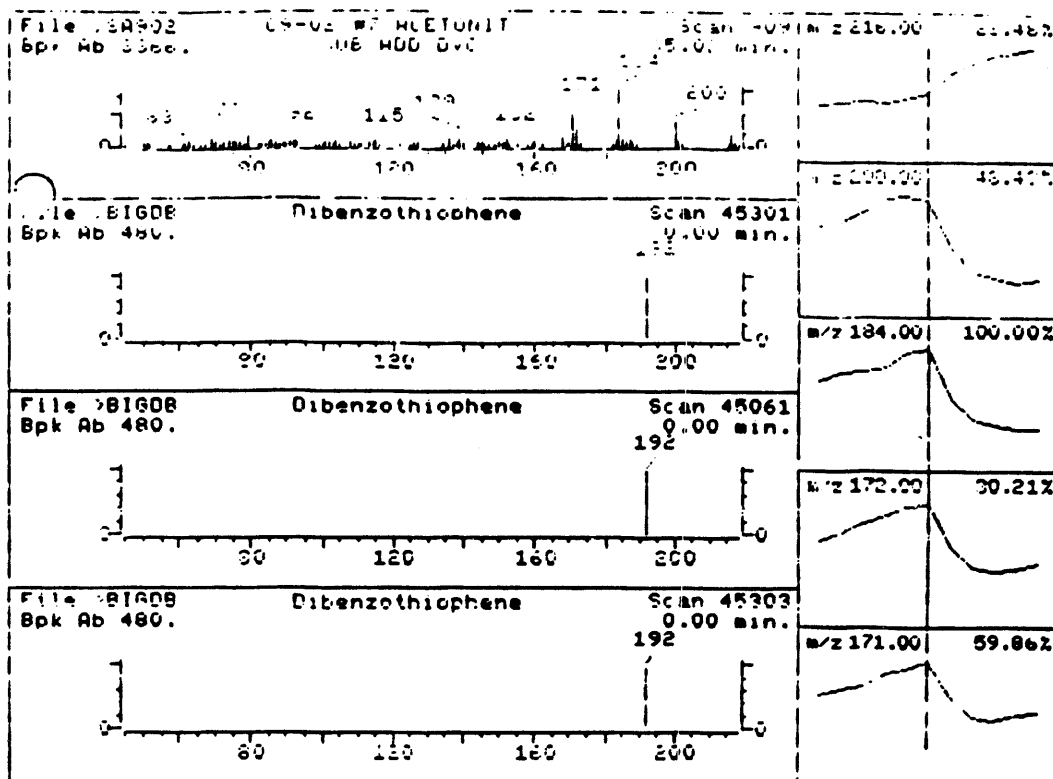
1. Dibenzothiophene
2. Dibenzothiophene
3. Dibenzothiophene
4. Dibenzothiophene, 5-oxide
5. Phenoxathiin

184 C12H8S
184 C12H8S
184 C12H8S
200 C12H8OS
200 C12H8OS

Sample file: SH902    Spectrum #:    909
Search speed: 1    Tilting option: 5    No. of ion ranges searched:   47

| | Prob. | CHS # | CON # | ROOT | K | DK | #FLG | TILT | % | CON | C_1 | H_1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 33* | 132050 | 45301 | "BIGDB | 61 | 40 | 0 | 0 | 88 | 60 | 12 | |
| 2. | 31* | 132650 | 45061 | "BIGDB | 78 | 55 | 2 | 0 | 87 | 60 | 8 | |
| 3. | 20* | 132050 | 45303 | "BIGDB | 48 | 55 | 0 | 0 | 85 | 60 | 8 | 60 |
| 4. | 24* | 1013236 | 45123 | "BIGDB | 52 | 72 | 0 | 0 | 85 | 60 | 7 | 5. |
| 5. | 20* | 262204 | 43901 | "BIGDB | 43 | 66 | 2 | 1 | 71 | 55 | | 1. |
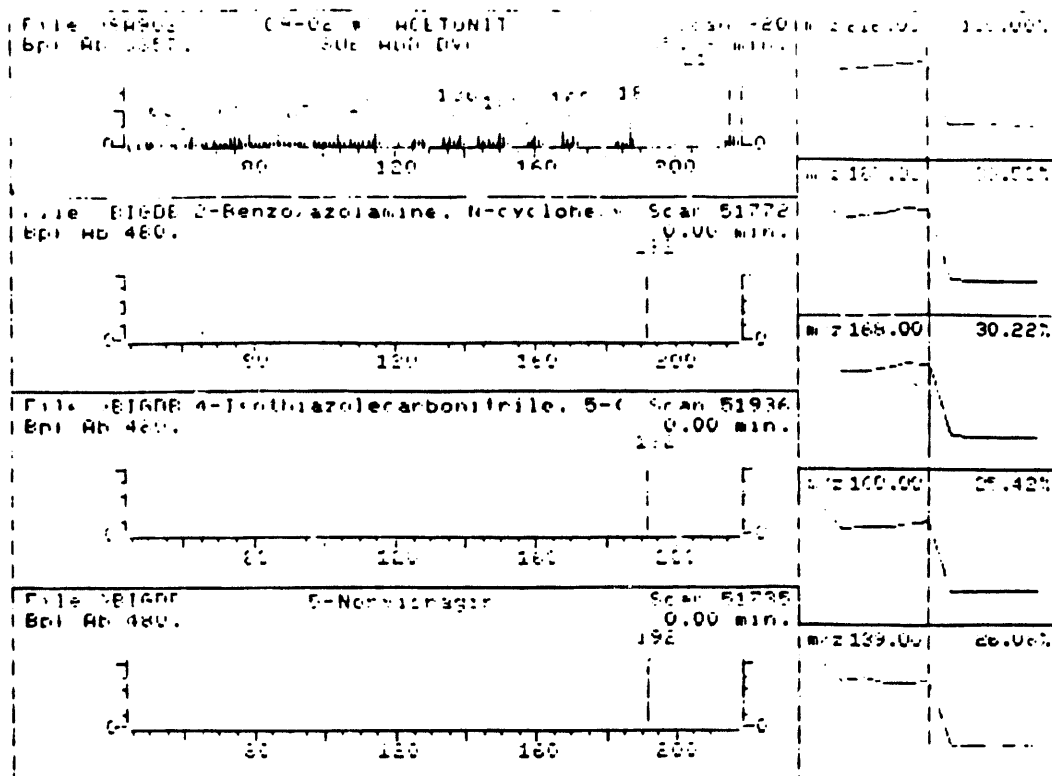
1. 2-Benzoxazolamine, N-cyclohexyl-
2. 4-Isothiazolecarbonitrile, 5-(ethylthio)-3-(methylth) io)-
3. 5-Norvisnagin
4. Di-(4-AMINOPHENYL)-SULFIDE
5. N-ACETYL,N-PROPYL LEUCINE

Sample file: SA902    Spectrum #:         920
Search speed: 1    Tilting option: S    No. of ion ranges searched:    49

| | Prob. | CAS # | CON # | ROOT | K | DK | #FLG | TILT | : | CON | L_I | R_II |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 25* | 10460110 | 51772 | "BIGDB | 23 | 100 | 3 | 0 | 15- | 47 | 7 | 1. |
| 2. | 25* | 2686260 | 51936 | "BIGDB | 31 | 125 | 3 | 0 | 100 | 46 | | 1. |
| 3. | 20* | 4481001 | 51735 | "BIGDB | 28 | 87 | 3 | 0 | 100 | 55 | 5 | 1. |
| 4. | 20* | 0 | 51795 | "BIGDB | 29 | 50 | 2 | 0 | 72 | 25 | 5 | 1. |
| 5. | 15* | 0 | 51699 | "BIGDB | 27 | 34 | 3 | 0 | 100 | 60 | | 1. |
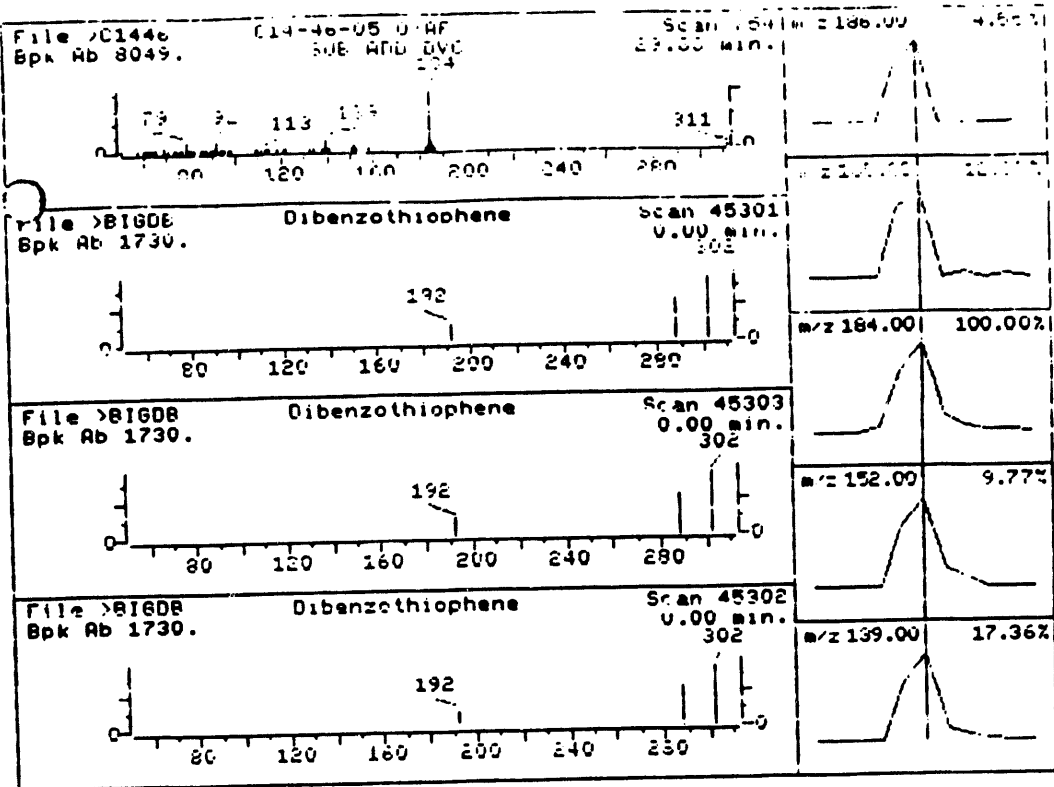
1. Dibenzothiophene
2. Dibenzothiophene
3. Dibenzothiophene
4. Dibenzothiophene
5. Dibenzothiophene

184  C12H8S
184  C12H8S
184  C12H8S
184  C12H8S
184  C12H8S

Sample file: C1446    Spectrum #:              754
Search speed: 1    Fitting option: 5         No. of ion ranges searched:  55

|    | Prob. | CAS # | CON # | ROOT | R | DR | #FLG | TILT | % | CON | C_I | R_I |
|----|-------|-------|-------|------|---|----|----|-----|---|----|-----|-----|
| 1. | 96*   | 132650 | 45501 | "BIGDB | 90 | 11 | 1 | 0 | 68 | 5 | 72 | 9 |
| 2. | 95*   | 132650 | 45503 | "BIGDB | 98 | 5 | 1 | 1 | 36 | 1 | 72 | 5 |
| 3. | 95*   | 132650 | 45502 | "BIGDB | 91 | 8 | 0 | 2 | 62 | 1 | 72 | 5 |
| 4. | 87*   | 132650 | 45504 | "BIGDB | 73 | 33 | 2 | 1 | 71 | 5 | 67 | 4 |
| 5. | 87*   | 132650 | 45501 | "BIGDB | 73 | 40 | 2 | 1 | 67 | 1 | 63 | 4 |
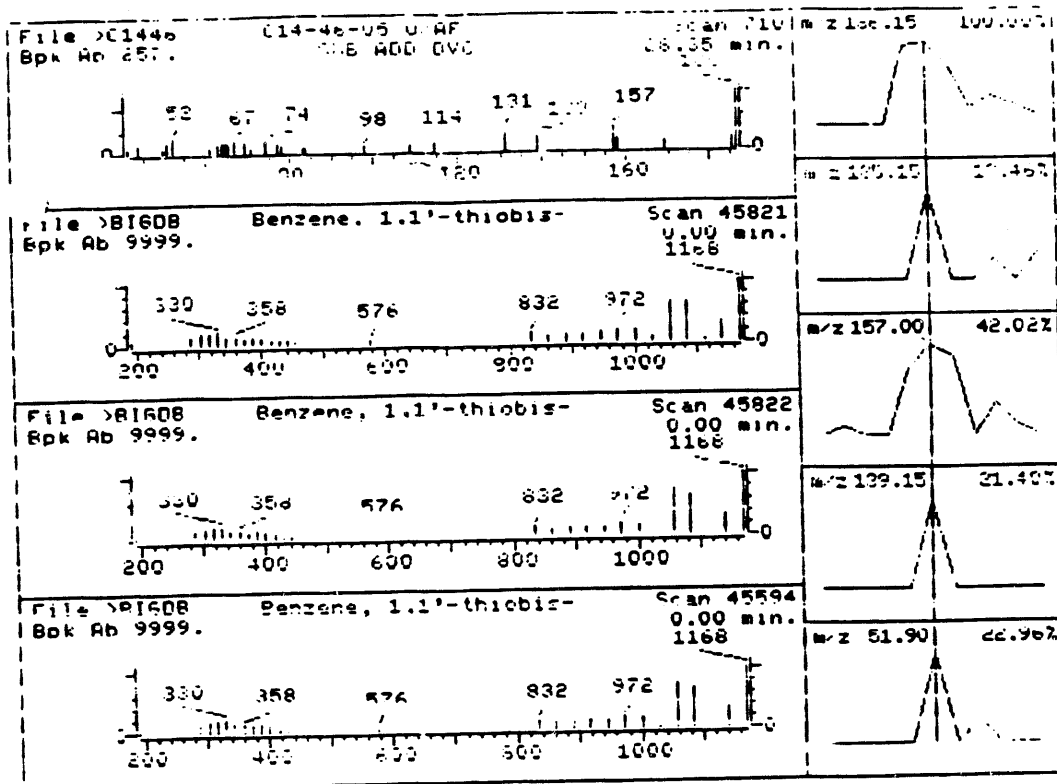
1. Benzene, 1,1'-thiobis-          186 C12H10S
2. Benzene, 1,1'-thiobis-          186 C12H10S
3. Benzene, 1,1'-thiobis-          186 C12H10S
4. Psoralene                       186 C11H6O3

Sample file: >C1440     Spectrum #:     710
Search speed: 1     Fitting option: S     No. of ion ranges searched:     45

| | Prob. | CHS # | CON # | ROOT | K | OK | #FLG | TILT | % | CON | C_I | R_I |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 15* | 137002 | 45821 | "BI60B | 29 | 85 | 3 | U | 100 | 58 | 3 | 1. |
| 2. | 15* | 137002 | 45822 | "BI60B | 22 | 92 | 3 | U | 100 | 58 | 3 | 1. |
| 3. | 15* | 137002 | 45594 | BI60B | 22 | 96 | 3 | U | 100 | 58 | 3 | 1. |
| 4. | 11* | 65777 | 45813 | "BI60B | 22 | 106 | 3 | U | 100 | 62 | 2 | 1. |

# APPENDIX C

## GC-MS SIMS Analyses for Five Standard Compounds

File: ;U0279    9999.0-0.0 amu. (1ml) >10
STD
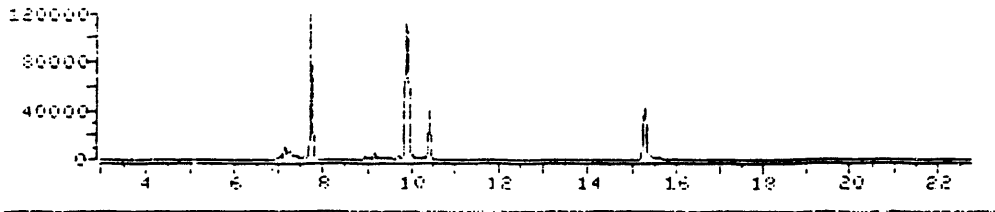
```
*---------------------------------------------------*
* Comp#  1   O-HYDROXYBIPHENYL              (Istd) *
*---------------------------------------------------*
```

Entering 3 ions over range:  6.90:   8.70 minutes.
Integrating mass 211.0.... slope:    .20  min area:    800

Peaks in user window (  7.80 +/-     .40 minutes):  4

| RT | Start | Max | Stop | Raw Area | CorrArea |
|------|------|------|------|---------|---------|
| 7.42 | 146 | 147 | 150 | 6182 | 5650 |
| 7.58 | 150 | 152 | 154 | 5791 | 5332 |
| 7.73 | 154 | 157 | 160 | 123709 | 122944 |
| 7.82 | 160 | 160 | 176 | 3162 | 2549 |

*--------------* Peak at RT:  7.42 minutes *--------------*

Rel abund error allowed:   50.00% (by AREA)

| Mass | Raw Area | Corr Area | Rel % | Target % |
|------|------|------|------|------|
| 211.0 Q | 6182 | 5650 | 100.00 | 100.00 |
| 227.2 | 5444 | 4886 | 86.48 | 98.00 |
| 242.2 | 3836 | 3461 | 61.26 | 65.00 |

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:  91

*--------------* Peak at RT:  7.58 minutes *--------------*

Rel abund error allowed:   50.00% (by AREA)

| Mass | Raw Area | Corr Area | Rel % | Target % |
|------|------|------|------|------|
| 211.0 Q | 5791 | 5332 | 100.00 | 100.00 |
| 227.2 | 5431 | 4942 | 92.69 | 98.00 |
| 242.2 | 3931 | 3606 | 67.63 | 65.00 |

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:  95

C-2

```
*--------------* Peak at RT:   7.73 minutes *--------------*

Rel abund error allowed:   50.00% (by AREA)
            Raw        Corr     Rel    Target
Mass        Area       Area      %       %

211.0 Q    123703     122944  100.00  100.00
227.2      116087     115283   93.77   98.00
242.2       83417      82876   67.41   65.00


All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:  96

  *------------* Peak at RT:   7.82 minutes *--------------*

Rel abund error allowed:   50.00% (by AREA)
            Raw        Corr     Rel    Target
Mass        Area       Area      %       %
------      ------    --------  ------  ------
211.0 Q      3162       2549  100.00  100.00
227.2        2823       2065   81.01   98.00
242.2        1983       1545   60.61   65.00


Maximization check failed for mass: 227.2
Maximization check failed for mass: 242.2

Peak FAILS qual criteria.

Auto Qdel: Largest area is hit number    3.
Istd: Removing all but best hit.

 1) *O-HYDROXYBIPHENYL          RT: 7.73 Area: 122944 1000.0          q: 96

*--------------------------------------------------------*
* Comp#  2  DBT                                          *
*--------------------------------------------------------*

    Entering 3 ions over range:   8.89: 10.69 minutes.
    Integrating mass 184.0.... slope:   .20  min area:     1500

Peaks in user window (  9.79 +/-    .40 minutes):  4

   RT   Start  Max   Stop  Raw Area  CorrArea
------  ------ ------ ----- --------  --------
 9.43    211   213    215    7353     1930
 9.52    215   216    220    7000     3255
 9.73    220   223    225    5982     2236
 9.88    225   228    231  433180   427538
```

```
*-------------* Peak at RT:   9.43 minutes *-------------*

Rel abund error allowed:   50.00% (by AREA)
           Raw       Corr     Rel    Target
Mass       Area      Area      %       %
-----      --------  --------  ------  ------
184.0 Q     7353       1930  100.00  100.00
152.0        679        191    9.90    8.80
139.8         92         30    1.55     .17

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:   96


*-------------* Peak at RT:   9.52 minutes *-------------*

Rel abund error allowed:   50.00% (by AREA)
           Raw       Corr     Rel    Target
Mass       Area      Area      %       %

184.0 Q     7000       3255  100.00  100.00
152.0        781        380   11.67    8.80
139.8         80         16     .49     .17

Maximization check failed for mass:  139.8

Peak FAILS qual criteria.

*-------------* Peak at RT:   9.73 minutes *-------------*

Rel abund error allowed:   50.00% (by AREA)
           Raw       Corr     Rel    Target
Mass       Area      Area      %       %
-----      --------  --------  ------  ------
184.0 Q     5982       2236  100.00  100.00
152.0        922        521   23.30    8.80
139.8        103         25    1.12     .17

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:   60


*-------------* Peak at RT:   9.88 minutes *-------------*

Rel abund error allowed:   50.00% (by AREA)
           Raw       Corr     Rel    Target
Mass       Area      Area      %       %
-----      --------  --------  ------  ------
184.0 Q   433180     427538  100.00  100.00
152.0      38555      37614    8.80    8.80
139.8        830        738     .17     .17

All ions pass maximization check.

Peak PASSES qual criteria.
Q-value:   99
```

2)   DBT                              RT: 9.43 Area:    1930 1930.0 NO CALIBq:  96
2)   DBT                              RT: 9.73 Area:    2236 2236.0 NO CALIBq:  60
2)   DBT                     C-4      RT: 9.88 Area:  427538 427538 NO CALIBq:  99

```
*------------------------------------------------------*
‹ Comp#  3  0-O'DIPHENOL DERRIV                        *
*------------------------------------------------------*
```

        Entering 2 ions over range:  9.50: 11.30 minutes.
        Integrating mass 330.2.... slope:    .20  min area:        800

        Peaks in user window ( 10.40 +/-       .40 minutes):   3

            RT    Start  Max    Stop   Raw Area  CorrArea
           ------ ------ ------ ------ --------- ---------
           10.07   232   234    237      5693      3014
           10.25   237   240    243      6539      3291
           10.40   243   245    248    107651    104884

        *-------------* Peak at RT: 10.07 minutes *-------------*

        Rel abund error allowed:  50.00% (by AREA)
                    Raw       Corr     Rel    Target
          Mass      Area      Area      %       %
          ------   --------  --------  ------  ------
          330.2 Q    5693      3014  100.00  100.00
          331.1      1561       954   31.65   28.00


        Peak PASSES qual criteria.
        Q-value:  93

        *-------------* Peak at RT: 10.25 minutes *-------------*

        Rel abund error allowed:  50.00% (by AREA)
                    Raw       Corr     Rel    Target
          Mass      Area      Area      %       %
          ------   --------  --------  ------  ------
          330.2 Q    6539      3291  100.00  100.00
          331.1      1764      1031   31.33   28.00

        All ions pass maximization check.

        Peak PASSES qual criteria.
        Q-value:  93

        *-------------* Peak at RT: 10.40 minutes *-------------*

        Rel abund error allowed:  50.00% (by AREA)
                    Raw       Corr     Rel    Target
          Mass      Area      Area      %       %
          ------   --------  --------  ------  ------
          330.2 Q  107651    104884  100.00  100.00
          331.1     28782     28162   26.85   28.00

        All ions pass maximization check.

        Peak PASSES qual criteria.
        Q-value:  97

  3)   0-O'DIPHENOL DERRIV          RT:10.07 Area:    3014   3014.0 NO CALIBq: 93
  3)   0-O'DIPHENOL DERRIV          RT:10.25 Area:    3291   3291.0 NO CALIBq: 93
  3)   0-O'DIPHENOL DERRIV          RT:10.40 Area:  104884 104884 NO CALIBq: 97

```
*-------------------------------------------------------*
* Comp#  4  DBT SULFOXIDE                               *
*-------------------------------------------------------*

     Entering 2 ions over range: 14.24: 16.04 minutes.
     Integrating mass 184.0.... slope:   .20  min area:      800

     Peaks in user window ( 15.14 +/-    .40 minutes):  1

        RT   Start  Max    Stop   Raw Area CorrArea
      ------ ------ ------ ------ -------- --------
      15.29   402    406    420     8498     7350

        *------------* Peak at RT: 15.29 minutes *------------*

     Rel abund error allowed:  50.00% (by AREA)
                 Raw      Corr     Rel    Target
       Mass      Area     Area      %       %
      ------ -------- -------- ------ ------
      184.0 Q    8498     7350 100.00 100.00
      200.0       749      585   7.96  33.00  XX

     All ions pass maximization check.

     Peak PASSES qual criteria.
     Q-value:  55

 4)  DBT SULFOXIDE                    RT:15.29 Area:    7350 7350.0 NO CALIBq: 55



* Comp#  5  DBT SULFONE                                 *
*-------------------------------------------------------*

     Entering 3 ions over range: 14.17: 15.97 minutes.
     Integrating mass 216.0.... slope:   .20  min area:      800

     Peaks in user window ( 15.07 +/-    .40 minutes):  1

        RT   Start  Max    Stop   Raw Area CorrArea
      ------ ------ ------ ------ -------- --------
      15.29   401    406    425   153330   152193

        *------------* Peak at RT: 15.29 minutes *------------*

     Rel abund error allowed:  50.00% (by AREA)
                 Raw      Corr     Rel    Target
       Mass      Area     Area      %       %
      ------ -------- -------- ------ ------
      216.0 Q  153330   152193 100.00 100.00
      136.0     53376    52458  34.47  35.00
      160.0     31415    30846  20.27  20.00

     All ions pass maximization check.

     Peak PASSES qual criteria.
     Q-value:  99

  5)  DBT SULFONE                      RT:15.29 Area: 152193 152193 NO CALIBq: 99

*----------------------*          C-6
* End QUANT Test Mode *
```

# APPENDIX D

## Samples of HPLC Analyses

(1) 0.1 mm o-OH-biphenyl std (RT of 9.83) in acetonitrile

(2) ICF incubated with 0.5 mm DBT in acetonitrile. (Sample was concentrated 10-fold prior to injection.)

(3) 0.1 mm '4S' standards in acetonitrile. The compounds with their retention times in parentheses are as follows: DBT sulfoxide (1.38), DBT sulfone (2.35) o,o'-biphenol (3-61), o-OH-biphenyl (6.15) and DBT (18.09)

(4) ECF incubated with 0.05 mm DBT in acetonitrile. (Sample was concentrated 20-fold prior to injection.)

(5) ECF incubated without DBT in acetonitrile. (Sample was concentrated 20-fold prior to injection.)

MF 1 FT 1        AZ 1
45   .59
RT 8 .91

FILE 3.     METHOD 0.     RUN 128

| PEAK# | AREA% | RT | AREA | BC |
|---|---|---|---|---|
| 1 | 1.409 | 0.45 | 8604 | 08 |
| 2 | 0.73 | 0.59 | 4459 | 06 |
| 3 | 2.095 | 0.75 | 12798 | 06 |
| 4 | 1.278 | 0.91 | 7805 | 07 |
| 5 | 51.887 | 9.83 | 316904 | 01 |
| 6 | 42.601 | 16.77 | 260188 | 01 |

....TOTAL....... 100. .............................610758........

9.83

(1)  0.1 mm o-oh-biphenyl std (RT of 9.83) in acetonitrile

RP 1 PT 1          AZ 1
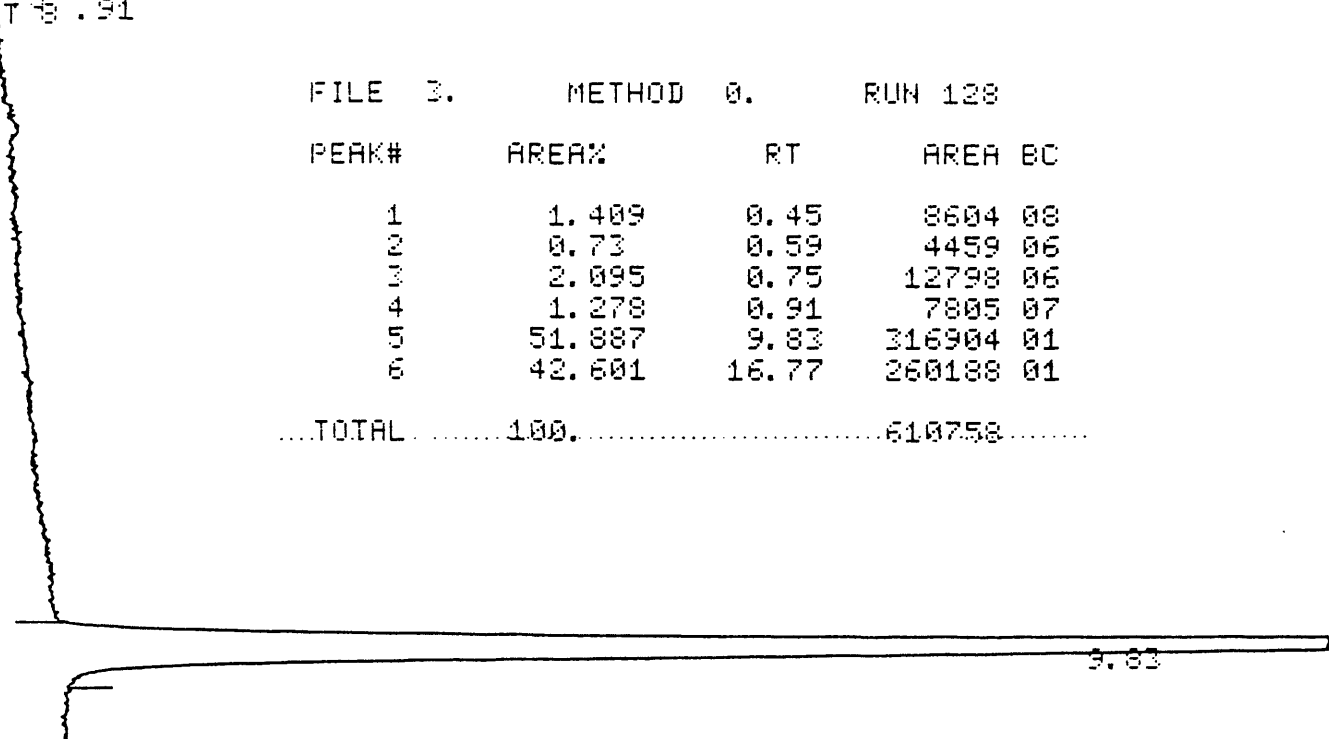
.74
1.07
.51  1.33
2.02

| PEAK# | AREA% | RT | AREA | BC |
|-------|-------|------|---------|----|
| 1 | 9.108 | 0.52 | 198664 | 08 |
| 2 | 3.94 | 0.66 | 85943 | 06 |
| 3 | 16.107 | 0.74 | 351310 | 07 |
| 4 | 12.453 | 0.94 | 271622 | 08 |
| 5 | 9.399 | 1.07 | 205012 | 06 |
| 6 | 8.215 | 1.15 | 179183 | 06 |
| 7 | 13.768 | 1.33 | 300308 | 06 |
| 8 | 7.482 | 1.51 | 163201 | 06 |
| 9 | 10.415 | 1.63 | 227158 | 06 |
| 10 | 8.462 | 2.02 | 184571 | 07 |
| 11 | 0.65 | 9.84 | 14177 | 01 |
| TOTAL | 100. | | 2181149 | |

9.84

(2)  ICF incubated with 0.5 mm DBT in acetonitrile.  (Sample
was concentrated 10-fold prior to injection.)

6.15

11.54

12.54
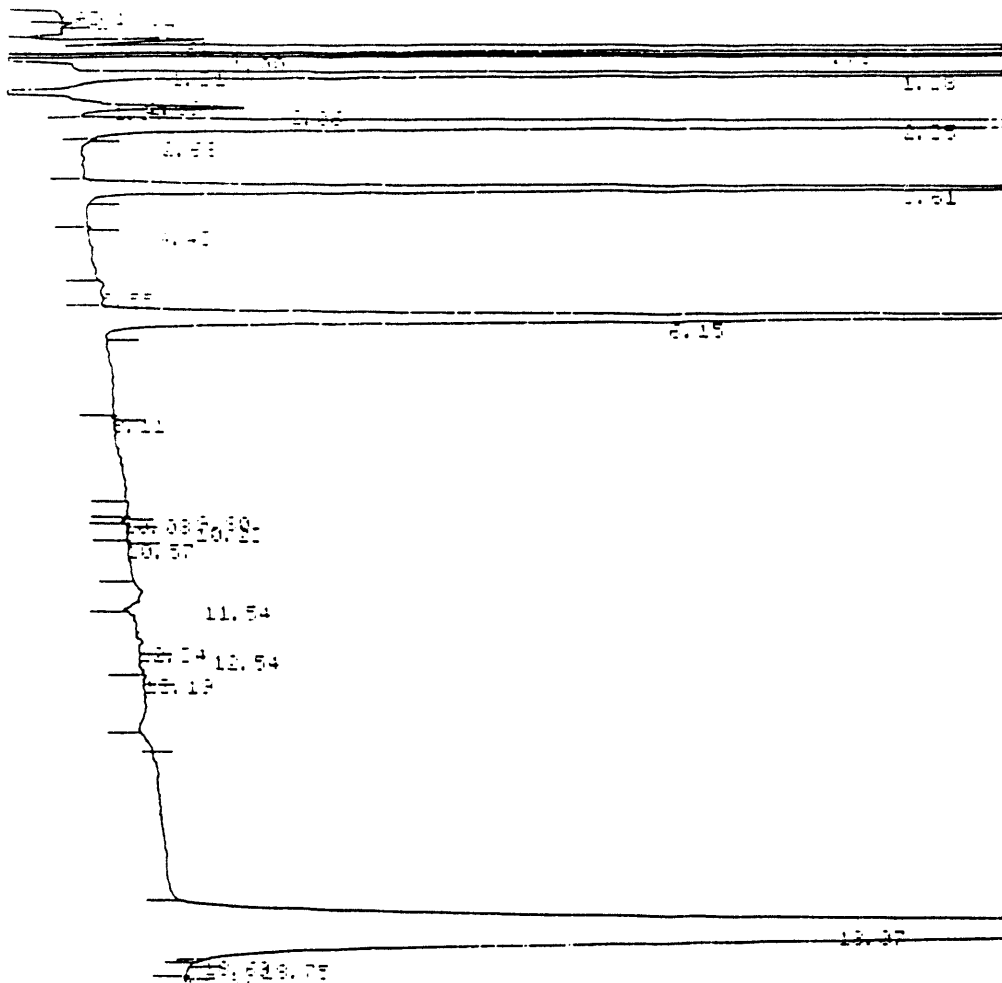
01/02/90 11:51:29     CH= "A"  PS=  1.

FILE I.    METHOD 0.    RUN 435    INDEX 435

PEAK #    AREA%    RT    AREA SC

       0.010    0.27       886  01
       1.191    0.62     30268  02
       8.400    0.78    568512  03
       4.721    1.      118233  01
       5.296    1.21    357013  02
      15.904    1.38   1072113  08
       1.501    1.89    101213  06
       0.860    1.97     58155  06
       1.293    2.06     87171  06
      29.173    2.35   1963843  08
       0.002    2.68       149  05
       4.333    3.61    292022  01
       0.003    4.43       174  01
       0.015    5.55      2378  02
       5.74     6.15    384939  02
       0.       6.11         6  01
       0.011    6.3       1103  02
       0.005    10.08      211  02
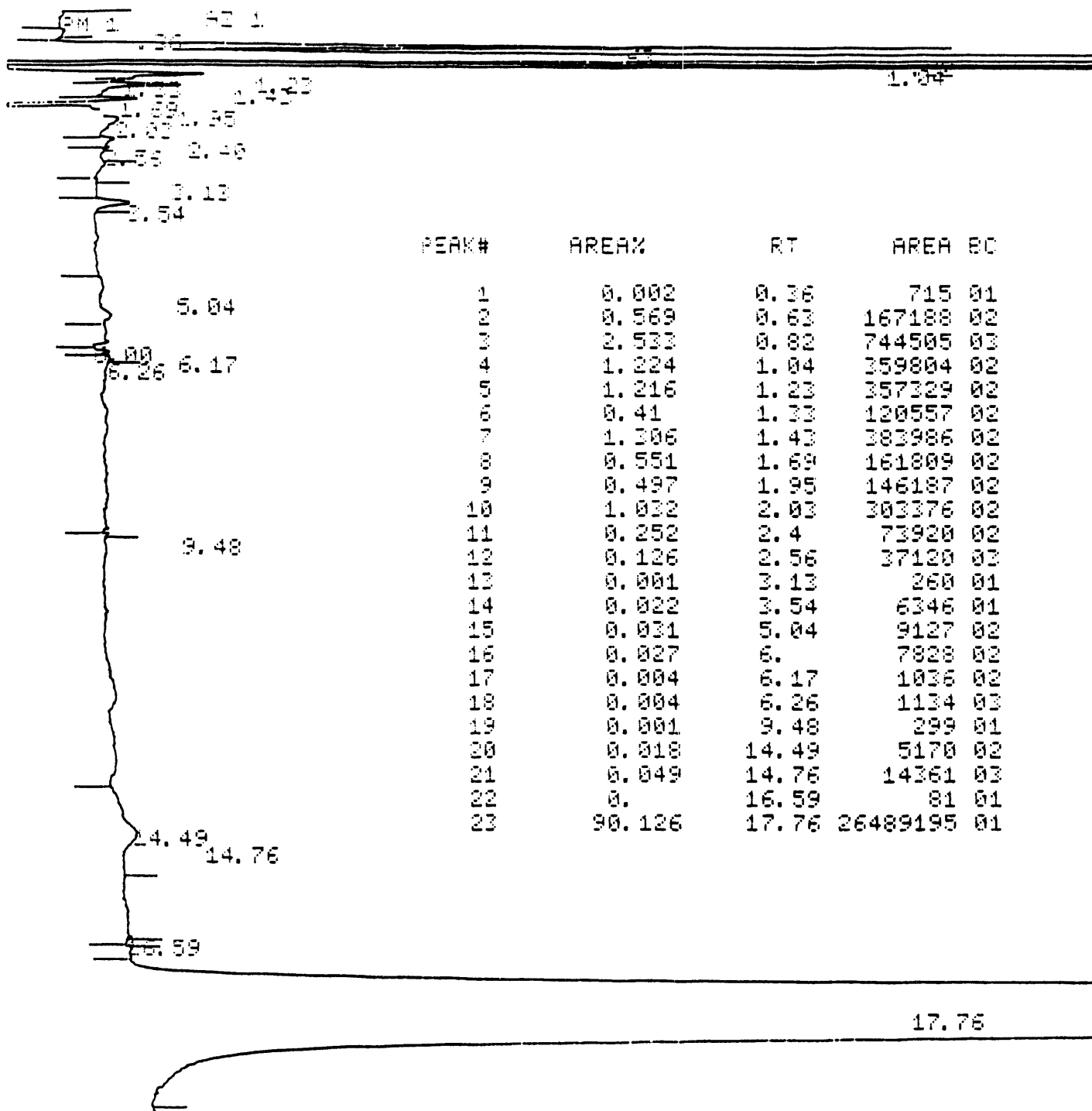       0.006    10.23      134  01
       0.002    10.57      145  01
       0.123    11.54     8551  02
       0.015    12.14     5774  02
       0.021    12.54     1340  02
       0.008    13.19      542  01
      20.421    18.09  1176791  02
       0.024    18.59     1713  02
       0.01     18.75      671  02
       0.002    19.04      167  01
       0.003    19.19      215  01
       0.815    20.18    54943  01

TOTAL    100.            6741252

(3) 0.1 mm '4S' standards in acetonitrile.
The compounds with their retention times
in parentheses are as follows:  DBT
sulfoxide (1.38), DBT sulfone (2.35)
o,o'-biphenol (3-61), o-oh-biphenyl (6.15)
and DBT (18.09)

D-4

PM 1       PC 1

1.04

| PEAK# | AREA% | RT | AREA | BC |
|-------|-------|-----|----------|----|
| 1 | 0.002 | 0.36 | 715 | 01 |
| 2 | 0.569 | 0.63 | 167188 | 02 |
| 3 | 2.533 | 0.82 | 744505 | 03 |
| 4 | 1.224 | 1.04 | 359804 | 02 |
| 5 | 1.216 | 1.23 | 357329 | 02 |
| 6 | 0.41 | 1.33 | 120557 | 02 |
| 7 | 1.306 | 1.43 | 383986 | 02 |
| 8 | 0.551 | 1.69 | 161809 | 02 |
| 9 | 0.497 | 1.95 | 146187 | 02 |
| 10 | 1.032 | 2.03 | 303376 | 02 |
| 11 | 0.252 | 2.4 | 73920 | 02 |
| 12 | 0.126 | 2.56 | 37120 | 02 |
| 13 | 0.001 | 3.13 | 260 | 01 |
| 14 | 0.022 | 3.54 | 6346 | 01 |
| 15 | 0.031 | 5.04 | 9127 | 02 |
| 16 | 0.027 | 6. | 7828 | 02 |
| 17 | 0.004 | 6.17 | 1036 | 02 |
| 18 | 0.004 | 6.26 | 1134 | 03 |
| 19 | 0.001 | 9.48 | 299 | 01 |
| 20 | 0.018 | 14.49 | 5170 | 02 |
| 21 | 0.049 | 14.76 | 14361 | 03 |
| 22 | 0. | 16.59 | 81 | 01 |
| 23 | 90.126 | 17.76 | 26489195 | 01 |

17.76

(4)  ECF incubated with 0.05 mm DBT in acetonitrile.  (Sample
was concentrated 20-fold prior to injection.)

| PEAK# | AREA% | RT | AREA | BC |
|-------|-------|------|---------|----|
| 1 | 0.008 | 0.31 | 212 | 01 |
| 2 | 4.858 | 0.58 | 122098 | 02 |
| 3 | 25.531 | 0.77 | 641712 | 03 |
| 4 | 12.98 | 0.99 | 326241 | 01 |
| 5 | 10.399 | 1.19 | 261366 | 02 |
| 6 | 5.159 | 1.25 | 129656 | 02 |
| 7 | 13.708 | 1.37 | 344538 | 02 |
| 8 | 4.732 | 1.61 | 118930 | 02 |
| 9 | 5.233 | 1.88 | 131522 | 02 |
| 10 | 16.295 | 1.97 | 409577 | 03 |
| 11 | 1.098 | 17.69 | 27585 | 01 |
| TOTAL | 100. | | 2513437 | |

17.69

(5) ECF incubated without DBT in acetonitrile. (Sample was concentrated 20-fold prior to injection.)

# END

DATE FILMED
3/6/92