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APPLICATIONS OF MICELLAR ENZYMOLOGY TO CLEAN COAL TECHNOLOGY

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Section 1

INTRODUCTION

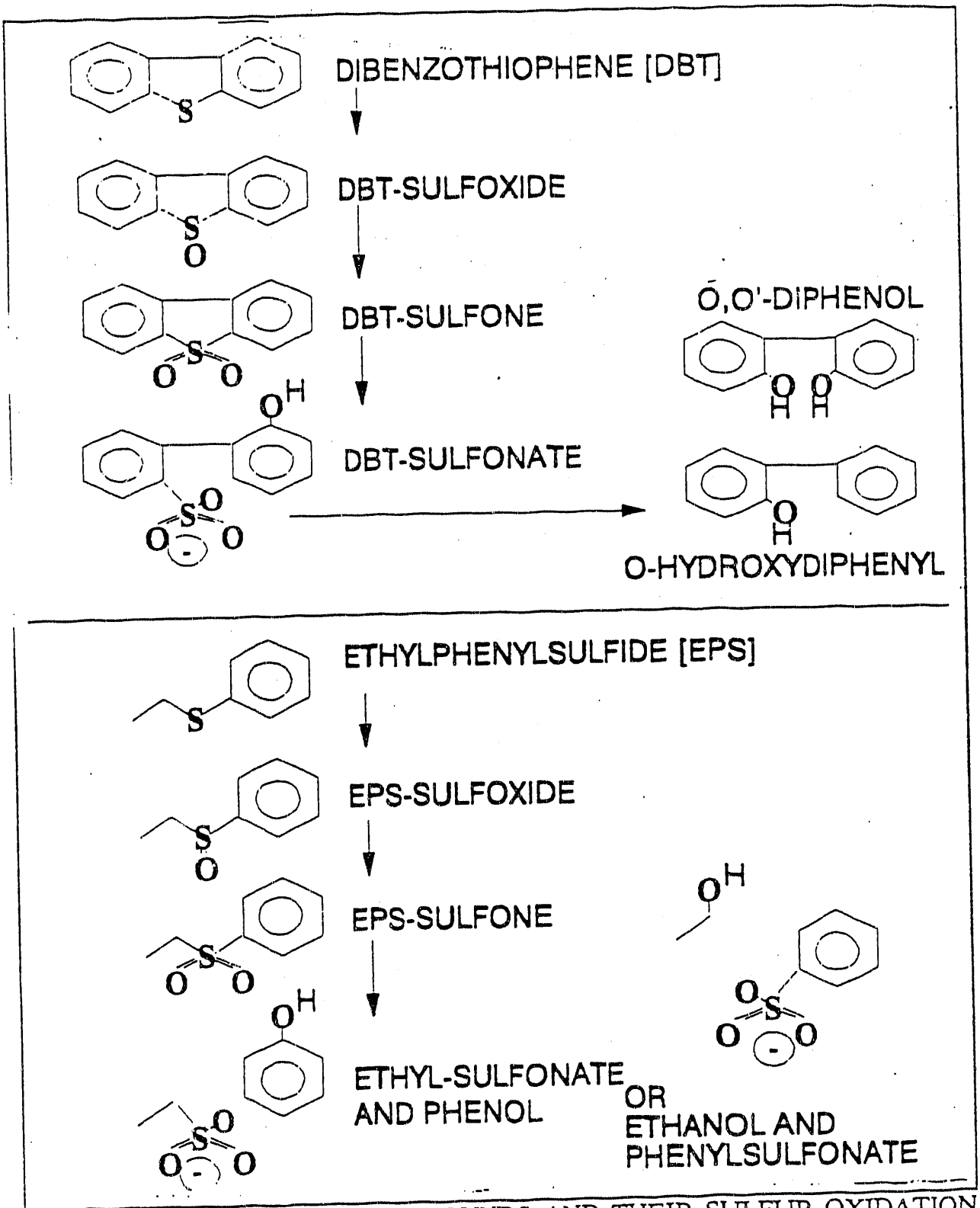
1.1 Program Overview

Full implementation of coal fuel sources will require more effective methods of providing "clean coal" as a fuel source. Methods must be developed to reduce the sulfur content of coal which significantly contributes to environmental pollution. This project is designed to develop methods for pre-combustion coal remediation by implementing recent advances in enzyme biochemistry. The novel approach of this study is incorporation of hydrophilic oxidative enzymes in reverse micelles in an organic solvent. Enzymes from commercial sources or microbial extracts are being investigated for their capacity to remove organic sulfur from coal by oxidation of the sulfur groups, splitting of C-S bonds and loss of sulfur as sulfuric acid (Figure 1). Dibenzothiophene (DBT) and ethylphenylsulfide (EPS) are serving as models of organic sulfur-containing components of coal in initial studies.

A goal of this project is to define a reverse micelle system that optimizes the catalytic activity of enzymes toward desulfurization of model compounds and ultimately coal samples. Among the variables which will be examined are the surfactant, the solvent, the water:surfactant ratio and the pH and ionic strength of the aqueous phase. Studies by several groups (Martinek et al., 1981; Kabanov et al., 1988; Martinek, 1989; Verhaert et al., 1990) have shown that the surfactant AOT over a broad concentration range in organic solvents produces micelles, comparatively uniform in diameter, which incorporate hydrophilic enzymes. The activity (kcat) of certain enzymes in this system is higher than in aqueous solution. This surfactant is therefore being examined first, although the potential disadvantages of an SO_3 containing molecule have been recognized. Other surfactants to be tested include Tritons, Tweens and Brij 35.

1.2 Summary of Results

FIGURE 1



THE MODEL COMPOUNDS AND THEIR SULFUR OXIDATION PRODUCTS

The ability of the oxidative enzymes, HRP and laccase, to oxidize the sulfur moiety in the model coal compound DBT was examined in the novel milieu of a reverse micelle solution. Experiments were chosen to retest conditions in which some evidence of sulfur oxidation had been previously detected but had not been consistently observed. Also studies were carried out with a purified form of DBT sulfoxide to determine its susceptibility to laccase oxidation. A preparation of HRP of higher specific activity (Rz=3.2) was also tested. To date we have not found evidence that DBT or its sulfur oxidation products are substrates for oxidation by HRP or laccase in AOT-isooctane reverse micelles. Studies were also conducted to test for DBT oxidation in a reverse micelle solution containing an intracellular extract from GB-1, a microorganism capable of oxidizing the model coal compound. Loss of DBT or production of sulfur oxidation products by the microbial extract was not observed under the conditions of the study. Control studies of the extract in an organic solution without micelles demonstrated less activity than previously observed in another laboratory and suggested the importance of concentrating the extract prior to incubations.

Results from the first year of the project were summarized in the thesis for the Masters degree submitted by Majid Yazdani. A new student, John Pezzullo, has begun work on this project.

Section 2

MATERIALS AND METHODS

2.1 Materials

Studies were carried out with HRP, Type I RZ=1.2 and Type VI RZ= 3.2 (Sigma Chemical Co.) and laccase from Polyporus versicolor (isolated by Reinhammer and obtained from Kirk). Substrates for HRP assays included hydrogen peroxide (H_2O_2, 30%, Fisher Scientific), DBT (Aldrich Chemical), DBT sulfoxide (K&K Laboratories),

and DBT sulfone (Aldrich Chemical Co.). Buffers included sodium phosphate (Fisher Scientific).

For formation of reverse micelle solution, the surfactant AOT, di(2-ethyl-hexyl)sodiurn sulphosuccinate, was obtained from Sigma Chemical Co. (The water content of the AOT has not been determined and therefore reported water content of micelle solutions do not account for the possible small contribution from this source.) Isooctane (Optima, Fisher Scientific) was used as organic solvent.

2.2 Methods

2.2.1 Enyme Assays in Reverse Micelle Solutions

AOT (MW 444) was dissolved in isooctane to produce a 0.1 M solution. For study of DBT (2.0 mM), DBT sulfoxide (0.02mM), and DBT sulfone (0.2 mM) in reverse micelle solutions, the compounds were added to 0.1 M AOT-isooctane. Reverse micelle solutions of laccase were formed with addition of enzyme in 32 ul of acetate:phosphate buffer (0.025 M:0.1M, 3:1, pH 5.0) per ml of organic phase. HRP (2.1 uM) and H2O2 (0.2 mM) were added in a total aqueous volume of 23 ul of phosphate buffer (pH 7.0, 0.025 M) per ml of organic solvent. Control samples contained no enzyme. Samples of two ml in 20-ml scintillation vials were shaken on a Scientific Industries Rotator for 24 hr.

2.2.2. Incubations of DBT with Microbial GB-1 Intracellular Fractions

DBT (0.05 or 1.0 mM) was incubated for 7 days in an organic solution, consisting of 90 % acetonitrile or 0.05 M AOT-isooctane with a 3.24 % aqueous phase. (The AOT concentration was reduced in an attempt to improve resolution of the DBT sulfoxide peak on HPLC.) The aqueous phases were either a control solution of Tris buffer (10 mM, pH 7.0) or an extract of cytosol from GB-1. Extracts were prepared by Sara Nochur, Ph.D. at DynaGen, Inc. and stored at -20 C prior to incubations (Nochur and

Kitchell, 1990). Incubates of 2 ml were placed in 20-ml scintillation vials and gently shaken on a Scientific Industries Rotator. After 7 days, samples were filtered and analyzed by HPLC as described below. Prior to analysis, filtrates from the acetonitrile study were transferred to 1-ml KIMAX Brand Accuform Microvials in 500 ul aliquots which were evaporated down to 50 ul.

2.2.3 Quantitative Analysis of DBT Metabolites: HPLC Procedure

DBT (0.1-2 mM), DBT sulfoxide (0.02 mM), DBT sulfone (0.02 mM) and dihydroxybiphenyl (0.02 M) were added to 0.1 M AOT in isooctane, alone or in combination to serve as standards. Standards and experimental samples were filtered through disposable syringe filter units (Rainin Instrument Co., No. 38-159, 0.2 u pore size, 3 mm diameter Nylon 66 membranes). Filtrates (4 ul) were analyzed at DynaGen, Inc. with a Waters HPLC system containing a C18 resolve (5 micron spherical) column. The mobile phase contained tetrahydrofuran:acetonitrile:water (23:20:57) at a programmed rate of 0.8-1.8 ml/min . The specific program was 0-10.5 min, 0.8 ml/min; 10.5-27.0 min, 1.8 ml/min; 27.0 min-, 0.8 ml/min. The detector was a Waters Lambda Max 481 LC spectrophotometer which was set at 280 nm. The method is a modification of procedures previously used by Dr. Kitchell at DynaGen, Inc. for analysis of DBT metabolites in organic solvents (Wyza et al., 1989).

Section 3
RESULTS

3.1 Activity of Laccase from Polyporus versicolor on DBT Sulfoxide After 24 hr in AOT Reverse Micelles

Initial studies with DBT sulfoxide as a possible substrate for laccase were carried out with the unpurified metabolite which was substantially contaminated with DBT sulfone.

These experiments were therefore replicated after purification of DBT sulfoxide by TLC as described in the Third Quaterly Report. The compound (approximately 0.02 mM) was incubated with laccase from Polyporus versicolor for 24 hr. The reverse micelle solution consisted of 0.1 M AOT in isooctane with a 3.2 % aqueous phase at pH 5 (3:1 0.025 M acetate buffer: 0.1 M phosphate buffer). The purity of the DBT sulfoxide was confirmed by subsequent HPLC analysis of the starting material. There was no evidence of significant decrease in the concentration of DBT sulfoxide in laccase-containing samples as compared to controls. No DBT sulfone or dihydoxybiphenyl was detected in either controls or enzyme-containing incubations (Table 1).

3.2 Activity of HRP Type I or VI in Reverse Micelle Solutions with DBT, DBT Sulfoxide or DBT Sulfone as Substrate

DBT, DBT sulfoxide and DBT sulfone were separately incubated for 24 hr in control reverse micelle solutions or solutions containing HRP. Aliquots were analyzed by HPLC. Neither HRP Type I or VI (2.1 uM) reduced the concentration of DBT or generated detectable levels of metabolites (Table 2). No conversion of DBT sulfoxide or sulfone was observed with HRP Type I.

3.3 Incubations of Intracellular Fractions from GB-1 Microbes with DBT in 90% Acetonitrile or AOT Reverse Micelles

Seven-day incubation of the intracellular fraction of GB-1 with DBT in a 0.05 M AOT-isooctane solution did not decrease the DBT concentration which remained similar to that in the control solution without extract (Table 3). No sulfur oxidation products were consistently produced . A small amount of DBT sulfone was detected in one extract-containing sample. The extract was reexamined in 90% acetonitrile, a condition that had previously been shown to result in DBT oxidation. In this experiment some problems with definitive resolution of DBT sulfoxide peaks occurred. In extract-containing samples no DBT sulfur oxidation products were consistently

TABLE 1 **Effect of 24-hr Incubation of DBT Sulfoxide with Laccase from Polyporus versicolor in Reverse Micelles**

	DBT Sulfoxide (mM)	DBT Sulfone	Dihydroxybiphenyl
CONTROL	0.049	-	-
	0.014	-	-
	0.014	-	-
LACCASE	0.013	-	-
	0.012	-	-
	0.013	-	-

- none detected

TABLE 2 **Effect of 24-hr Incubation of DBT, DBT Sulfoxide, and DBT Sulfone with HRP in Reverse Micelles**

SUBSTRATE	DBT	DBT SULFOXIDE	DBT SULFONE	DIHYDROXY-BIPHENYL
		(mM)		
DBT				
Control	1.93 + 0.06[a]	-[b]	-	-
HRP Type I	1.99 + 0.15	-	-	-
HRP Type VI	2.20 + 0.10	-	-	-
DBT SULFOXIDE				
Control	NA	0.016 + 0.000	-	-
HRP Type I	NA	0.017 + 0.000	-	-
DBT SULFONE				
Control	NA	-	0.020 + 0.001	-
HRP Type I	NA	-	0.021 + 0.001	-

a mean + SD, n=2

b none detected

NA not assayed

TABLE 3 Effect of 7-day Incubation of DBT with Intracellular Fraction from GB-1 in 90% Acetonitrile or 0.1 M AOT-Isooctane

	DBT	SX	SN	DI-OH	MONO-OH
90% ACETONITRILE					
Control DBT	0.150+0.014[a]	1.201 n=1	0.0007 n=1	-	0.002
Control ICF	0.002+0.00[a]	1.62+0.3[a]	-	-	0.003
DBT + ICF	0.107+0.07[b]	0.064 n=1	-	-	0.011
REVERSE MICELLES					
Control DBT	1.29+0.03[b]	0.90 n=1	-	-	-
Control ICF	-	1.05 n=1	0.0008 n=1	-	-
DBT + ICF	1.35+0.19[b]	-	0.002 n=1	-	-

SX=DBT Sulfoxide
SN=DBT Sulfone
DI-OH=Dihydroxybiphenyl
MONO-OH=Monohydroxybiphenyl

a mean + SD, n=2

b mean + SD, n=3

detected.

3.4 Evaluation of Solubility and UV Absorbance Characteristics of EPS, EP Sulfoxide and EP Sulfone

EPS, EP sulfoxide, and EP sulfone were solubilized in isooctane at 50 mM. At this concentration EPS remained in solution at room temperature, while EP sulfoxide and EP sulphone went into solution only after gentle heating to temperatures of 55 °C- 65 °C. The sulfides were analyzed by UV spectrophotometry. EPS and the metabolites were found to be essentially indistinguishable from each other in absorbance characteristics, all showing a single strong peak in the 254 nm range at concentrations of 0.05 mM and 0.5 mM (Figures 2-4). The extinction coefficient was least for the sulfone, approximately one-tenth that of EPS, and one-fifth that of the sulfoxide.

The absorbance of EPS in isooctane was linearly related to concentrations between 0.05 mM and 0.5 mM , as was EPS solubilized in AOT/ isooctane at the same concentrations (Figure 5).

Section 4

PLANS FOR THE FIFTH QUARTER

1.) Examination of oxidative activity of HRP and laccase in a reverse micelle solution with a non-sulfur containing surfactant, CTAB.

2.) Examination of enzymes in reverse micelles which may oxidize sulfur in EPS based on recent studies of sulfide oxidation by bacterial and mammalian enzymes (Holland, 1988,Kobayashi et al., 1986, and Rettie et al., 1990).

3.) Further work to enhance the DBT sulfur oxidizing activity of GB-1 including additional studies with reverse micelles.

Figure 2

LKB WAVELENGTH SCAN

Operator: EPS in toluene
Date: 10-10-1990
Plot title: 0.5mM
Mode Abs/%T: Abs
Plot scale: 2
Plot offset: 0
Start W/length: 230
Stop W/length: 430
Scan speed,nm/s: 3

SAMPLE NUMBER: 4

By WAVELENGTH :		By Peak Height:	
Peak WAVELENGTH	Peak Height	Peak WAVELENGTH	Peak Height
No peaks			

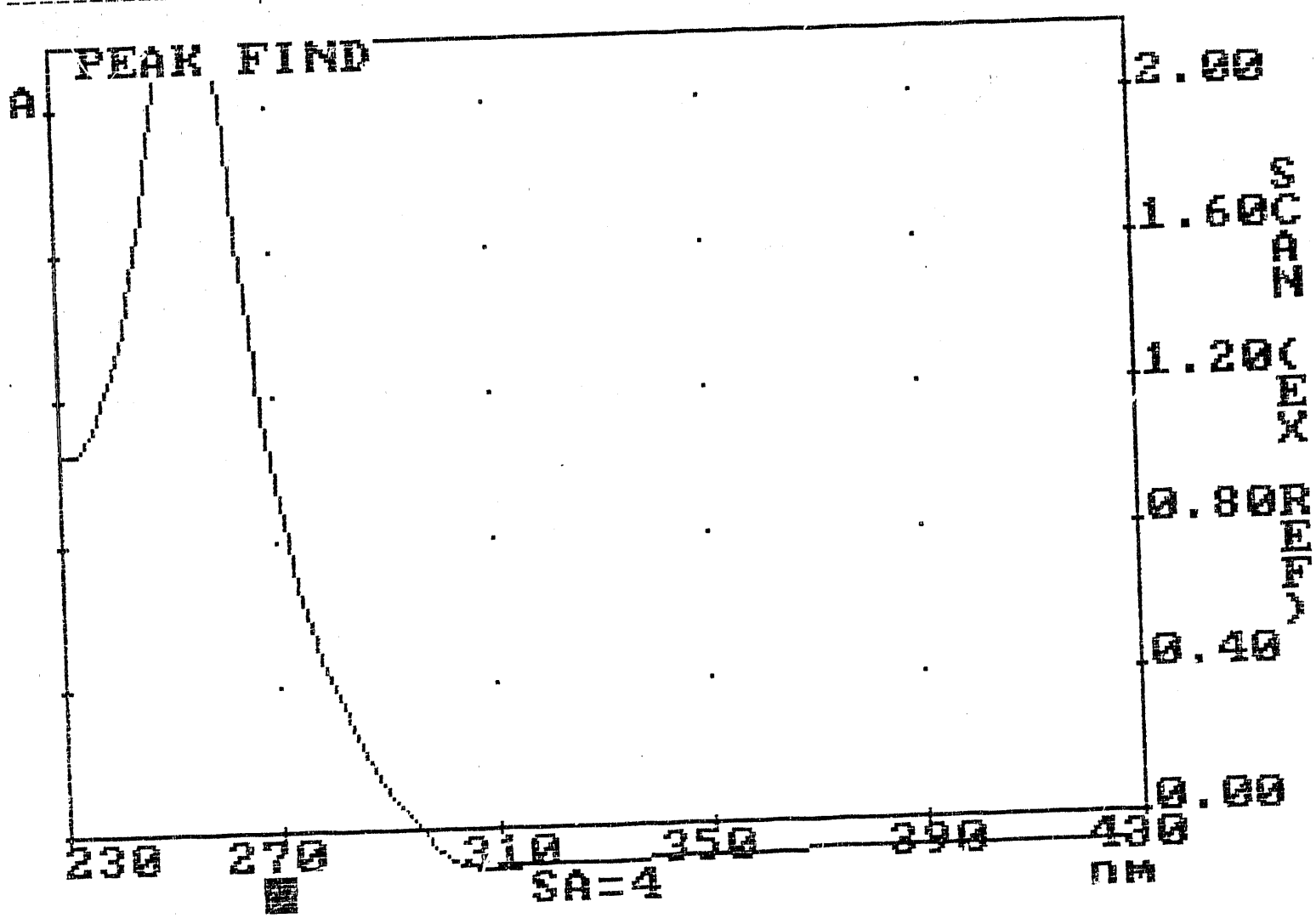


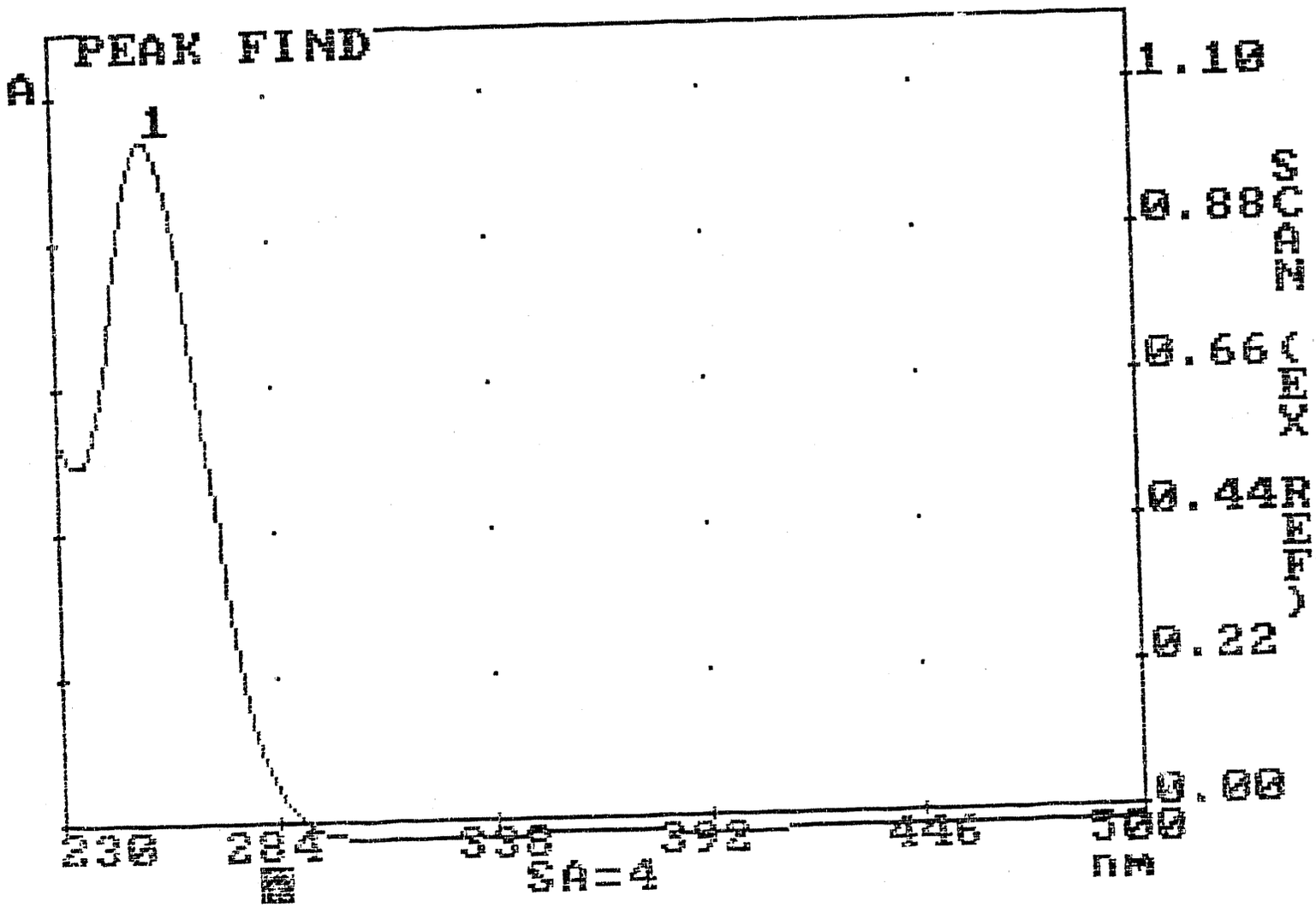
FIGURE 3

LKB WAVELENGTH SCAN

Operator: EP SULFOXIDE
Date: 10-22-1990
Plot title: 0.5mM
Mode Abs/%T: Abs
Plot scale: 2.7
Plot offset: 0
Start W/length: 230
Stop W/length: 500
Scan speed,nm/s: 3

SAMPLE NUMBER: 4

By WAVELENGTH : Peak	WAVELENGTH nm	Peak Height Abs	By Peak Height: Peak	WAVELENGTH nm	Peak Height Abs
1	254.5	1.034	1	254.5	1.034



LKB WAVELENGTH SCAN

Operator: EP SULPHONE
Date: 10-22-1990
Plot title: 0.5mM
Mode Abs/%T: Abs
Plot scale: 2
Plot offset: 0
Start W/length: 230
Stop W/length: 500
Scan speed,nm/s: 3

SAMPLE NUMBER: 4

By WAVELENGTH : Peak	WAVELENGTH nm	Peak Height Abs	By Peak Height: Peak	WAVELENGTH nm	Peak Height Abs
1	261.5	0.314	1	261.5	0.314

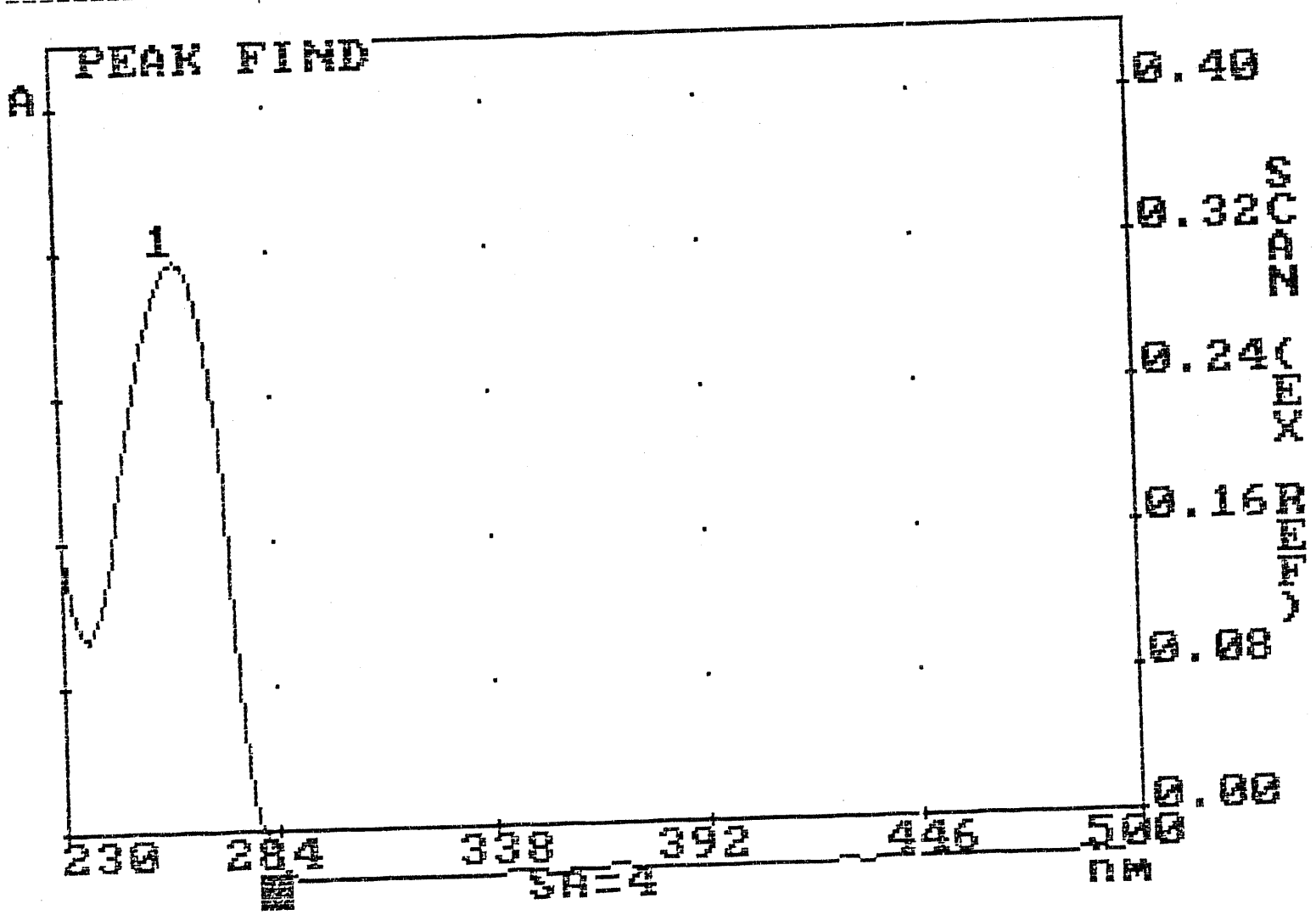
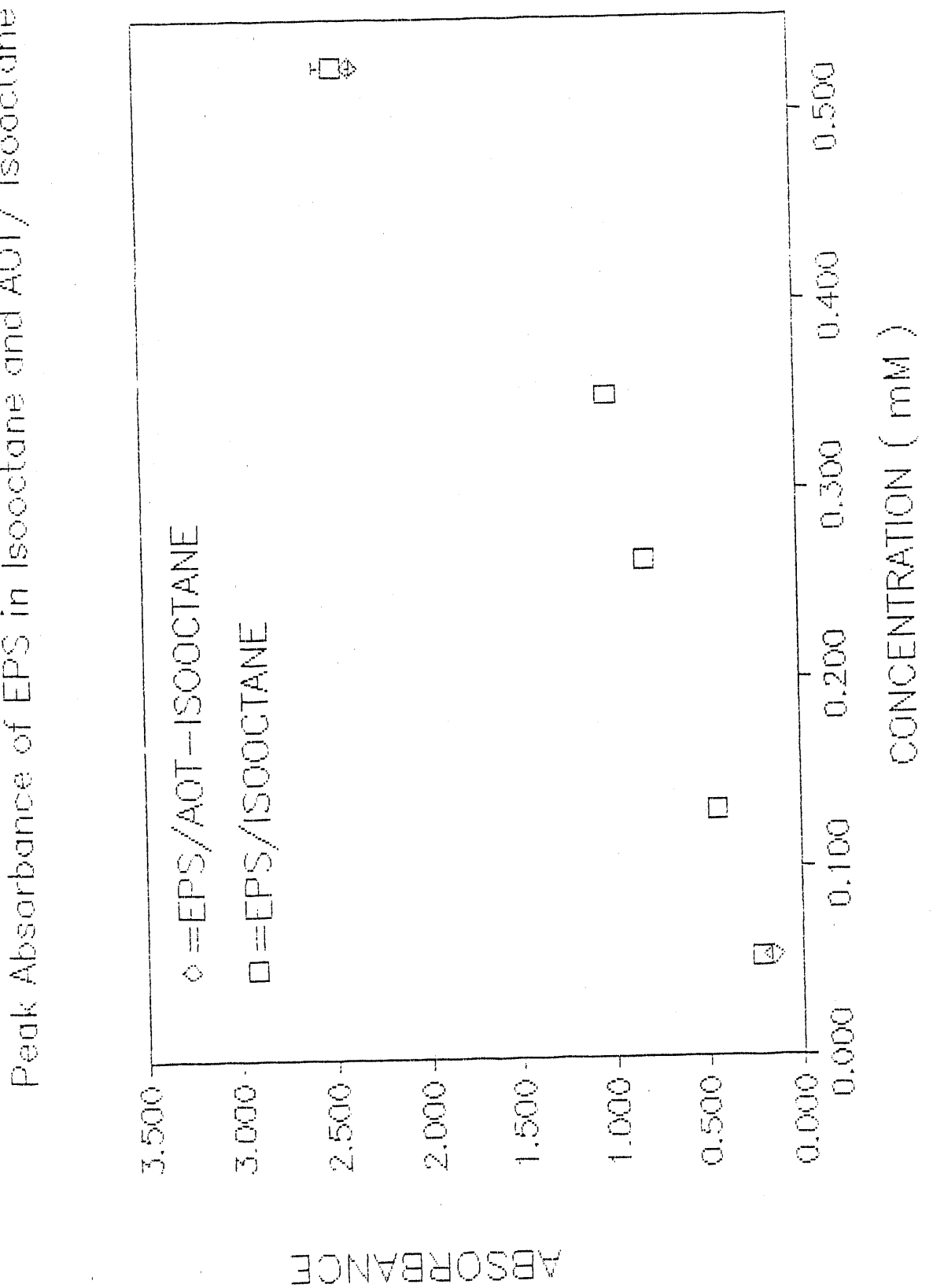


FIGURE 5


Peak Absorbance of EPS in Isooctane and AOT/ Isooctane
◇ =EPS/AOT-ISOOCTANE
□ =EPS/ISOOCTANE
ABSORBANCE
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.000
0.100
0.200
0.300
0.400
0.500
CONCENTRATION (mM)

Section 5

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