Final Report

# Regulation of Coal Polymer Degradation by Fungi

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## Disclaimer

## Abstract

A variety of lignin degrading fungi mediate solubilization and subsequent biodegradation of coal macromolecules (a.k.a. coal polymer) from highly oxidized low rank coals such as leonardites. It appears that oxalate or possibly other metal chelators (*i.e*., certain Krebs Cycle intermediates) mediate solubilization of low rank coals while extracellular oxidases have a role in subsequent oxidation of solubilized coal macromolecule. These processes are under nutritional control. For example, in the case of *P. chrysosporium*, solubilization of leonardite occurred when the fungi were cultured on most but not all nutrient agars tested and subsequent biodegradation occurred only in nutrient nitrogen limited cultures. Lignin peroxidases mediate oxidation of coal macromolecule in a reaction that is dependent on the presence of veratryl alcohol and hydrogen peroxide. Kinetic evidence suggests that veratryl alcohol is oxidized to the veratryl alcohol cation radical which then mediates oxidation of the coal macromolecule. Results by others suggest that Mn peroxidases mediate formation of reactive $Mn^{3+}$ complexes which also mediate oxidation of coal macromolecule. A biomimetic approach was used to study solubilization of a North Dakota leonardite. It was found that a concentration ~75 mM sodium oxalate was optimal for solubilization of this low rank coal. This is important because this is well above the concentration of oxalate produced by fungi in liquid culture. Higher local concentrations probably occur in solid agar cultures and thus may account for the observation that greater solubilization occurs in agar media relative to liquid media. The characteristics of biomimetically solubilized leonardite were similar to those of biologically solubilized leonardite. Perhaps our most interesting observation was that in addition to oxalate, other common Lewis bases (phosphate/hydrogen phosphate/dihydrogen phosphate and bicarbonate/carbonate ions) are able to mediate substantial solubilization of leonardite at physiological pH values. Lastly, we present evidence that some fungi appear to possess coal solubilization ability in which the initial events of solubilization is not mediated by oxalate ion.

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## Executive Summary

A variety of wood rotting fungi and other fungi secrete oxalate. This is of importance because oxalate is able to solubilize highly oxidized low rank coals such as leonardite. Many of the coal macromolecules in low rank coal are held together by ionic linkages with metal ions. When these metal ions are chelated by oxalate, the ionic linkages are broken and the relatively polar coal macromolecules become water soluble. A biomimetic approach was used to study solubilization of leonardite. It was shown that optimal solubilization occurred at sodium oxalate concentrations of ~75 mM. This is of importance because such concentrations are higher that those typically found in liquid cultures of wood rotting fungi and may account for the fact only moderate amounts of coal was solubilized in such cultures. In contrast, substantial leonardite solubilization occurred when fungi (*P. chrysosporium* and *T. versicolor*) were cultured on most of the agar media tested. This is probably due to the fact that oxalate diffuses slowly in such media, leading to oxalate concentrations that are more amenable to leonardite solubilization. Biomimetically solubilized coal macromolecule was shown to be similar in many respects to coal macromolecule solubilized biologically. Possibly the most interesting observation made during these investigations is that common Lewis bases such as phosphate/hydrogen phosphate/dihydrogen phosphate and bicarbonate/carbonate ions mediate substantial solubilization of leonardite at physiological pH values. This finding may be of importance in studies aimed at using solubilized leonardite for methane production by anaerobic fermentation. Coal solubilization and subsequent biodegradation (*i.e*., oxidation, depolymerization or decolorization) are distinct events in wood rotting fungi. These processes are also under nutritional control. For example, coal solubilization occurs in a variety nutrient agars. However, at least in the case of *P. chrysosporium,* subsequent biodegradation as assayed by decolorization appears to occur only in nutrient nitrogen limited cultures; a condition which promotes expression of

extracellular lignin degrading enzymes that are also involved in biodegradation of soluble coal macromolecule. Chelation of metal ions by oxalate (or possibly other metal chelators) appears to be the predominant mechanism whereby solubilization of leonardite is achieved. The role of extracellular fungal oxidases in the solubilization process appears to be minimal. In contrast, extracellular peroxidases appear to have an important role in subsequent biodegradation of soluble coal macromolecule. The mechanism by which subsequent biodegradation occurs involves production of reactive intermediates such as veratryl alcohol cation radical and $Mn^{3+}$ complexes which appear to function as oxidants of soluble coal macromolecule. Reactive oxygen species may also be involved in this process. We have also studied solubilization of higher rank coals. In general, subbituminous and bituminous coals are resistant to oxalate mediated solubilization; even when these coals are preoxidized. However, a number of preoxidized subbituminous and bituminous coals are solubilized by certain fungi. These findings suggest that there may be mechanisms other than oxalate mediated solubilization that are responsible for solubilization of such coals.

# Introduction

## i. Solubilization and Depolymerization of Coal by Microorganisms: A Brief Historical Perspective.

Coal resources are abundant in the United States (Faison, 1993). Unfortunately, substantial amounts of this vital resource is low rank coal which possesses low heating capacity. Furthermore this material often contains relatively high amounts of sulfur. These factors combine to lessen the economic value of low rank coal relative to bituminous and anthracite coals. Thus there is considerable interest in coal processing technologies which produce cleaner burning and cost effective fuels from low rank coal. Lumpkin (1988) has reviewed briefly several physicochemical methods used for successful coal liquification and fuel production. Although successful, it was concluded that such processes are not yet economical; mainly due to the fact that all these processes are energy intensive.

In addition to physicochemical processes, there has been substantial interest in biological processes that result in solubilization of coals. Fakoussa (1981) was the first to demonstrate that a variety of microorganisms could use German bituminous coals as their sole source of carbon and energy. Shortly thereafter, Cohen and Gabrielle (1982) reported that two lignin degrading fungi, *Trametes versicolor* and *Poria monticola*[1] were able to solubilize substantial amounts of a North Dakota lignite; forming a black liquid. In their investigation the authors noted that lignite and lignin possess some similarities in structure and it was suggested that the lignin degrading system of these fungi might be responsible for the solubilization observed. In the

[1] Fungal systematic is rather complicated. The species *Coriolus versicolor* studied by Cohen and Gabrielle (1982) is also known as *Polyporus versicolor* and *Trametes versicolor.* Currently the latter name appears to be the one that is most widely accepted. Similarly, *Poria monticola* is also known as *Poria placenta.*

ensuing years, a variety of other fungi and bacteria were shown to be able to solubilize selected coals (Table 1). Of interest is the fact that several microorganisms that are not generally associated with the ability to degrade lignin were, nevertheless, shown to be able to solubilize low rank coals at least as well as the lignin degrading fungi. For example, *Aspergillis spp., Penicillium spp*. and *Candida spp.* are not known for their lignin degrading ability, yet representatives of all three genera were shown to be able to mediate extensive solubilization of selected low rank coals (Stewart *et al.* 1990; Scott *et al.,* 1986). Table 1 is a representative summary of fungi that have been shown to be able to solubilize coals. The mechanism by which low rank coal is solubilized has been the subject of considerable scrutiny. Despite initial reports suggesting involvement of extracellular enzymes that are secreted by lignin degrading fungi (Cohen *et al.* 1987, Pyne *et al.* 1987), it is now known that a major mechanism of coal solubilization involves secretion of oxalate ions (or possibly other substances) which function as metal chelators (Cohen *et al.*, 1990, Fredrickson *et al.* 1990). Apparently, oxalate ions are able to chelate metal ions in low rank coals. This is important because in some low rank coals metal ions form bonds which link individual coal macromolecules. However, once these ionic linkages are broken by the metal chelator, the relatively polar coal macromolecules become soluble in water. In retrospect, it is not surprising that *Aspergillus spp.* and *Penicillium spp.* are able to solubilize some low rank coals as many members of these genera are known to secrete oxalate ions (Hodgkinson, 1977, and references therein). Similarly, many wood rotting fungi also secrete oxalate ions (Dutton *et al*., 1993). Interestingly, oxalate ion has an important role in lignin degradation (Barr and Aust, 1994). However, oxalate is clearly not unique to the lignin degrading system and is secreted by a variety of microorganisms that have little or no capacity to degrade lignin.

## Table 1

### Representative List of Fungi Reported to be able to Solubilize Coals.

| Coal Type | Microorganism | References |
|---|---|---|
| Nigerian Preoxidized Subbituminous coal | *Phanerochaete chrysosporium* | Achi (1993) |
| North Dakota Lignite | *Trametes versicolor* | Cohen & Gabrielle (1982)<br>Torzilli and Isbister (1994) |
| North Dakota Lignite | *Candida sp.*<br>*Aspergillus sp.*<br>*Paecilomyces sp.*<br>*Penicillium waksmanii* ML20<br>*Sporothrix sp.*<br>*Trametes versicolor* | Scott et al. (1986) |
| Mississippi Lignite | *Aspergillis sp.*<br>*Candida sp.*<br>*Penicillium waksmanii* ML20<br>*Trametes versicolor* | Scott et al. (1986) |
| Preoxidized Beulah Zap II Lignite | *Cunninghamella sp.* | Stewart et al. (1990) |
| Preoxidized Pennsylvania Upper Freeport Bituminous Coal | *Penicillium sp.*<br>*Cunninghamella sp.* | Stewart et al. (1990) |
| Preoxidized Illinois *6 Bituminous Coal | *Penicillium sp.*<br>*Cunninghamella sp.* | Stewart et al. (1990) |

Following solubilization, it is reasonable to expect that solubilized coal macromolecules are further acted upon biologically and undergo processes which result in a decrease in molecular weight. Although such processes would be analogous to depolymerization, it should be appreciated that soluble coal macromolecule is a heterogeneous material with respect to chemical composition and molecular weight. Furthermore, there are probably no distinct monomers linked one to another in a predictable and repetitive manner in the sense that, for example, amino acids monomers are linked together by amide bonds to form proteins and glucose monomers are linked together by glycosidic linkages to form cellulose. It should also be appreciated that depolymerization of coal macromolecule might be more correctly thought of as a decrease in the average molecular weight of a mixture of components than as true depolymerization.

Several studies have been published which address the ability of lignin degrading enzymes to depolymerize solubilized coal macromolecule. Wondrack *et al.* (1989) reported that lignin peroxidase from *Phanerochaete chrysosporium* was able to mediate substantial disappearance of base solubilized coal macromolecule obtained from German subbituminous coal and from a North Dakota lignite. In these experiments depolymerization was monitored by high performance Gel permeation column chromatography (GPC-HPLC) which provides information concerning the relative decrease in amount and size (relative molecular weight) of coal macromolecule present before and after treatment with lignin peroxidase. However, minimal amounts of low molecular weight material, which would have been indicative of monomer formation, was observed. The authors (Wondrack *et al*., 1989) noted that apparent depolymerization was enhanced when veratryl alcohol (the natural substrate for lignin peroxidases) was present in reaction mixtures and that coal macromolecule was a competitive inhibitor of lignin peroxidase mediated veratryl

alcohol oxidase activity.

Ralph and Catcheside (1994) showed that nitrogen limited cultures of *P. chrysosporium* were able to mediate extensive degradation of base solubilized Morwell brown coal. More recently, Ralph *et al.* (1996) studied the ability of several brown rot and white rot fungi to degrade base solubilized macromolecules from Morwell brown coal. Those fungi (*Gloeophyllum trabeum*, *Lentinus lepideus* and *Trametes versicolor*) that produced little or no lignin peroxidase, Mn peroxidase or laccase under the incubation conditions used caused no change in the solubilized coal macromolecule. Two fungi (*Ganoderma applanatum* and *Pycnoporus cinnabarinus*) that also produced little or no lignin peroxidase, Mn peroxidase or laccase caused an increase in the absorbance of the coal macromolecule (monitored at 400 nm). Additionally, *G. applanatum* caused an increase in apparent molecular weight of the soluble coal macromolecule. Two fungi, *Merulis tremellosus* and *Perenniporia tephropora*, which secreted Mn peroxidase and laccase, both appeared to polymerize coal macromolecule. However, incubation with *M. tremellosus* decreased the amount of material that absorbed at 400 nm while incubation with *P. tephropora* did not. Incubation of soluble coal macromolecule with *P. chrysosporium* which secreted lignin peroxidase and Mn peroxidase resulted in decreased absorbance and an increase or a decrease in molecular weight depending on the culture volume. It was concluded that increased oxygen availability in shallow 10 mL cultures favored catabolism (*ie.*, depolymerization) over polymerization. Taken together these observations provide strong evidence for the involvement of fungal extracellular oxidases in the biodegradation of soluble coal macromolecules.

Most recently, Hofrichter and Frtitsche (1997) have presented evidence implicating the involvement of Mn peroxidase from *Nematoloma frowardii* in the depolymerization of

solubilized low rank coal. However, it is interesting to note that depolymerization occurred over the course of several days.

### ii. Objectives of the present investigation.

1) To test the hypothesis that coal (leonardite) solubilization and the subsequent depolymerization of the solubilized coal macromolecules are distinct events in lignin degrading fungi. In addition to *T. versicolor, Phanerochaete chrysosporium*, another lignin degrading fungus that also has the ability to solubilize coal, will be studied.

2) To test the hypothesis that the processes of coal (leonardite) solubilization and coal macromolecule depolymerization in lignin degrading fungi can be regulated by altering the nutritional status of the microorganism. Coal solubilization is expected to occur in nutrient rich media whereas depolymerization of solubilized coal macromolecules is expected to occur in nutrient limited media.

3) To determine the role of extracellular enzymes (laccases, lignin peroxidases and Mn peroxidases) that are secreted by lignin degrading fungi during coal solubilization or coal macromolecule depolymerization.

4) To assess the role of enzymatically generated oxygen radicals, non-radical active oxygen species, veratryl alcohol radicals and $Mn^{+++}$ complexes in coal macromolecule depolymerization.

5) To characterize products of coal solubilization and coal macromolecule depolymerization that are formed by *T. versicolor* and *P. chrysosporium* and their respective extracellular enzymes. Solubilization products formed using oxalic acid and other metal chelators will also be characterized and compared.

## Methods and Materials

*Chemicals.* Leonardite is an oxidized North Dakota lignite. This material was supplied as a fine powder by the American Colloid Company, Skokie, IL and was dried for 24 h at 105$^{o}$C before use.

*Microorganisms.* Cultures of *Phanerochaete chrysosporium* (ATCC 24725) and *Trametes versicolor* (ATCC 12679) were purchased from the American Type Culture Collection, Rockville, MD. Fungi were subcultured and maintained on Malt Agar and Sabouraud Agar, respectively, on Petri plates and on slants in culture tubes. Liquid cultures of fungi were grown in Fahraeus-Reinhammar medium or in the nutrient nitrogen limited liquid medium used by Bumpus and Aust (1987), which is a modification of the medium described by Kirk *et al.* (1977) and Fenn and Kirk (1979). Nutrient nitrogen sufficient medium was made by increasing ten fold the ammonium tartrate concentration.

*Biological solubilization of leonardite by wood rotting fungi.* Petri plates containing several different solid media were used. Powdered leonardite (20 mg) was sprinkled as evenly as possible over each Petri plate and cultures were inoculated using an agar plug from stock cultures of each fungus. Periodically, cultures were visually examined for signs of solubilization. Solubilization was scored as follows: 4+ extensive solubilization in which a brown to black liquid diffused from the solid coal and covered or nearly covered the entire plate; 3+ solubilized coal covered approximately one half to three quarters of the plate; 2+ solubilization occurred but solubilized coal covered less than one half of the plate; 1+ a small amount of solubilization occurred but solubilized coal covered less than one quarter of the plate; - no solubilization was observed.

*Biomimetic solubilization of leonardite.* Leonardite (1 g) was placed in a 125 mL Erlenmeyer flask containing 100 mL of water and 1 g of sodium oxalate. This mixture was stirred for 24 h after which it was centrifuged briefly (1400 xg, 15 min) to remove most of the particulate material. The dark brown solution was then dialyzed (12-14 kda MWCO) extensively against distilled deionized water, filtered through a 0.22 µm cellulose acetate filter (Corning Costar Corp., Cambridge, MA), lyophilized and stored at room temperature. This material was characterized by UV-visible, IR and $^{1}$H-NMR spectroscopy and the molecular weight range was determined by GPC-HPLC (Polman and Quigley, 1991). The relationship between absorbance and mass of soluble leonardite macromolecule at 600 nm was found to be 1.71 AU = 1.0 mg/mL. This relationship was used in a number of experiments to estimate concentration of soluble coal macromolecule in aqueous solution.

The effect of sodium oxalate concentration on its ability to solubilize leonardite was assessed over a concentration range which varied from 0 to 300 mM sodium oxalate. Mixtures containing sodium oxalate and 20 mg leonardite in 10 mL water in 20 mL vials were agitated at 200 rpm on a rotary shaker for 24 h. The amount of coal that was not solubilized was assayed using a gravimetric procedure in which insoluble particulate material was collected by vacuum filtration and its mass determined on dry tared 47 mm, 0.45 µm nylon membrane filters (Whatman). The vials were washed with 10 mL of water which was then passed over the nylon membranes. The membranes were again dried and the mass of the dry membrane and coal that was not solubilized was determined. The amount of coal solubilized was then calculated. Preliminary experiments revealed that the nylon filters lost a substantial amount of their mass during this procedure and were thus unsuitable for gravimetric determinations unless they were washed prior to use. Thus, in our experiments, nylon

filters were pretreated by soaking them in ~500 mL of water for about 30 minutes. During this time the filters were gently agitated and the water was changed once. The filters were then dried at 105$^{o}$ C, 30 min. Filters pretreated in this manner were suitable for use in the gravimetric procedure described above.

The effect of leonardite concentration on its own solubilization was assessed in a similar manner. In these experiments, 74.5 mM (1g/100 mL) sodium oxalate in water containing leonardite concentrations from 50 mg/L to 50 gm/L were used. At the highest concentrations studied, the nylon filters became plugged. Thus, these technical difficulties prevented us from using the same volume for each concentration studied. The concentrations studied follow. The volume in parentheses is the volume that was used: 50 g/L (10 mL), 40 g/L (10 mL), 30 g/L (10 mL), 25 g/L (10 mL), 20 g/L (10 mL), 10g/L (20 ml), 5 g/L (20 mL), 1 g/L (20 mL), 0.5 g/L (20 mL), 0.1 g/L (200 mL), 0.05 g/L (200 mL). In other experiments, the effect of time versus leonardite solubilization was assessed at 0, 1, 10 and 74.6 mM sodium oxalate.

The effect of pH on the ability of several Lewis bases to solubilize leonardite was assessed. For sodium oxalate, a concentration of 74.6 mM (1 g/100 mL) was used. For potassium phosphate/hydrogen phosphate/dihydrogen phosphate and sodium bicarbonate/carbonate, a concentration of 75 mM was used. Mixtures containing 10 mL of these solutions at varying pH values and 20 mg leonardite were shaken for 24 h on a rotary shaker (200 rpm) after which time 1 mL aliquots were centrifuged (4 min, 11,000 xg) in an Eppendorf microcentrifuge and the absorbance at 600 nm was acquired and plotted against pH. The effect of sodium hydroxide concentration on leonardite solubilization was assessed in a similar manner. Except for sodium bicarbonate/carbonate, pKa values were corrected for ionic strength to calculate the concentration of acid and conjugate base for each pH value.

Solubilization was also assessed gravimetrically at selected pH values.

*Laccase production by Trametes versicolor : Isolation and assay of laccase.*

To produce laccase, a fungal mat of *T. versicolor* was homogenized in an Osterizer blender with 100 ml of sterile deionized water. Of this mixture 10 mL was placed in 250 mL Fahraeus-Reinhammar medium and allowed to grow at ambient room temperature, with agitation on a rotary shaker (200-250 rpm) for 3-4 days (Fahraeus & Reinhammar 1967). Laccase production was induced by adding 2.5 ml of a solution of xylidine (0.024g in 10 ml ethanol) followed by continued incubation for another 3-4 days as described (Fahraeus & Reinhammar 1967). Laccase activity was assayed as follows. One hundred microliters of the extracellular fluid was placed in a 1.5 mL cuvette with 1.0 mL of a solution of catechol (0.11g in 10 ml sodium acetate, pH 5.0) and the rate of change of absorbance at 400 nm was monitored. Enzyme activity is expressed by the increase in absorbance at 400 nm per minute/mL. In some experiments, solid leonardite or soluble coal macromolecule was added to preparations of this enzyme to assess the role of laccase in leonardite solubilization and coal macromolecule decolorization and depolymerization. To isolate laccase, the extracellular fluid from a 260 mL culture of *T. versicolor* was centrifuged for 30 minutes at ~1400 xg. The supernatant was then concentrated by ammonium sulfate precipitation, lyophilization or centrifugation in Centriprep tubes (Amicon) (10,000 molecular weight cutoff). The concentrated fluid (2 mL) was then chromatographed in 50 mM potassium phosphate, pH 6.0 on a PD-10 column (Pharmacia, Uppsala). Fifteen 1.5 ml fractions were collected. Each fraction was assayed for laccase activity, and the absorbance at 280 nm was acquired for each fraction. Most of the laccase activity was associated with fraction 3. According to Fahraeus and Reinhammar (1967), two laccase isozymes represent essentially all of the proteins present in the

extracellular fluid of *T. versicolor* that is grown on the growth medium used in this investigation. This method represents a rapid purification by which reasonable amounts of laccase can be obtained. If individual isozymes are required, they can be separated by anion exchange chromatography as described (Fahraeus and Reinhammar, 1967).

## Results and Discussion

### A. Solubilization of Low Rank Coal.

### i. Solubilization of Leonardite by *Phanerochaete chrysosporium* and *Trametes versicolor*.

The ability of fungi to solubilize low rank coals has been demonstrated by a number of investigators (Achi, 1993; Cohen and Gabrielle, 1982; Cohen and Grey, 1993; Scott *et al.*; 1986; Stewart *et al.*; 1990; Torzilli and Isbister, 1994; Wilson et al., 1987). In Table 2 we confirm that *P. chrysosporium* and *T. versicolor* are among those fungi able to mediate solubilization of leonardite when grown on selected solid agar media.

**Table 2**

**Solubilization of Leonardite by *Phanerochaete chrysosporium* and *Trametes versicolor* grown on several types of agar media**

| Microorganism | Sabouraud Agar | Modified Sabouraud Agar | Yeast Malt Agar | Potato Dextrose Agar | Malt Agar |
|---|---|---|---|---|---|
| P. chrysosporium | ++++ | ++++ | ++++* | ++++* | - |
| T. versicolor | ++++ | ++++ | ++++ | ++ | - |

Twenty milligrams of Leonardite was placed on Petri plates containing several types of agar media. Following inoculation with *P. chrysosporium* or *T. versicolor* plates were incubated at room temperature in a humidified chamber for 50 days at which time they were visually examined for solubilization. Solubilization was scored as described in the Methods and Materials section.

*Although coal solubilization occurred and the Petri plate was completely covered, it was apparent that the color intensity was substantially less than that observed for other plates that received ++++ scoring.

Of interest is the observation that solubilization did not occur when the fungi were grown on Malt agar. In our hands, *T. versicolor* does not grow well on Malt agar and this might be responsible, in part, for the fact that solubilization was not observed when *T. versicolor* was cultured on this medium. *P. chrysosporium,* however, does grow quite well on Malt agar and did not solubilize leonardite under these conditions. This observation clearly shows that the nutrient medium on which the fungi are cultured is important to the solubilization process. Although leonardite solubilization was extensive when performed in solid agar media such as Sabouraud agar, only minimal amounts of solubilization occurred in liquid media. We believe that this is a function of oxalate concentration. In liquid cultures, Dutton *et al.* (1993) showed that the oxalate concentration in liquid cultures of white rot fungi varies between 0 and 10 mM. This is well below the concentration of 75 mM oxalate that we have shown is near optimum for leonardite solubilization (Bumpus *et al.* 1998). It appears that solubilization is more effective in solid media not because more oxalate is secreted, but because of the low amounts of water present and the fact that oxalate diffusion would occur relatively slowly. These factors would combine to produce sufficiently high local concentrations of oxalate for leonardite solubilization.

Several experiments were performed in an attempt to increase the amount of oxalate produced in liquid cultures of *P. chrysosporium*. In nitrogen limited cultures *P. chrysosporium* began to produce oxalate on day two of incubation and reached a peak on day 3 of 2.8 mM. In nutrient nitrogen sufficient cultures oxalate production was not observed until day eight of incubation and peak oxalate concentration was not reached until day eleven and was never greater that 0.07 mM. It is known that oxalate production by several fungi is affected by the substrates on which they are grown. We showed that when succinate was present as the sole carbon source, oxalate production by *P. chrysosporium* was observed after two days of growth. However, a

maximum oxalate concentration of only 1.1 mM was observed. When glucose and succinate were both present in culture, a substantial increase in oxalate concentration was observed. After 3 days of incubation, a peak oxalate concentration of 16.7 mM was observed after which oxalate concentration declined to 11.1 mM on day 12 at which time the experiment was terminated. Although supplementation of fungal cultures with sodium succinate enhances the amount of oxalate produced, this would not likely be a cost effective strategy to enhance solubilization of low rank coal in liquid culture. Furthermore we have found that several Krebs cycle intermediates are able to mediate solubilization of low rank (Table 3). This is important as there are

**Table 3.**

**Solubilization of Leonardite by Krebs Cycle Intermediates**

| Krebs Cycle Intermediate | Percent Solubilization |
|---|---|
| Malate | 34% |
| Fumarate | 4% |
| α−Ketoglutarate | 9.5% |
| Oxaloacetate | 38.5% |
| Succinate | 7% |
| Citrate | 44% |
| Isocitrate | 40.5% |

Solutions (75 mM) of Krebs Cycle intermediates in water were prepared from the free acid or their sodium salts. The pH of each solution was then adjusted to pH 7 with HCl or NaOH. The ability of each compound to solubilize leonardite was then determined as follows. To each of three 20 mL scintillation vials was added 20 mg of leonardite and 10 mL of one of the Krebs Cycle intermediate solutions. These mixtures were agitated for twenty four hours after which time the mass of the insoluble material was determined gravimetrically. The amount of material solubilized was then calculated by difference.

several fungi are able to over express these intermediates. For example, a strain of *Aspergillis niger* over expresses production of citrate when grown on low-sugar media

and on cotton waste (Kiel *et al.* 1981). Thus fungi have already been described which secrete sufficiently high concentrations of metal chelators (*i.e.,* certain Krebs cycle intermediates) capable of mediating solubilization of low rank coal.

It appears that oxalate has an important role in solubilization by fungi of low rank coals such as leonardite. There are several techniques available to measure oxalate in aqueous solution. We used the procedure described by Dutton *et al* . (1991). In this procedure oxalate concentration is measured by high performance liquid chromatography. Production of oxalate in pelleted agitated cultures of *P. chrysosporium* and *T. versicolor* grown in Fahraeus Reinhammar medium (1967) is presented in Figures 1 and 2.

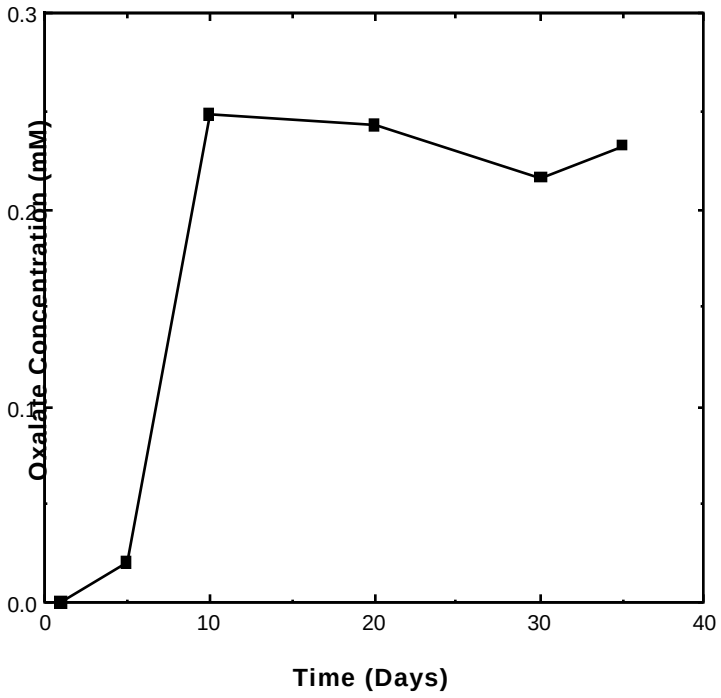



Figure 1. Oxalate production in agitated pelleted cultures of *P. chrysosporium.* Fungal cultures (100 mL) were grown in Fahraeus-Reinhammar medium.

The highest concentrations (~0.25 and 0.6 mM, respectively) of oxalate produced were found to be substantially less than that known to be near optimum (*i.e.,* ~75 mM oxalate) for leonardite solubilization and were less than those values reported by Dutton *et al*. (1993).

In summary these investigations have shown that in some fungi, solubilization of low rank coal is dependent on its nutritional status. Specifically, nutritional conditions that would promote secretion of oxalate ion or other metal chelators would also be expected to promote solubilization of low rank coal.

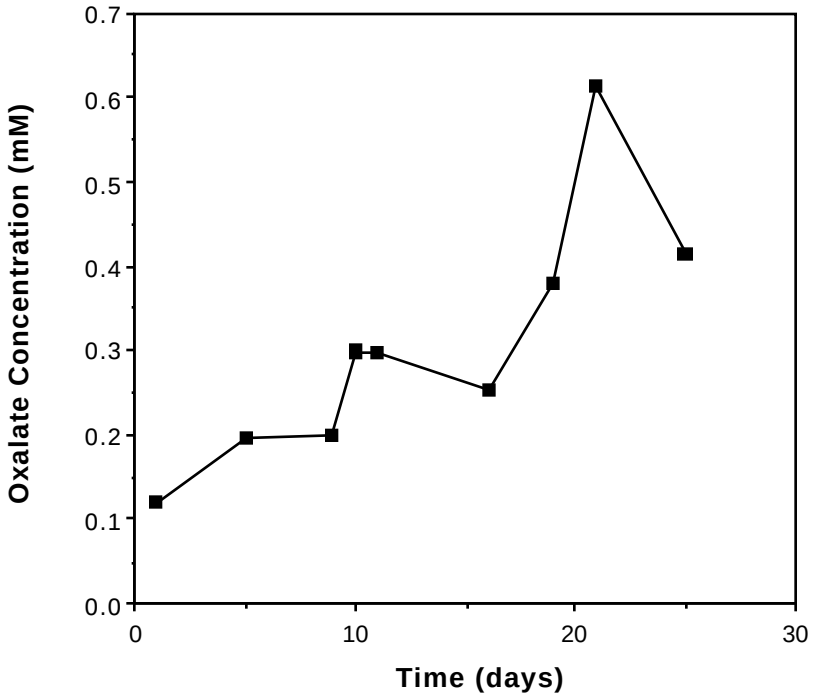



Figure 2. Oxalate production in agitated pelleted cultures of *T. versicolor.* Fungal cultures (100 mL) were grown in Fahraeus-Reinhammar medium.

### ii. Biomimetic Solubilization of Leonardite.

Cohen *et al.* (1990) showed that oxalate ion secreted by *T. versicolor* was responsible for solubilization of low rank coal. Although the involvement of oxalate in low rank coal solubilization by fungi *in vivo* is now generally accepted; when our studies were initiated there was limited information concerning the efficiency, rate and optimal conditions of oxalate mediated coal solubilization. Therefore, we studied solubilization of a North Dakota leonardite using sodium oxalate (Bumpus *et al*., 1998). Because oxalate ions are responsible for leonardite solubilization *in vivo*, solubilization by an oxalate salt *in vitro* may be considered to be a biomimetic process.

Initial investigations focused on assessing the amount of low rank coal that was solubilized. Oxalate treatment results in solubilization of considerable amounts of a brown black material. In many cases, the absorbance of this material at 600 nm in aqueous solution could be acquired without diluting the sample. Therefore this wavelength was used in an attempt to develop an assay to quantitate the degree of solubilization that occurred in our experiments. Unfortunately, absorbance was shown to be pH dependent. Furthermore, material that was not truly solubilized (*i.e.*, fine colloidal material) by oxalate or other metal chelators sometimes remained suspended or was easily resuspended following centrifugation to remove particulate material. In any case, these problems led to over estimation of the amount of low rank coal that was solubilized. To overcome these problems we developed a gravimetric procedure that was substantially more labor intensive but clearly more accurate for assessing the amount of coal solubilized in our experiments. Although the spectrophotometric assay had its shortcomings, it was still useful in a number of experiments and was regularly used in conjunction with the gravimetric procedure.

A sodium oxalate concentration of approximately 75 mM was shown to be near optimal when the leonardite concentration was 2 mg/mL (Figures 3 and 4).

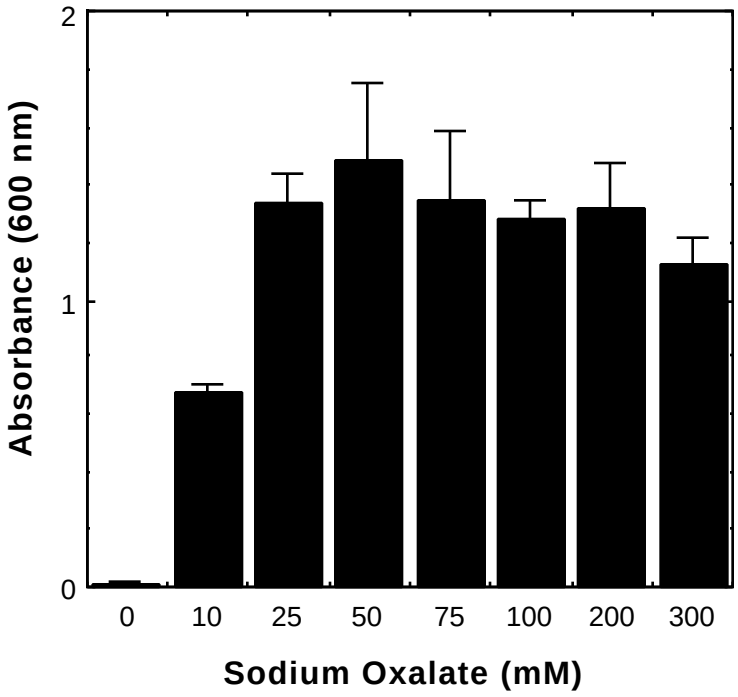
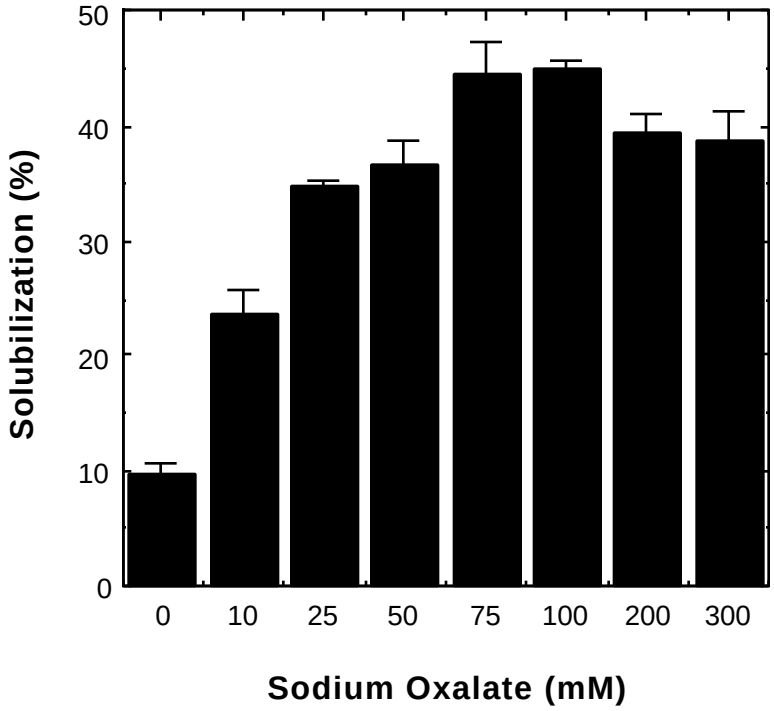



Figure 3. Effect of sodium oxalate concentration on leonardite solubilization determined by absorbance at 600 nm. In 20 mL reaction vials each reaction mixture contained 20 mg of leonardite and 10 mL of sodium oxalate at the indicated concentrations. Reaction vials were placed on a rotary shaker for 24 h. Particulate material was then collected on 0.45 µm filters and washed with 10 mL of water. The absorbance of the filtrate at 600 nm was then acquired, multiplied by two to account for the dilution which occurred during washing and plotted against sodium oxalate concentration. Experiments were performed in triplicate.

This was of interest because, as noted above, oxalate concentrations in liquid cultures of wood rotting fungi are typically well below this concentration. Nevertheless, substantial amounts of leonardite were solubilized at oxalate concentrations more typical of those found in liquid cultures of these fungi.




(Figure 4). Effect of sodium oxalate concentration on leonardite solubilization determined gravimetrically. In 20 mL reaction vials each reaction mixture contained 20 mg of leonardite and 10 mL of sodium oxalate at the indicated concentrations. Reaction vials were placed on a rotary shaker for 24 h. The mass of the particulate material was determined gravimetrically as described in methods and materials. These values were plotted versus sodium oxalate concentration. Experiments were performed in triplicate.

The effect of leonardite on its own concentration was also investigated (Figure 5). It was shown that at a sodium oxalate concentration of ~75 mM, the amount of leonardite solubilized increased linearly over a range in which the concentration of this low rank coal increased from 50 mg/L to 50 g/L. The relative percent of low rank coal solubilized initially increased but then decreased over the concentration range studied.

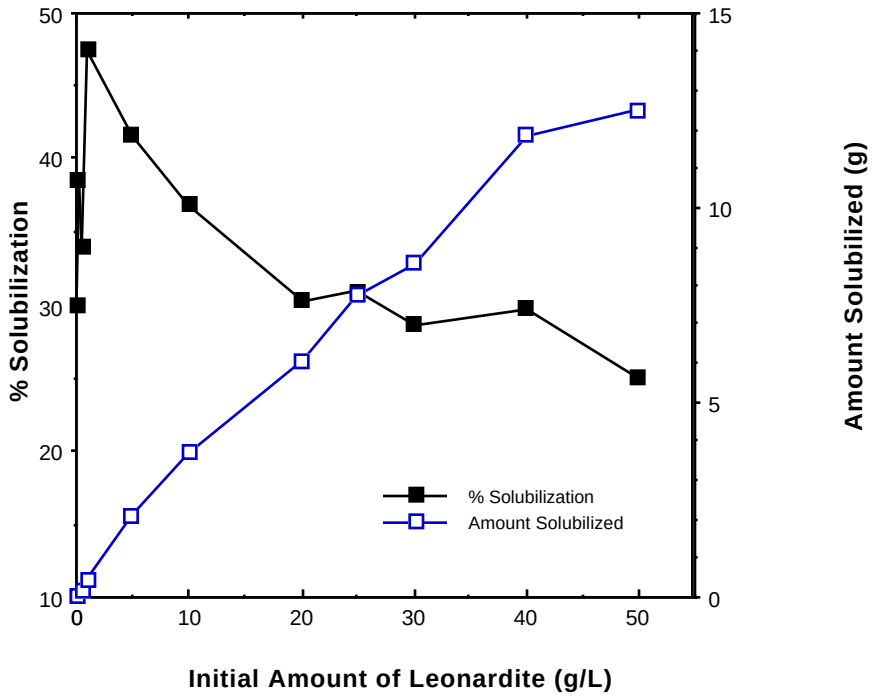



Figure 5. Effect of leonardite on its own solubilization. Reaction vessels containing the requisite amounts of leonardite and volumes of 74.5 mM sodium oxalate were placed on a rotary shaker for 24 h after which time the amount of leonardite solubilized was determined gravimetrically. Experiments were performed in triplicate.

The time course for solubilization of low rank coal by sodium oxalate at three different oxalate concentrations (1 mM, 10 mM and 74.5 mM) is presented in Figure 6. Following an initial rapid solubilization, continued solubilization at a slower rate occurred for the duration of the time course as measured by an increase in absorbance at 600 nm. Results obtained using 1 mM and 10 mM sodium oxalate were relatively straightforward. However, when 74.5 mM sodium oxalate was used a second rapid increase in absorbance was observed after several days of incubation. The precise

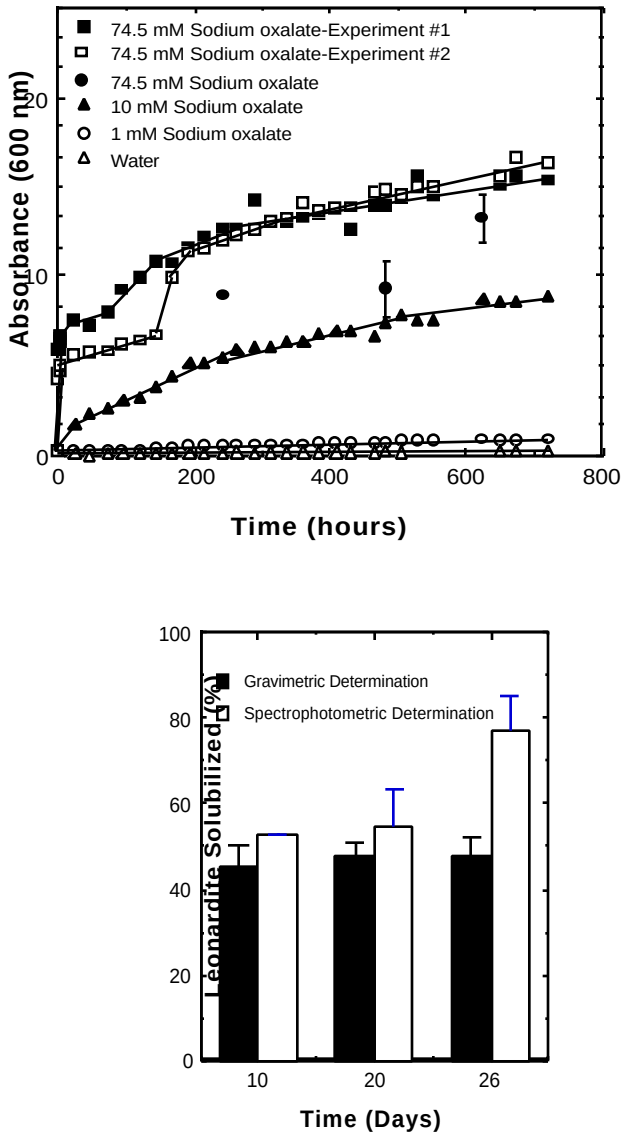



Figure 6. Effect of incubation time on oxalate mediated solubilization of leonardite. Reaction mixtures contained 1 gm of leonardite in 100 mL of water or sodium oxalate in water at the indicated concentrations. Reaction mixtures were stirred on a magnetic stirrer and the $A_{600}$ was determined at the times indicated (top). In three experiment (closed squares, open squares, closed circles) the initial concentration of sodium oxalate was 74.5 mM. In other experiments, incubation mixtures contained 10 mM sodium oxalate (closed triangles), 1 mM sodium oxalate (open circles) and water (open triangles). In one experiment (bottom) solubilization was determined spectrophotometrically (using the relationship $A_{600}$/mg = 1.71) and gravimetrically. Spectrophotometrically acquired data is represented by the open columns in the bottom graph while gravimetrically acquired data is represented by the solid columns. Error bars represent $\pm$ 1 standard deviation. In this experiment, triplicate samples containing 1 gm of leonardite in 100 mL of 74.5 mM sodium oxalate were stirred for 10, 20 and 26 days. Samples were centrifuged for 30 min at 11,000 xg. The $A_{600}$ of the supernatant of each sample was then determined. The pellets were then dried. The mass of each pellet was determined and the amount of leonardite solubilized was calculated. See text for further explanation.

time at which this second rapid increase occurred, however, was not predictable. Experiments were performed in which solubilization was measured gravimetrically. In these experiments the percent solubilization of low rank coal after 10, 20 and 26 days was shown to be 45.3 $\pm$ 0.05, 48 $\pm$ 0.03 and 48.05 $\pm$ 0.04, respectively. Of interest was the observation that following 26 days of incubation the $A_{600}$/mg/mL of soluble coal macromolecule in water was found to be 2.75, which is considerably greater than the relationship that we had determined and used in our spectrophotometric assay to calculate the concentration of soluble coal macromolecule. Subsequent studies showed that the increase in this ratio was due, at least in part, to an increase in pH from 5.2 to ~8.9 that occurred during the incubation. Thus, these studies show that calculations based solely on absorbance data can lead to overestimation of the amount of low rank coal solubilized during studies which require prolonged incubation.

In another study (Figure 7) 1 g of low rank coal was subjected for ten days to daily incubation and extraction with 100 mL of 74.5 mM sodium oxalate.

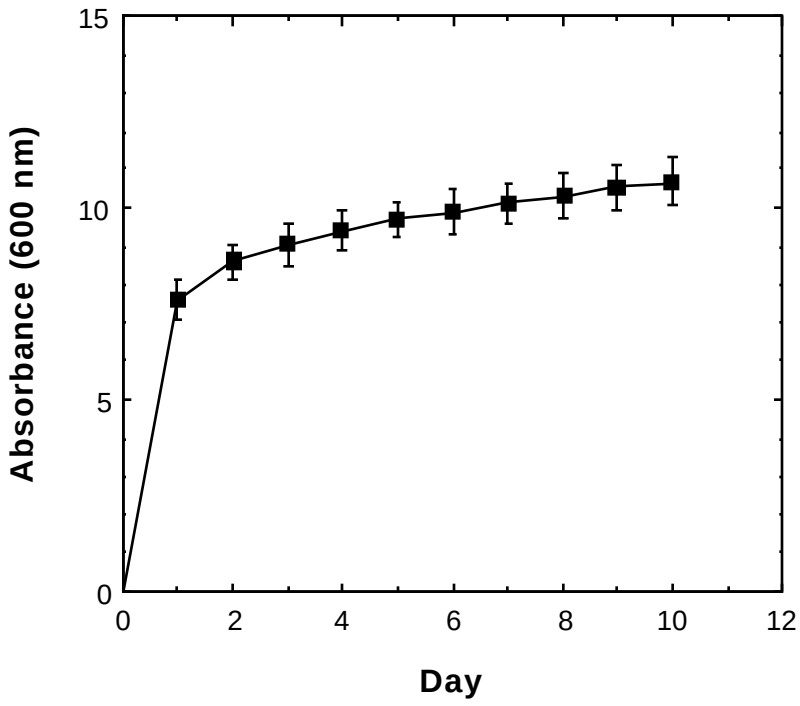



Figure 7. Effect of repeated oxalate treatment on leonardite solubilization. Reaction mixtures contained 1 gm of leonardite and 100 mL of 74.5 mM sodium oxalate in water. Reaction mixtures were stirred for 24 h and centrifuged (1400 xg, 15 min). A 1 mL aliquot was then removed, centrifuge at 11,000 x g for 4 min and the $A_{600}$ of the supernatant was determined. The pellet was then resuspended in 100 mL of 74.5 mM sodium oxalate and this process was repeated nine more times.

In these experiments it was demonstrated that even on the tenth day of this treatment a measurable amount of leonardite was solubilized; again confirming that after an initial rapid rate, oxalate-mediated solubilization of low rank coal is a relatively slow process.

The effect of pH on low rank coal solubilization in the presence of sodium oxalate, sodium bicarbonate/carbonate and potassium phosphate/hydrogen phosphate/dihydrogen phosphate is presented in Figures 8 and 9. The effect of sodium hydroxide concentration on low rank coal solubilization is also presented in

Figures 8 and 9**.**

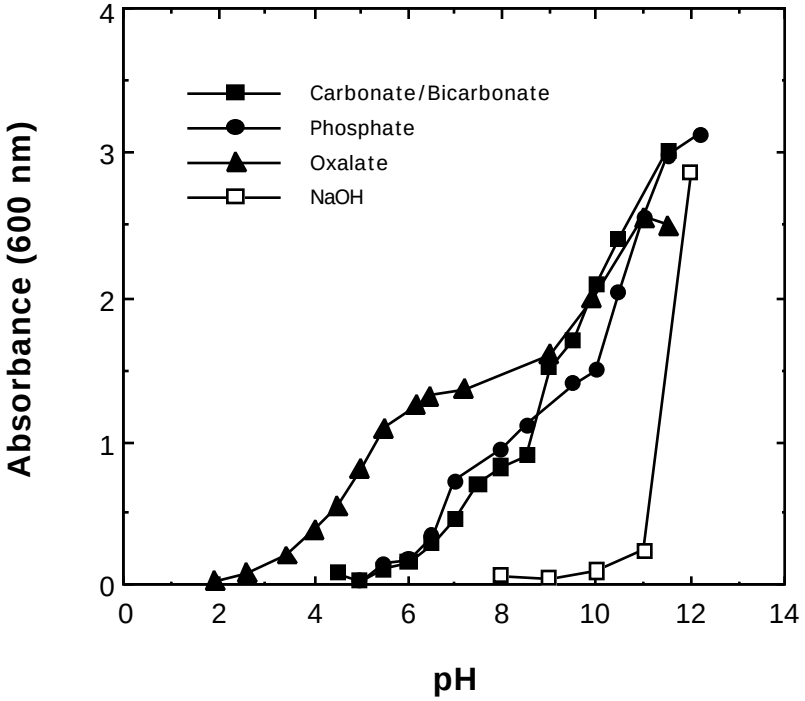



Figure 8. Effect of pH on leonardite solubilization in the presence of several Lewis bases determined by absorbance at 600 nm. In 20 mL reaction vials each reaction mixture contained 20 mg of leonardite and, at the pH values indicated, 10 mL of sodium oxalate (74.5 mM), potassium phosphate/hydrogen phosphate/dihydrogen phosphate (75 mM), sodium bicarbonate/carbonate (75 mM) or the indicated concentration of sodium hydroxide. Reaction vials were placed on a rotary shaker for 24 h after which time aliquots were centrifuged and the absorbance at 600 nm was determined . In separate experiments the amount of leonardite solubilized was determined gravimetrically (Figure 9).

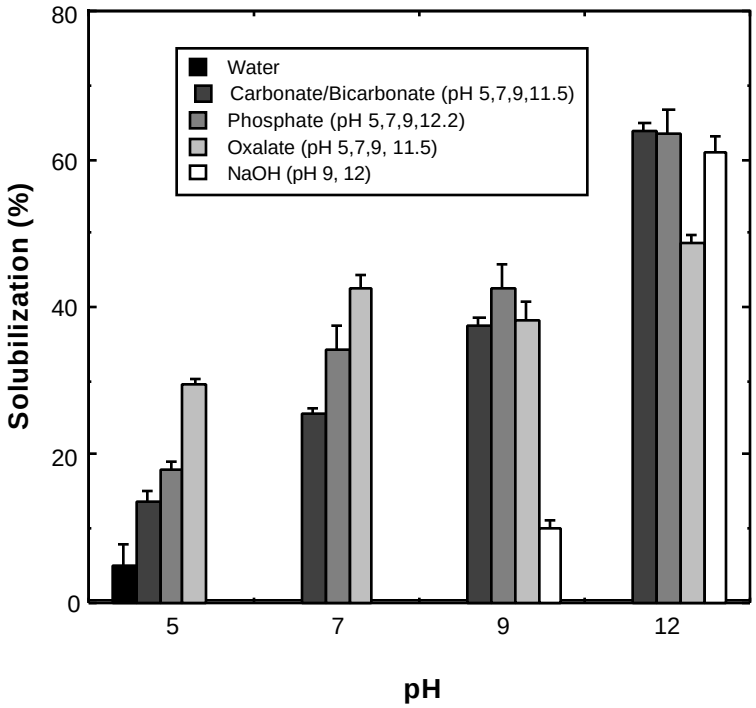



Figure 9. Effect of pH on leonardite solubilization in the presence of several Lewis bases determined gravimetrically. In 20 mL reaction vials each reaction mixture contained 20 mg of leonardite and, at the pH values indicated, 10 mL of sodium oxalate (74.5 mM), potassium phosphate/hydrogen phosphate/dihydrogen phosphate (75 mM), sodium bicarbonate/carbonate (75 mM) or the indicated concentration of sodium hydroxide. Reaction vials were placed on a rotary shaker for 24 h after which time the amount of leonardite solubilized was determined gravimetrically. Gravimetric experiments were performed in triplicate.

The phosphate/hydrogen phosphate/dihydrogen phosphate and the bicarbonate/carbonate systems were studied because of their importance in environmental systems and sodium oxalate, of course, was selected for study because because of its documented role in solubilization of low rank coal *in vivo*. Sodium hydroxide was also studied because it has long been known that low rank coal is solubilized in the presence of strong base (Fowkes and Frost, 1960) and it is important to be able to distinguish between base-mediated solubilization that is caused by high

concentrations of hydroxide ion and solubilization that is mediated by oxalate, phosphate/hydrogen phosphate/dihydrogen phosphate and bicarbonate/carbonate ions functioning as Lewis bases (*i.e*., as metal chelators). In every case, solubilization increased as pH increased and for each Lewis base optimal solubilization occurred at pH values at which the species under investigation existed as the divalent (or higher valent) anion. The most important observation is that substantial solubilization occurred at pH values in the physiological range; well below those pH values at which hydroxide-mediated solubilization takes place. The effect of pH on low rank coal solubilization by each of the three Lewis bases studied is presented individually in Figures 10, 11 and 12, respectively.

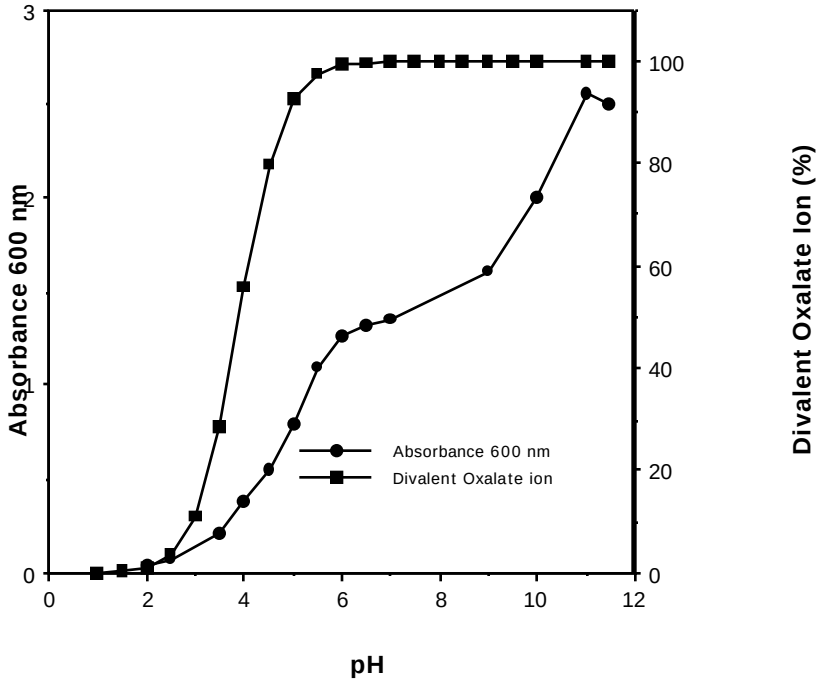



Figure 10. Effect of pH on leonardite solubilization in the presence of sodium oxalate. The data describing oxalate mediated solubilization of leonardite from Figure 8 was replotted to illustrate the effect of ionization on solubilization.

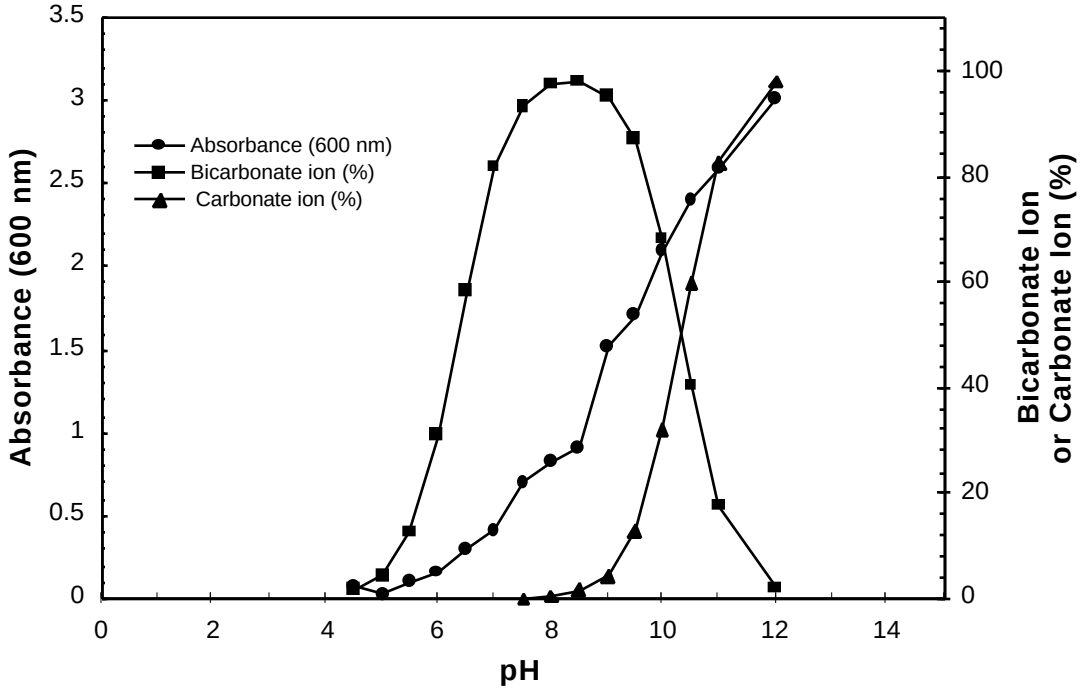



Figure 11. Effect of pH on leonardite solubilization in the presence of sodium bicarbonate/carbonate. The data describing bicarbonate/carbonate mediated solubilization of leonardite from Figure 8 was replotted to illustrate the effect of ionization on solubilization.

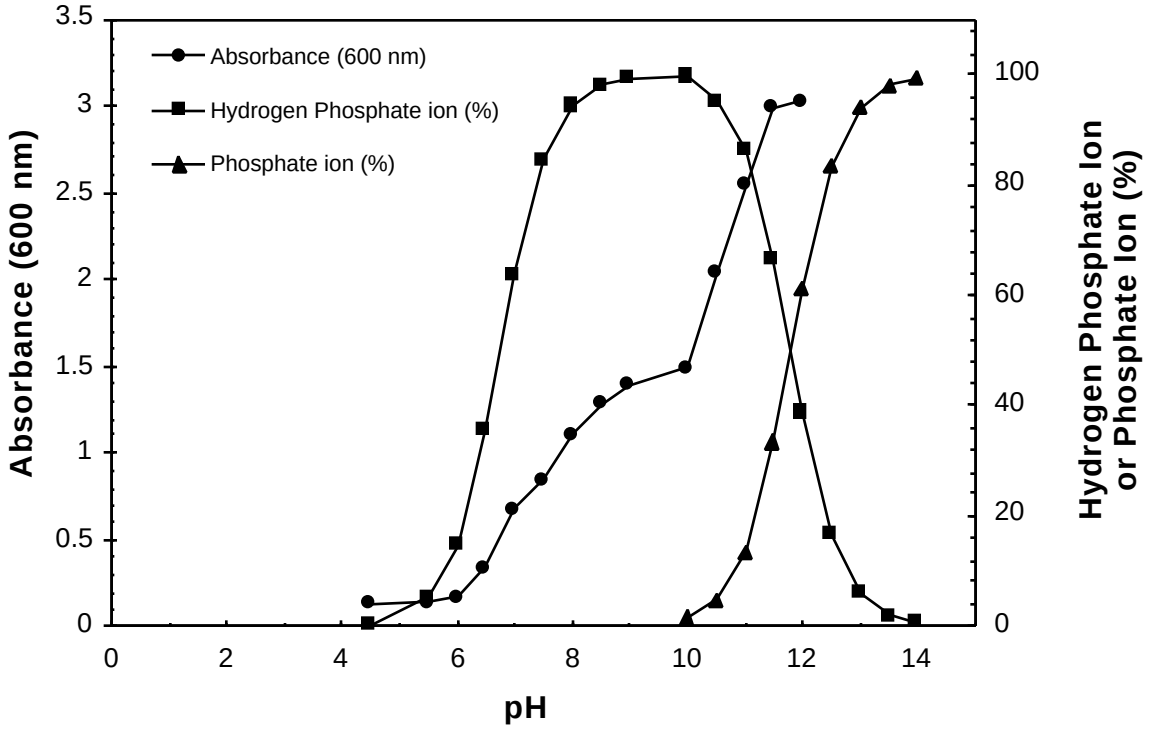



Figure 12. Effect of pH on leonardite solubilization in the presence of potassium phosphate.The data describing phosphate/hydrogen phosphate/dihydrogen phosphate mediated solubilization of leonardite from Figure 8 was replotted to illustrate the effect of ionization on solubilization.

### iii. Characterization of Biomimetically Solubilized Coal Macromolecule.

The spectral characteristics (data not shown) of biomimetically solubilized coal macromolecule were very similar to those reported for coal macromolecule solubilized biologically by *T. versicolor* (Wilson et al., 1987). These characteristics are consistent with those of humic acid, an acidic aromatic polymer normally found in peat, brown coal and lignite.

The molecular weight of soluble coal macromolecule was estimated by GPC-HPLC using proteins of known molecular weight as standards (Figure 13).

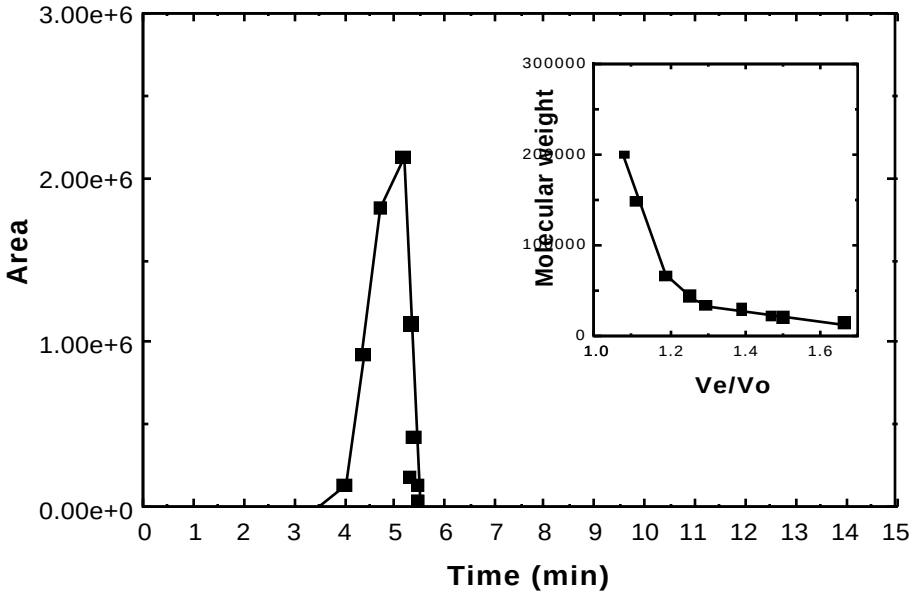



Figure 13. Estimation of the molecular weight range of soluble coal macromolecule. During GPC-HPLC of a 20 µL aliquot of solubilized coal macromolecule (2.1 mg/mL), fractions were collected every 0.5 min. Aliquots (20 µL) of fractions containing material have UV absorbance at 280 nm were then reinjected into the HPLC and the average molecular weight of material in each fraction was determined. Inset graph: standard curve used to estimate average molecule weight of soluble coal macromolecule. Molecular weight standards used to construct this standard curve included sweet potato ß-amylase (MW 200,000), yeast alcohol dehydrogenase (MW 150,000), bovine serum albumin (MW 66,000), chicken egg ovalbumin (MW 45,000), carbonic anhydrase (MW 29,000), bovine pancreas trypsinogen (MW 24,000), soybean trypsin inhibitor (MW 20,100), bovine ß-lactoglobulin (MW 18,400 (elutes as a dimer)) and chicken egg white lysozyme (MW 14,300).

In these assays, coal macromolecules elute as a relatively symmetrical peak. However, unlike the protein standards, it is known coal macromolecule is a mixture rather than a homogeneous entity and the retention time of the peak is only a rough estimate of the average molecular weight of the coal macromolecules that comprise this mixture. In order to estimate the molecular weight range of coal macromolecules that are present in this mixture, a 20 µl aliquot of concentrated coal macromolecule was assayed by GPC-HPLC and fractions were collected at one-half minute intervals. Aliquots (20 µl) of these fractions were then individually assayed by GPC-HPLC. Results (Figure 13) demonstrated that the molecular weight of solubilized coal macromolecule ranged from about 14,000 (the molecular weight cut-off of the dialysis tubing used to prepare the sample) to approximately 66,000.

After extensive treatment with sodium oxalate, a substantial amount (~40%) of leonardite was refractory to solubilization. It would be logical to suggest that the refractory material might represent a more “mature” fraction of the coal, *i.e.,* a fraction that might resemble a bituminous coal. If this is true, such a fraction would be expected to be more highly reduced than the parent leonardite. The elemental analysis of leonardite and the insoluble material remaining after extraction of leonardite by sodium oxalate solution is presented in Table 4 and shows that the ratio of hydrogen to carbon is increased in the extracted sample relative to the parent leonardite. However, the ratio of oxygen to carbon is also increased. It should also be noted that the amount of sulfur in the insoluble material is substantially reduced.

**Table 4.**

**Elemental Analysis of Leonardite and the Insoluble Residue that Remains Following Extensive Extraction of Leonardite with Sodium Oxalate Solution.**

| Analyte | Leonardite | Residue |
|---|---|---|
| Carbon | 48.60% | 40.46% |
| Hydrogen | 3.31% | 3.90% |
| Nitrogen | 1.03% | 0.60% |
| Sulfur | 2.11% | 0.64% |
| Oxygen | 23.73% | 24.68% |
| Ash | 21.22% | 29.71% |

In some of our investigations, solubilization of leonardite was estimated by monitoring the increase in absorbance that occurs in the visible range of this material. The UV visible spectrum of this material is nondescript, gradually increasing in intensity through the visible spectrum into the UV. There are no distinct peaks.

A number of investigations, including our own, focus on the ability of fungi to solubilize low rank coal and/or degrade soluble coal macromolecule. Typically, such experiments involve monitoring changes in molecular weight of soluble coal macromolecule following treatment by fungi or fungal enzymes. A variety of techniques can be used to estimate the molecular weight of coal macromolecules. These include light scattering procedures, vapor-phase osmometry, mass spectrometry and gel permeation high performance column chromatography (GPC-HPLC). Unfortunately, all of these procedures appear to possess characteristic drawbacks that are not necessarily due to the technique *per se*, but to the heterogeneity and complexity of the coal macromolecules under investigation.

Because of its ease of use, GPC-HPLC has often be selected as the technique of choice for many investigations. However, some GPC-HPLC procedures suffer from the fact that soluble coal components sometimes bind reversibly or irreversibly to certain GPC-HPLC matrices. Furthermore, molecular weight is generally estimated using protein molecular weight standards that may not be suitable for estimating molecular weight of coal macromolecules. Wondrack *et al.* (1989) used GPC-HPLC (SynChropak GPC 300 column) to assess the ability of lignin peroxidase from *Phanerochaete chrysosporium* to depolymerase solubilized coal macromolecule from a North Dakota Lignite and a German subbituminous coal. In these studies, however, the possibility of coal macromolecule interacting with the column matrix or with growth media constituents was not addressed. These potential problems were addressed by Polman and Quigley (1991). In their investigations it was shown that coal macromolecule constituents did not bind to SynChropak GPC 300 columns during GPC-HPLC in 20 mM potassium phosphate buffer, pH 7.1. Furthermore, these investigators showed that a number of common media constituents had little effect on the elution of coal macromolecules from this column.

In our investigations we have used GPC-HPLC to estimate the molecular weight of oxalate solubilized coal macromolecule prepared from leonardite and we have discovered two factors that must be considered when using GPC-HPLC to estimated the molecular weight of coal macromolecules. We have shown that the composition of the aqueous solution in which the coal macromolecule is dissolved can have a dramatic effect on the apparent molecular weight determined. We have also shown that when potassium phosphate is added to buffer an aqueous solution of soluble coal macromolecule, the apparent molecule weight of the coal macromolecule undergoes time dependent changes in the magnitude of the apparent molecular

weight. A SynChropak GPC 100 column was used in these experiments.

Oxalate solubilized leonardite that had been extensively dialyzed against water, filtered through a 0.22 µm filter and lyophilized was redissolved in water. The GPC-HPLC chromatogram of this material is presented in Figure 14.

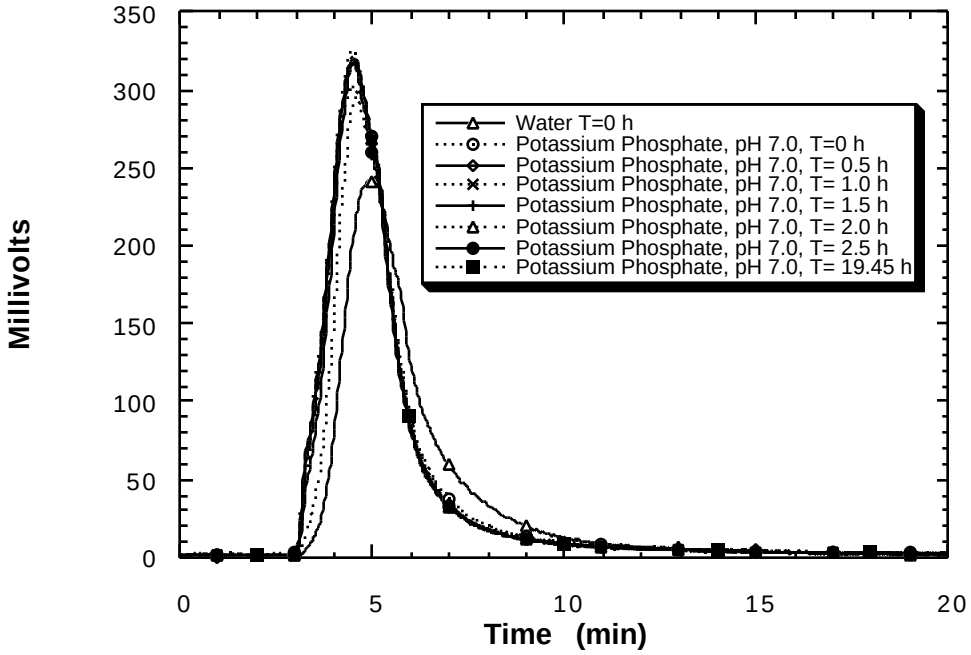



Figure 14. Effect of phosphate buffer on the GPC-HPLC elution profile of oxalate solubilized coal macromolecule. Coal macromolecule (5.6 mg) was dissolved in 2.5 mL of water. To a 2 mL centrifuge tube was added 0.25 mL of this coal macromolecule solution and 0.25 mL of water. The solution (1.12 mg coal macromolecule/mL water was rapidly mixed and a 20 µL aliquot was immediately analyzed by GPC-HPLC. To another 2 mL centrifuge tube 0.25 mL of the coal macromolecule solution and 0.25 mL of 400 mM potassium phosphate, pH 7.0, was added to give a final concentration of 1.12 mg coal macromolecule/mL 200 mM potassium phosphate. The solution was rapidly mixed and a 20 µL aliquot was immediately analyzed by GPC-HPLC. Aliquots (20 µL) were also analyzed after 0.5, 1.0, 1.5, 2.0, 2.5 and 19.45 h. The GPC-HPLC elution solvent was 200 mM potassium phosphate, pH 7.0. The flow rate was 0.5 mL/min and elution of coal macromolecule was monitored at 350 nm. Separation was achieved using a Synchropak GPC-100 column (250 mm x 4.6 mm).

This material had a peak retention time of 5.025 min which was very near the peak retention time for carbonic anhydrase (MW 29,000). The chromatogram of an identical freshly prepare solution of oxalate solubilized coal macromolecule in 200 mM

potassium phosphate, pH 7.0, is also presented in Figure 14. However, this freshly prepared solution had a peak retention time of 4.60 to 4.65 min. Furthermore, the peak retention time decreased with time such that after 19.45 h a peak retention time of 4.45 to 4.50 min was observed. This retention time is similar to that of horseradish peroxidase, a calibration protein that has a retention time of 4.375 min, corresponding to a molecular weight of ~45,000.

A problem with GPC-HPLC is that minor variations in peak retention times reflect rather large variations in molecular weight. Thus, in order to better support our conclusion that the average molecular weight of the coal macromolecule mixture is increasing with time, we also examined closely, the appearance of high molecular weight coal macromolecule. This data is presented in Figure 15, using the same data presented in Figure 14, but on a scale that more clearly illustrates the formation of very high molecular weight coal macromolecule as a function of time.

The effect of incubation time on the appearance of high molecular weight coal macromolecule aggregations that have a retention time identical to Thyroglobulin (MW 669,000, RT= 3.4 min) is also illustrated (Figure 16). The effect of incubating coal macromolecule with water only did not result in formation of higher molecular weight coal aggregates. Indeed, prolonged incubations resulted in formation of lower molecular weight aggregations.

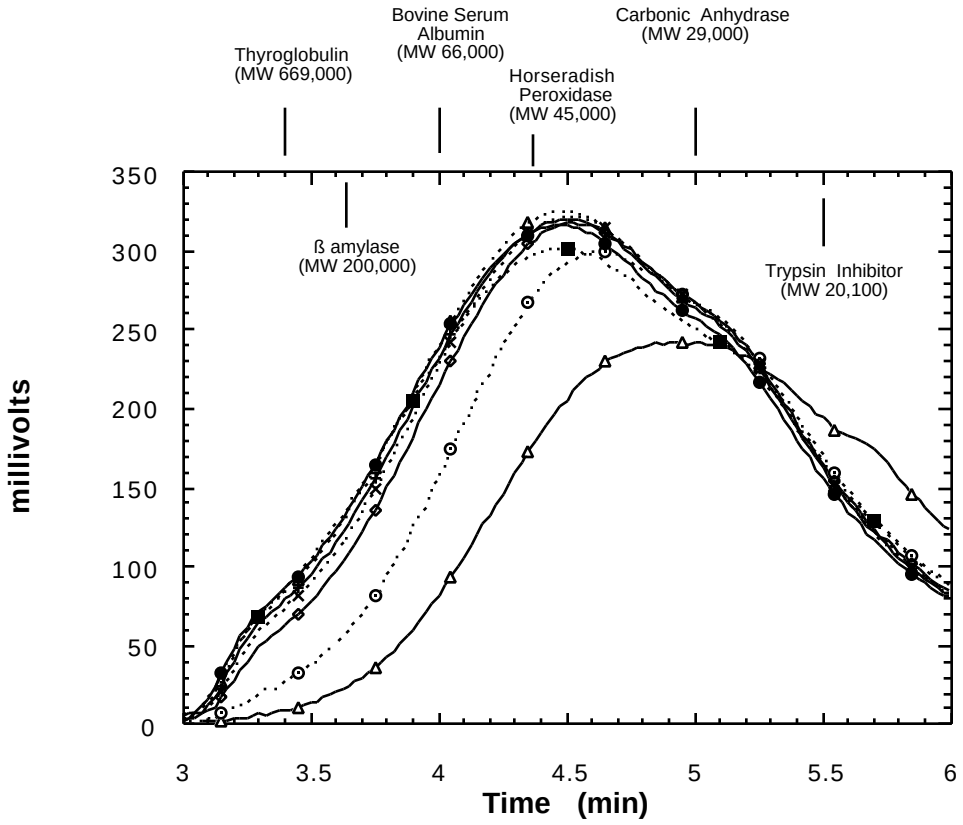



Figure 15. Effect of phosphate buffer on the GPC-HPLC elution profile of oxalate solubilized coal macromolecule. The same data presented in Figure 1 is reillustrated to show more clearly the time dependent increase in high molecular weight material that occurs. The peak elution times for several proteins that are used as molecular weight standards are presented for comparison. The symbols used to designate times of injection are also the same as those used in Figure 14.

Taken together, these results show clearly that the composition of the aqueous solvent in which coal macromolecules are dissolved will affect the magnitude of the apparent molecular weights that are determined. In the case of oxalate solubilized coal macromolecule, incubation with 200 mM potassium phosphate, pH 7.0 resulted in the time dependent formation of higher molecular weight aggregates.

In addition to the oxalate solubilized coal macromolecule used in the above described experiments, we have also isolated another soluble fraction during the oxalate mediated solubilization of leonardite. This fraction was not retained during dialysis using a membrane with a 14,000 MWCO. It was, however, retained during dialysis

using a membrane having a 3,000 MWCO. Thus, the apparent molecular weight of this fraction was estimated to be between 3,000 and 14,000.

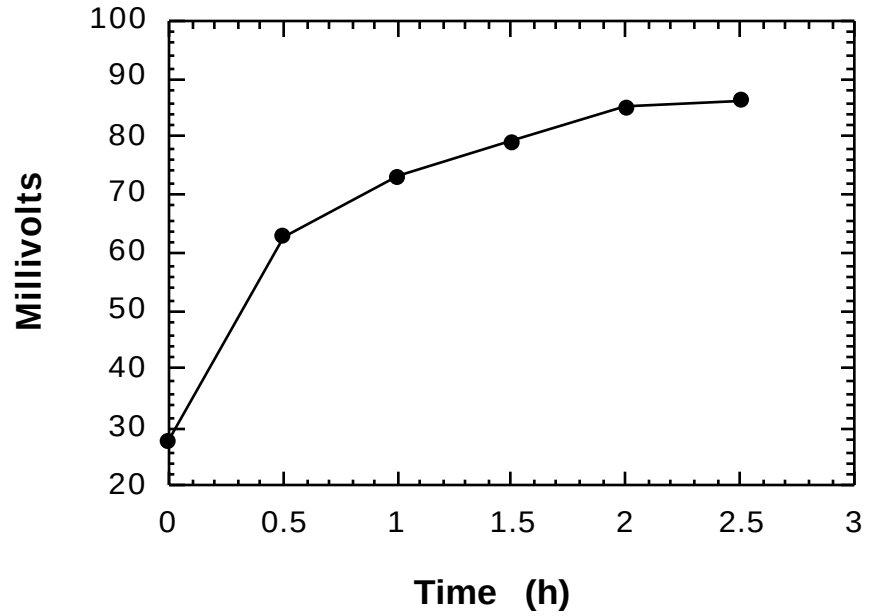



Figure 16. Time dependent increase of high molecular weight material (retention time = 3.4 m) formed following addition of potassium phosphate to an aqueous solution of soluble coal macromolecule. The same data presented in Figure 14 is reillustrated to show the time dependent increase in high molecular weigh material that occurs at a single retention time. The retention time of 3.4 m is the same as that of thyroglobulin, a high molecular weight (MW 669,000) protein standard.

The GPC-HPLC chromatogram of this material is illustrated in Figure 17 (open triangles, solid line) and was fairly consistent for material of this molecular weight range.

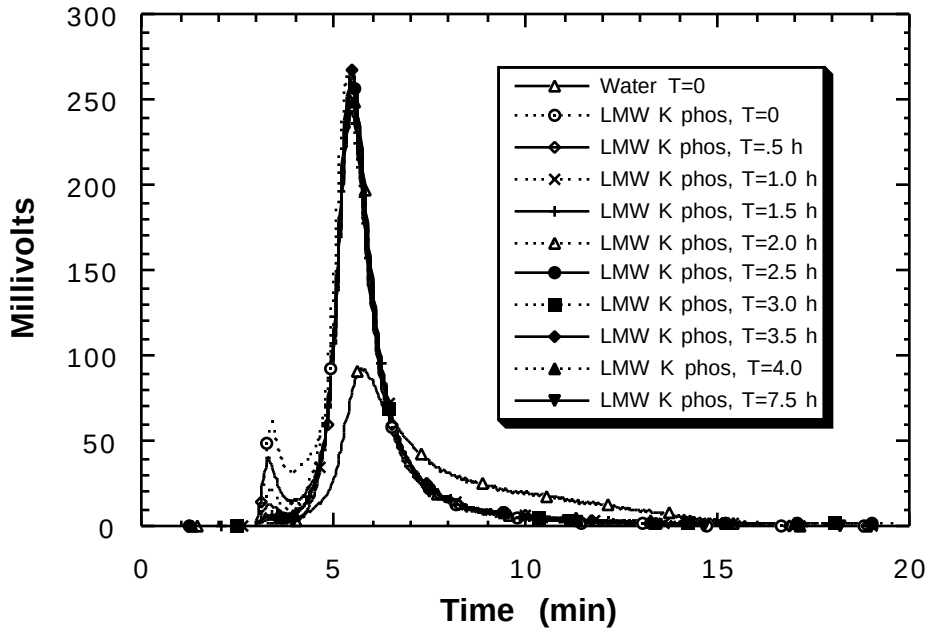



Figure 17. Effect of phosphate buffer on the GPC-HPLC Elution profile of oxalate solubilized low molecular weight coal macromolecule (LMWCM). LMWCM (5.6 mg) was dissolved in 2.5 mL of water. To a 2 mL centrifuge tube was added 0.25 mL of this LMWCM solution and 0.25 mL of water. The solution (1.12 mg LMWCM/mL water) was rapidly mixed and a 20 µL aliquot was immediately analyzed by GPC-HPLC. To another 2 mL centrifuge tube 0.25 mL of the LMWCM solution and 0.25 mL of 400 mM potassium phosphate, pH 7.0, were added to give a final concentration of 1.12 mg LMWCM/mL of 200 mM potassium phosphate. The solution was rapidly mixed and a 20 µL aliquot was immediately analyzed by GPC-HPLC. Aliquots (20 µL) were also analyzed after 0.5, 1.0, 1.5, 2.0, 2.5 , 3.0, 3.5, 4.0 and 7.5 h. The flow rate was 0.5 mL/min and elution of coal macromolecule was monitored at 350 nm. Separation was achieved using a Synchropak GPC-100 column (250 mm x 4.6 mm).

The peak retention time of this material was 5.8 min and was accompanied by substantial tailing of the peak. When dissolved in 200 mM potassium phosphate, pH 7.0, two peaks were observed. The major peak was relatively symmetric and had a peak retention time of 5.425 min . A smaller peak was also observed that had a peak retention time of 3.375 min, similar to the retention time for the thyroglobulin standard. Of interest was the observation that this peak slowly disappeared over the course of several hours. Figure 18 is a reillustration of the high molecule weight region of the

chromatogram presented in Figure 17. And Figure 19 illustrates the disappearance of the absorbance of this peak at a retention time of 3.4 min as a function of time.

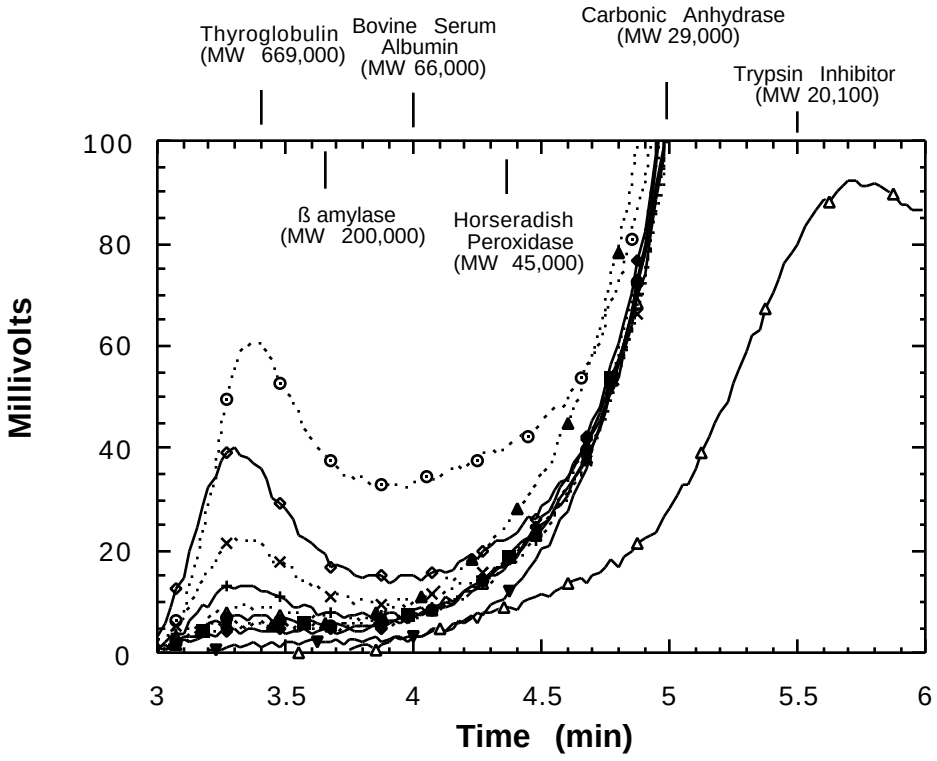



Figure 18. Effect of phosphate buffer on the GPC-HPLC elution profile of oxalate solubilized LMWCM. The same data presented in Figure 17 is reillustrated to show more clearly the time dependent decrease in high molecular weight material that occurs. The peak elution times for several proteins that are used as molecular weight standards are presented for comparison. The symbols used to designate times of injection are also the same as those used in Figure 17.

The use of GPC-HPLC to estimate rapidly the molecular weight of soluble coal macromolecules will continue to be a valuable technique in studies directed toward achieving a better understanding of the molecular architecture of soluble coal and in studies focusing on how coal and soluble coal derived macromolecules are attacked by microorganisms and enzymes. Polman and Quigley (1991) have shown that the molecular weight of coal macromolecules may be conveniently estimated via GPC-HPLC using a SynChropak GPC-300 column. These authors also demonstrated that a

variety of medium components and fungal metabolites did not affect the elution behavior of soluble coal macromolecule on the column. In contrast they did show that sodium doedecyl sulfate increased the apparent molecular weight by ~50% and that if protein was present during coal precipitation (a pretreatment step) it would become bound to the coal macromolecule. They also showed that acid precipitation and resolubilization increased the apparent molecular weight by ~25%, but this effect disappeared with time. In our investigations we have discovered several other factors

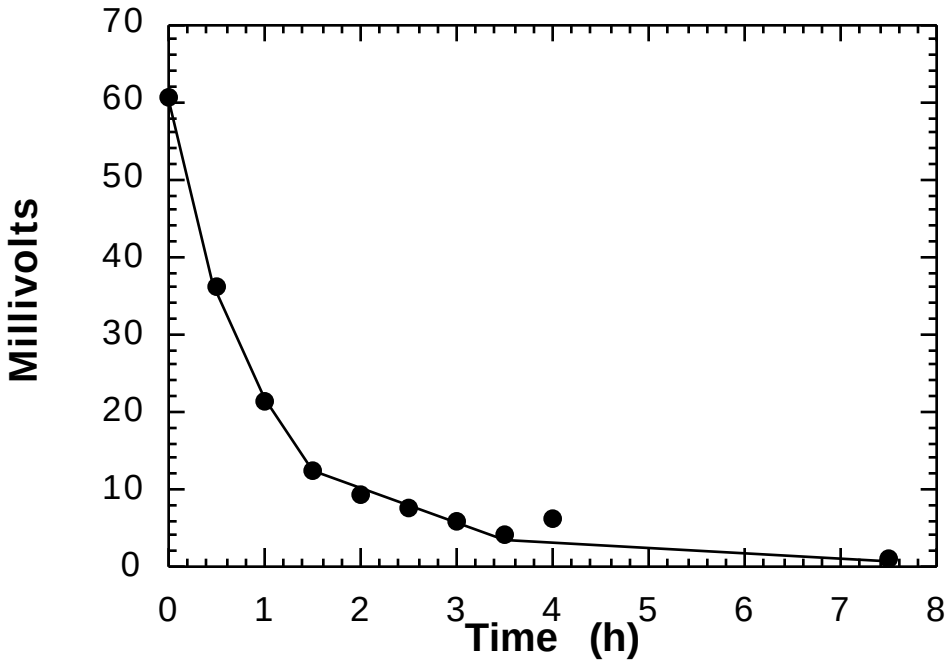



Figure 19. Time dependent decrease of high molecular weight material (retention time = 3.4 m) formed following addition of potassium phosphate to an aqueous solution of LMWCM. The same data presented in Figure 4 is reillustrated to show the time dependent decrease in high molecular weigh material that occurs at a single retention time. The retention time of 3.4 min that was used is the same as that of thyroglobulin, a high molecular weight (MW 669,000) protein standard.

that must be taken into consideration when estimating the molecular weight of soluble coal macromolecules. Specifically, we have shown that the nature of the aqueous solution in which the coal macromolecule is dissolved can cause time dependent

changes in the observed molecular weights. Furthermore, we showed that the nature of the soluble coal derived material is important in this process. The high molecular weight oxalate solubilized coal macromolecule increased in apparent molecular weight in a time dependent manner when the water in which it was dissolved was adjusted to 200 mM potassium phosphate pH 7.0. In contrast, when the low molecular weight fraction was dissolved in this buffer a new high molecular weight peak rapidly appeared and then slowly disappeared. These factors are important when assessing biodegradation of soluble coal macromolecules. Later in this report we note that nitrogen limited cultures of *Phanerochaete chrysosporium* were able to mediate substantial decolorization of soluble coal macromolecule. However, no distinct depolymerization products (*i.e.,* monomers) were observed during GPC-HPLC of extracellular fluid of incubated cultures that contained soluble coal macromolecule. Furthermore, the differences in the chromatograms of extracellular fluid of incubated cultures and controls appeared to reflect mainly the fact that coal macromolecule was metabolized during the incubation period. We also noted that samples of coal macromolecule that had been incubated with *P. chrysosporium* possessed slightly smaller molecular weights than controls. This may be correct. However, our recent investigations suggest that it is probably not correct to envision soluble coal macromolecule as a mixture of noninteracting species. In our view, some coal macromolecules must form noncovalent complexes whose intermolecular attractions are sufficiently strong that they are not separated during GPC-HPLC. Furthermore, our data suggests that even when these complexes undergo enzymatic modification, the resultant products do not necessarily undergo dissociation and still elute with retention times similar to or the same as the nonoxidized material. This interpretation is completely consistent with the results of our coal macromolecule biodegradation experiments that were described above.

### iv. Solubilization of Oxidized and Nonoxidized Argonne Premium Coals by Sodium Oxalate.

Because leonardite is solubilized by oxalate ion, it was logical to ask if other coals are solubilized by this natural chelating agent. The effect of oxalate on the solubilization of several oxidized and nonoxidized Argonne Premium Coals is presented in Figure 20. Results show that, relative to leonardite, only very small amounts of these coals are solubilized by oxalate ion.

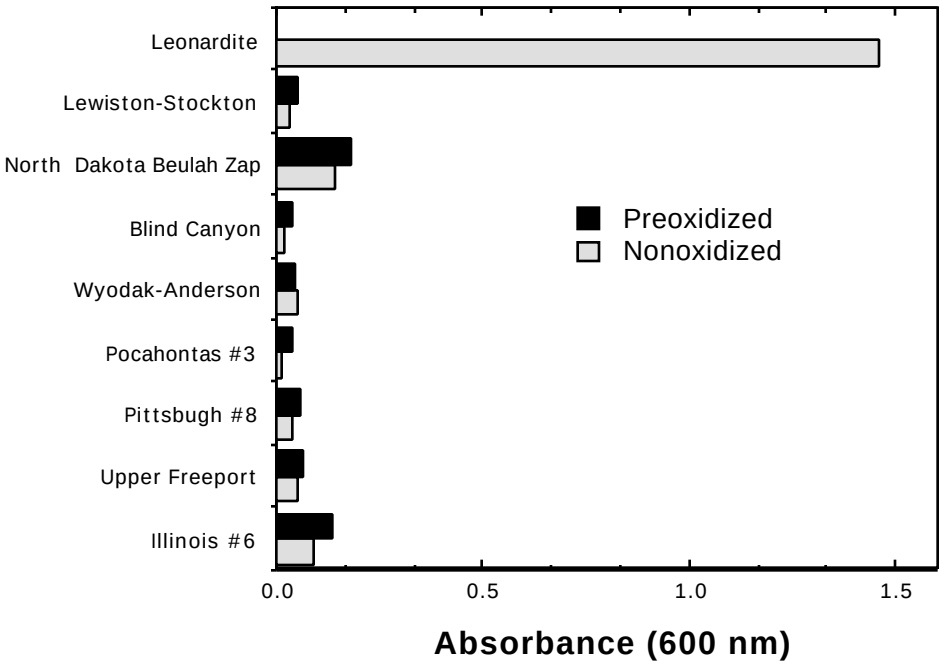
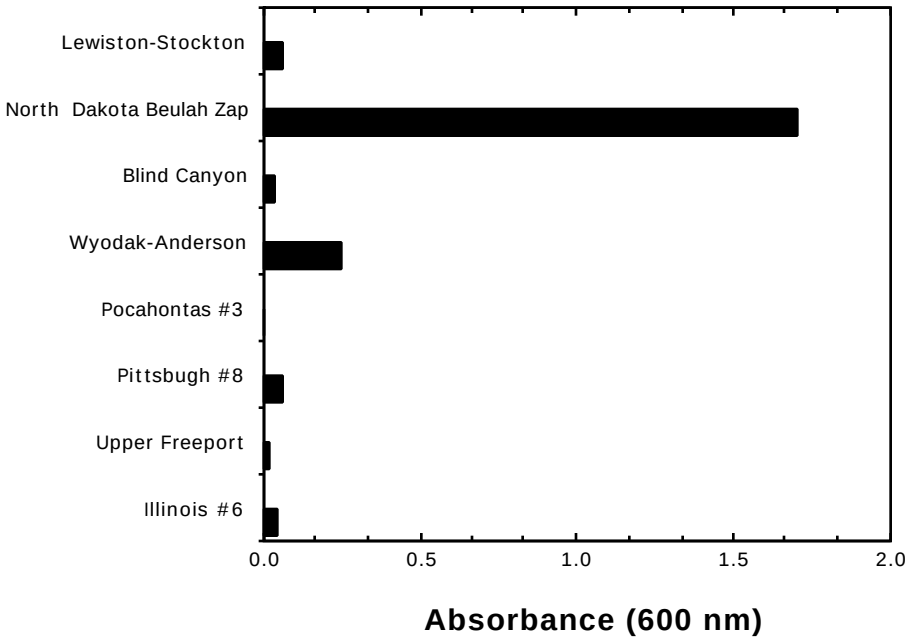



Figure 20. Solubilization of Argonne Premium Coals and leonardite by 75 mM sodium oxalate. For each coal, a 10 mL suspension containing twenty mg of each coal and 10 mL of 74.6 mM sodium oxalate was prepared and mixed for 24 h. An aliquot was then centrifuged and the absorbance of the sample at 600 nm was determined.

These results also suggest that only the most highly oxidized coals are susceptible to substantial oxalate mediated solubilization.

Studies of oxalate solubilization of coal can be used as an investigative tool to partially assess the mechanism by which coals are solubilized by fungi. As a case in point, Stewart and associates (1990) showed that substantial solubilization of Pittsburgh #8 bituminous coal by *Penicillium sp.* and *Cunninghamella sp.* occurred only after preoxidation of the coal by exposure to heat (150$^{o}$C) for seven days. Of interest is the fact that neither *P. chrysosporium* nor *T. versicolor* solubilized this material. Of further interest is the fact that our studies show that oxalate solubilization of preoxidized Pittsburgh #8 bituminous coal is, at best, minimal. Taken together these studies indicate that certain fungi must possess a mechanism for coal solubilization that is not based on the action of oxalate ion.

The ability of sodium oxalate to solubilize preoxidized Argonne Premium Coals was further studied. Because of the dependence of solubilization on pH, solubilization studies were performed using pH adjusted (pH ~7) aqueous solutions of these coals containing sodium oxalate as illustrated in Figure 21. The GPC-HPLC of material solubilized from representative Argonne Premium Coals by aqueous solutions of sodium oxalate is also illustrated in Figure 21. Of the eight Argonne Premium Coals, only the Beulah-Zap North Dakota Lignite was extensively solubilized by this process.



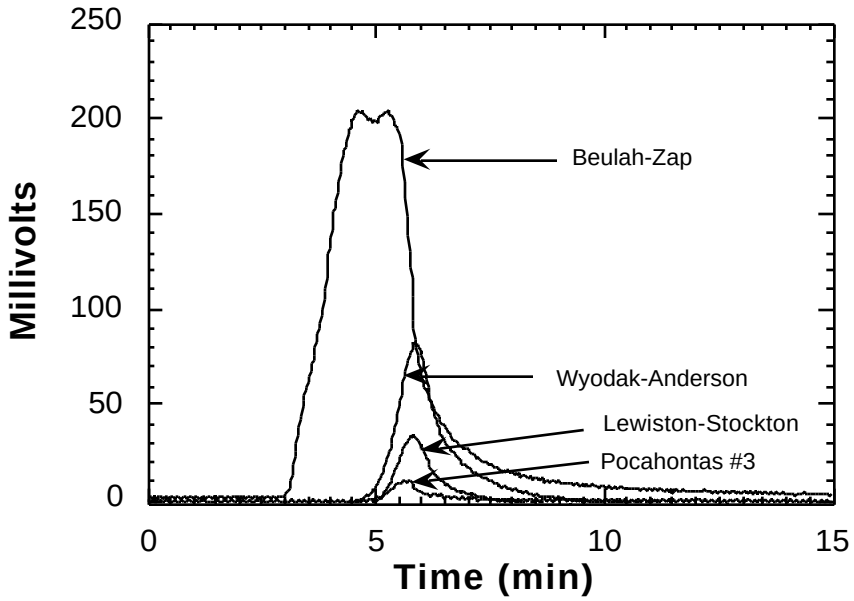




Figure 21. Absorbance at 600 nm (top) and GPC-HPLC elution profiles (bottom) of material solubilized from Argonne Premium Coals by an aqueous solution of sodium oxalate. Soluble material from the Argonne Premium Coals which were not illustrated in GPC elution profile had elution profiles very similar to that of soluble material from Pocahontas #3. In these experiments, 50 mg of dry coal was incubated with 10 mL of 75 mM sodium oxalate, pH 7.0 on a rotary shaker (200 rpm) for 48 h. Samples were then centrifuged and absorbance at 600 nm was determined and 20 µL aliquots were assayed by GPC-HPLC. The material solubilized from the Beulah-Zap North Dakota lignite was diluted 4:1 prior to analysis. Absorbance was monitored at 350 nm.

It should be noted that in Figure 21 the solubilized material obtained from the Beulah-Zap North Dakota Lignite was diluted 4:1 prior to analysis whereas the water soluble material from the other coals was not diluted.

### v. Solubilization of Preoxidized and Nonoxidized Argonne Premium Coals by Sodium Hydroxide and by Two Wood Rotting Fungi.

In general, Argonne Premium Coals were relatively resistant to base mediated solubilization. However, when these coals were preoxidized (150$^{o}$C for seven days),

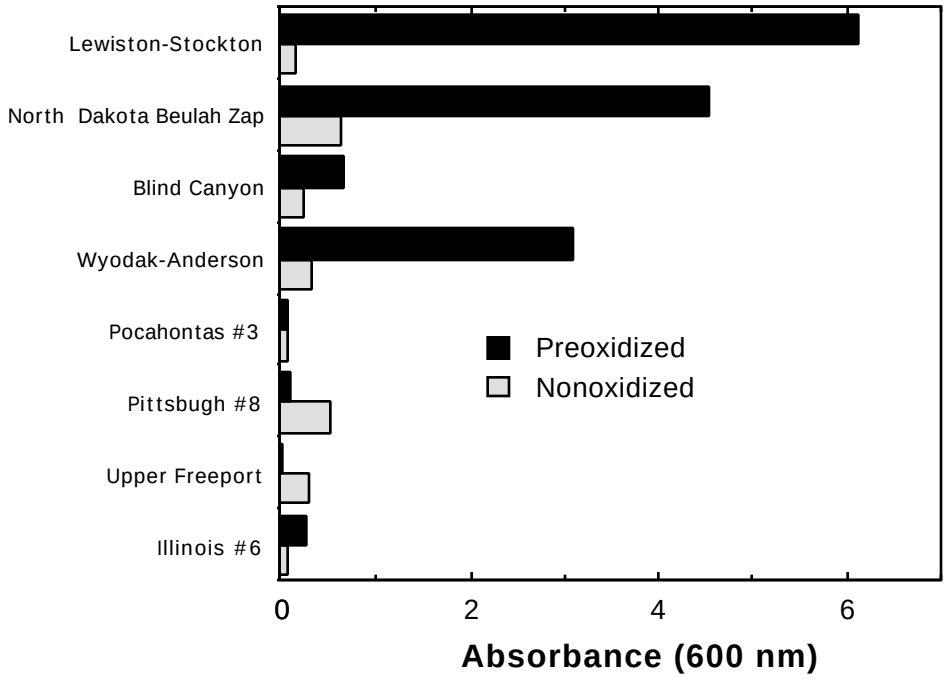



Figure 22. Solubilization of Argonne Premium Coals by Sodium hydroxide (pH 12). For each coal, a 20 mL suspension of 1 M sodium hydroxide and 20 mg of each coal was prepared and mixed for 24 h. An aliquot was then centrifuged and the absorbance of the sample at 600 nm was determined.

substantial amounts of several coals were solubilized. Most affected were the Lewiston-Stockton bituminous coal, the Beulah-Zap Lignite and the Wyodak-Anderson Subbituminous coal. Argonne Premium Coals that were not preoxidized were also resistant to solubilization by *P. chrysosporium* and *T. versicolor.* However, these fungi were able to solubilize substantially, preoxidized Lewiston-Stockton bituminous coal, Beulah-Zap lignite and Wyodak-Anderson subbituminous coal. Figure 20 shows that relative to leonardite, Argonne Premium Coals and preoxidized Argonne Premium Coals were resistant to sodium oxalate mediated solubilization. However, preoxidation did appear to have a marginally beneficial effect on oxalate mediated solubilization. Of the preoxidized Argonne Premium Coals tested the one of lowest rank, the Beulah-Zap lignite was most affected by preoxidation and most amenable to oxalate mediated solubilization.

Our studies dealing with coal solubilization by fungi are of most interest. That the Beulah-Zap lignite was solubilized can be explained by the fact that of all the Argonne Premium Coals, this coal was the most amenable to oxalate mediated solubilization. The fact that the Lewiston-Stockton bituminous coal and the Wyodak-Anderson subbituminous coal were solubilized by *P. chrysosporium* and *T. versicolor* is important because these coals are only marginally amenable to oxalate mediated solubilization and these results suggest that other factors, possible degradative enzymes, are involved in solubilization of these higher rank coals. There are only a few studies dealing with the solubilization of subbituminous and bituminous coals by wood rotting fungi. Achi (1993) showed that several members of the Basidiomycetes were able to mediate solubilization of a preoxidized Nigerian subbituminous coal and Scott *et al.* (1986) reported that *T. versicolor* solubilized trace amounts of a Wyodak subbituminous coal. Stewart *et al.* (1990) reported that *P. chrysosporium* was able to mediate solubilization of trace amounts of Pennsylvania Upper Freeport Bituminous

Coal, but did not solubilize a similar sample that had been preoxidized. Our observation that *P. chrysosporium* and *T. versicolor* solubilized substantially, a bituminous coal (preoxidized Lewiston-Stockton bituminous coal) is a relatively uncommon observation for a member of the Basidiomycetes. It should be noted, however, that certain Ascomycetes (*Penicillium sp*. and *Cunninghamella sp*.) have been reported to solubilize three bituminous coals (Illinois # 6, Pittsburgh # 8 and Pennsylvania Upper Freeport bituminous coals) (Stewart *et al*., 1990). Taken together, these reports and our observations suggest that oxalate mediated solubilization is important only for the initial solubilization of highly oxidized low rank coals. For subbituminous and bituminous coals that are amenable to fungal solubilization it appears that some process other that metal chelation and concomitant cleavage of ionic linkages is required as an initial event in solubilization of such coals. Although oxalate is not responsible for substantial initial solubilization of subbituminous and bituminous coals, it is possible that oxalate may still have a role in aiding or increasing solubilization of such coals.

**vi. Effect of laccase on Coal Solubilization.**

It has been suggested that the laccase from *Trametes versicolor* may have a role in solubilization of low rank coal (Cohen *et al.*, 1987; Pyne *et al.* 1987)). However, a more recent assessment suggests that laccase has a limited role in solubilization of leonardite (Quigley, 1993). Because of conflicting data in the literature, we assessed the ability of the laccase from *T. versicolor* to solubilize leonardite. In these experiments, 20 mg of leonardite was added to a 20 mL scintillation vial containing 1.8 mL of 100 mM potassium phosphate buffer, pH 6.0 and 200 µL of a laccase preparation. The reaction mixture was mixed gently and allowed to incubate at room temperature for 48 h. The initial laccase activity of this preparation was approximately

four times that which would be present in induced cultures of *T. versicolor*. During this incubation, no coal solubilization occurred as evidenced by the fact that an increase of absorbance at 600 nm did not occur in treated samples.

### vii. Solubilization of A Nitric Acid Oxidized North Dakota Lignite Using Sodium Oxalate.

Wondrack *et al.* (1989) prepared for their investigations a soluble fraction (III-B) from a nitric acid oxidized North Dakota Lignite using a procedure that involves solubilization with a strong base. We showed that our procedures using sodium oxalate is also effective in solubilizing this material. One gram of sodium oxalate was added to one gram of nitric acid oxidized North Dakota Lignite in 100 mL of water and stirred. The pH of this material was approximately pH 4.6. Visual examination of this mixture suggested that little or no solubilization was taking place. Sodium hydroxide (1 M) was then added dropwise to the solution until a pH of 6.8 was achieved. The solution then turned dark brown and was stirred overnight. Following centrifugation to remove particulate material, the solution was dialyzed extensively against water and stored. These results are of interest because they demonstrate that like the leonardite used in most of our studies, the nitric acid oxidized North Dakota Lignite used by Wondrack *et al*. (1989) can also be solubilized using less caustic procedures.

## B. Biodegradation of solubilized macromolecule from low rank coal.

### i. Depolymerization of Biomimetically Solubilized Coal by *P. chrysosporium.*

*P. chrysosporium* was shown to be able to decolorize extensively coal macromolecule

that had been solubilized biomimetically (*i.e.,* using sodium oxalate) from low rank coal. Of interest is the fact that decolorization occurred in nutrient nitrogen limited but not in nutrient nitrogen sufficient malt agar cultures. This is important because the lignin degrading system of *P. chrysosporium* is expressed in nutrient limited cultures and is suppressed in nutrient nitrogen sufficient cultures (Kirk *et al.* 1978). Thus, these experiments strongly suggest that the lignin degrading system is involved in this process.

Substantial decolorization of solubilized low rank coal macromolecule also occurred in nutrient nitrogen limited liquid cultures of *P. chrysosporium*. In stationary and agitated cultures (Initial concentration of soluble coal macromolecule = 290 mg/L) 83.8 $\pm$ 2.3% and 89.6 $\pm$ 1.0% decolorization, respectively, was observed after 8 days of incubation.

In similar experiments in which incubated samples (initial concentration of soluble coal macromolecule = ~1000 mg/L) were incubated for 12 days, 46.2 $\pm$ 3.0 % (N=9) decolorization at 600 nm was observed. The UV-visible spectrum of a representative sample and control is presented in Figure 23. Figure 24 is a replot of the spectral data in which the absorbance of incubated samples is expressed as a percentage of the absorbance of nonincubated controls. These results show the degree of decolorization is a function of the wavelength at which decolorization is monitored. They also show that loss of absorbance occurs in the ultraviolet range indicating that aromatic rings as well as chromophoric substructures are attacked. The apparent resistance to decolorization of chromophoric material at 800 nm is an artifact due to the fact that negligible absorbance by coal macromolecule occurs at this wavelength.

When these samples were analyzed by GPC-HPLC (Figure 25), it was shown that although a substantial decrease in absorbance had occurred, a very small decrease in

the molecular weight had occurred and no low molecular weight material that could be properly termed monomers could be identified.

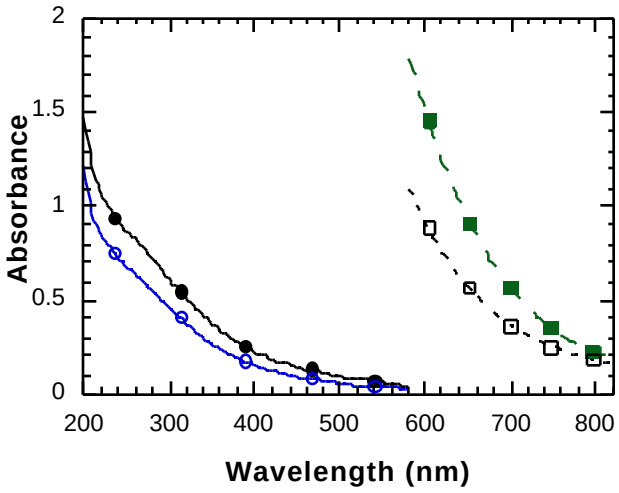



Figure 23. Decolorization of soluble coal macromolecule by a ligninolytic culture of *P. chrysosporium*. The UV-visible spectrum (open symbols) of the extracellular fluid of a ligninolytic culture of *P. chrysosporium* that had been incubated with oxalate solubilized coal macromolecule for twelve days was acquired. This spectrum was then compared with a similar spectrum (closed symbols) of a nonincubated sample that was removed immediately following addition to the culture. The absorption spectra from 200 to 580 nm were acquired using a sample that had been diluted 40:1 with water. The absorption spectra from 580 nm to 820 nm were acquired using the undiluted sample.

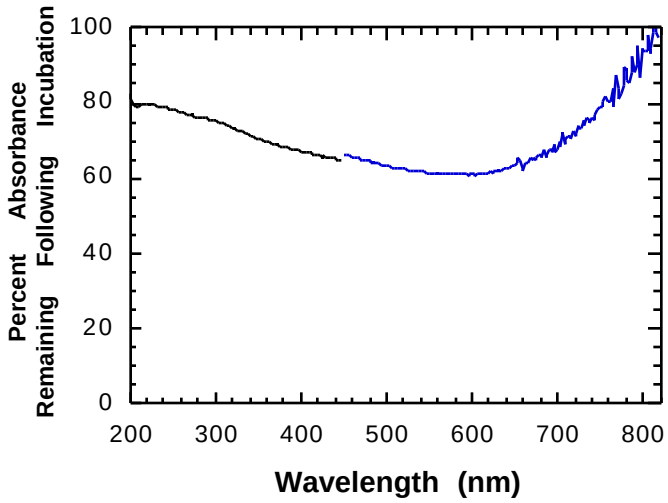



Figure 24. Decolorization of soluble coal macromolecule by a ligninolytic culture of P.*chrysosporium* expressed as a percentage. Absorbance data were acquired on the same sample used to generate Figure 23 and expressed as the percent of absorbance due to coal macromolecule remaining after incubation with *P. chrysosporium* for twelve days. The data from 200 to 450 nm was acquired using samples that had been diluted 40:1 whereas the data from 450 nm to 820 nm was acquired using samples that had been diluted 4:1

In summary, this set of experiments demonstrates that biomimetically solubilized coal macromolecule is degraded by *P. chrysosporium* and the lignin degrading system of this fungus is involved in the biodegradation process. A major goal of our research is to determine if coal depolymerization occurs in this system. Although we observed an increase in the peak retention time of coal macromolecule incubated with *P. chrysosporium*, indicative of a decrease in molecular weight, we did not observe the appearance of distinct fragments indicative of low molecular weight substructures. This could be due to the possibility that low molecular weight material does not accumulate because it is readily metabolized by the fungus. That low molecular weight metabolites self associate (or remain associated) to form higher molecular weight aggregates is another possibility.

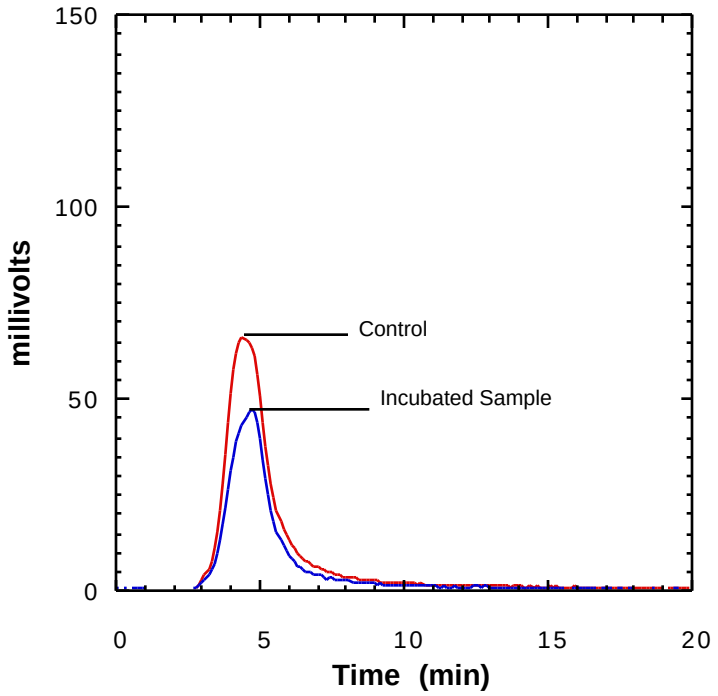



Figure 25. Effect of incubation of soluble coal macromolecule with a ligninolytic culture of *P. chrysosporium* for twelve days. Incubated samples and nonincubated controls were assayed by GPC-HPLC.

### ii. Oxidation by Lignin Peroxidase of Biomimetically and Base Solubilized Coal Macromolecule .

Lignin peroxidases are involved in the initial oxidative depolymerization of lignin. Given the fact that coal macromolecule is, in part, derived from lignin it is reasonable to suspect that coal macromolecule might also be oxidized by lignin peroxidases. However, as noted elsewhere in this report, it should be appreciated that there are important differences as well as similarities between lignin and soluble coal macromolecule.

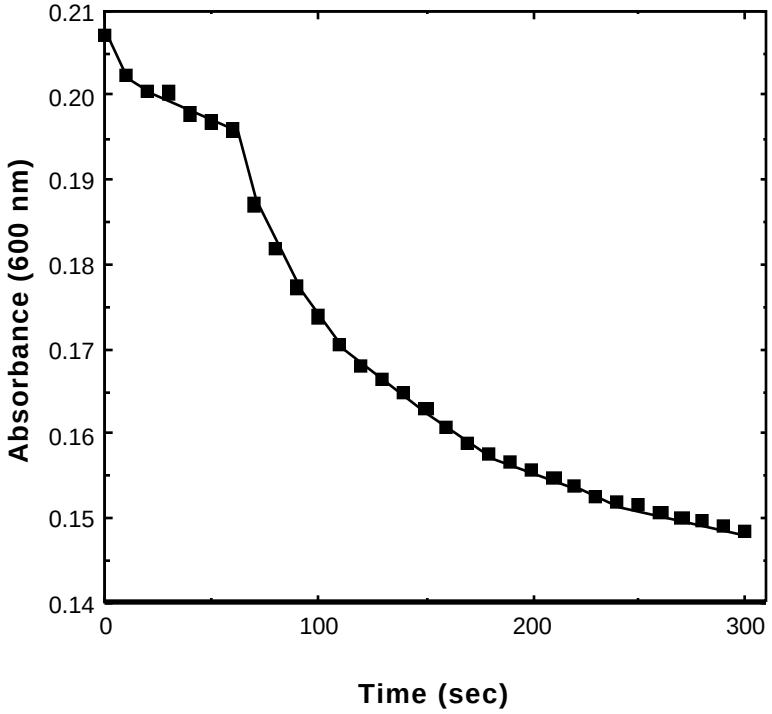



Figure 26 Decolorization of biomimetically solubilized coal macromolecule by lignin peroxidase H8 from *Phanerochaete chrysosporium* . The reaction mixture contained 116 nM lignin peroxidase H8, coal macromolecule , 1.5 mM veratryl alcohol and 1.0 mM hydrogen peroxide. All components of the reaction mixture except for coal macromolecule were dissolved in 220 mM sodium tartrate buffer, pH 4.5. Coal macromolecule was added in 50 µL of water. The total reaction volume was 1.05 mL. Reactions were initiated by the addition of lignin peroxidase and monitored at 600 nm at room temperature. In control incubations in which hydrogen peroxide and/or enzyme was omitted no decolorization was observed.

Figure 26. shows that biomimetically solubilized coal macromolecule can be decolorized by lignin peroxidases. However, it should be noted that decolorization is not very extensive and, in addition to hydrogen peroxide, depends on the presence of veratryl alcohol, a natural substrate for lignin peroxidase that is secreted by *P. chrysosporium*.

Figure 27 shows that biomimetically solubilized coal macromolecule is also a good inhibitor of lignin peroxidase mediated veratryl alcohol oxidase activity. Wondrack et al. (1989) reported that base solubilized coal macromolecule was a competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidation. Initial attempts to determine if biomimetically solubilized coal macromolecule was also a competitive inhibitor of lignin peroxidase revealed that in the presence of this material a concentration dependent lag period occurred (Figure 27). Interestingly, such a lag also

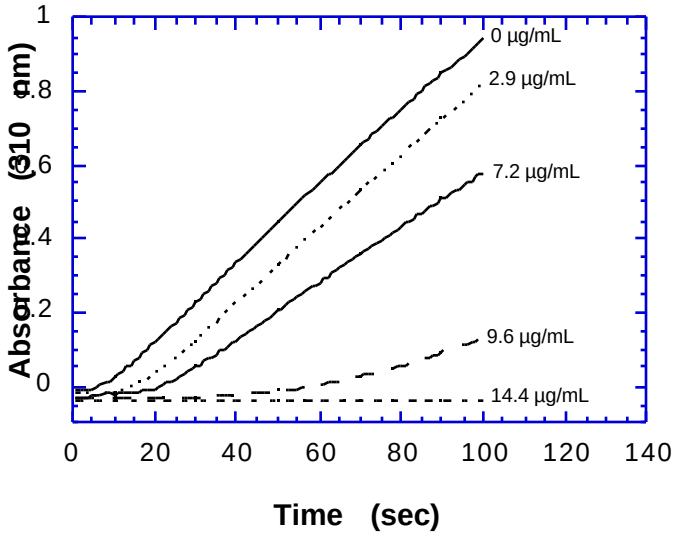



Figure 27. Effect of increasing concentration of biomimetically solubilized coal macromolecule on lignin peroxidase activity. The reaction took place in a volume of 1 ml in 220 mM sodium tartrate buffer, pH 4.5, containing 1.67 mM veratryl alcohol, 8.8 µg lignin peroxidase and 0.5 mM hydrogen peroxide. The concentration of oxalate solubilized coal macromolecule present varied from 0 to 14.4 µg/mL.

occurred when fraction B-III, the base solubilized material studied by Wondrack *et al.*

was substituted in this reaction mixture. Subsequent experiments revealed that the lag period was observed only at relatively low concentrations of lignin peroxidase and soluble coal macromolecule. When the concentrations of both lignin peroxidase and soluble coal macromolecule were increased at the same ratio, the lag period disappeared (Figure 28).

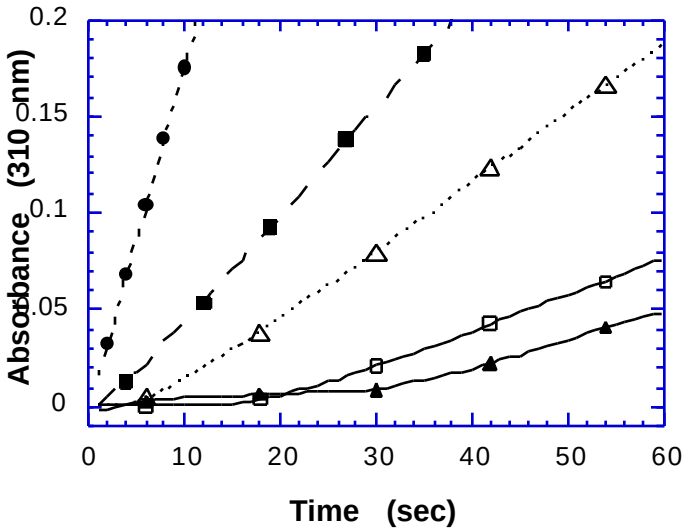



Figure 28. Effect on enzyme activity of increasing the amount of soluble coal macromolecule and lignin peroxidase at a constant ratio. Solid triangles-50 µg soluble coal macromolecule and 0.88 µg lignin peroxidase; Open squares-200 µg soluble coal macromolecules, 3.53 µg lignin peroxidase; open triangles-250 µg soluble coal macromolecule,4.4 µg lignin peroxidase; solid squares-350 µg soluble coal macromolecule, 6.16 µg, lignin peroxidase; Solid circles-0 ug soluble coal macromolecule, 0.88 µg lignin peroxidase. The reaction took place in a volume of 1 ml in 220 mM sodium tartrate buffer, pH 3.5, containing 1.67 mM veratryl alcohol and 0.5 mM hydrogen peroxide. For this experiment, base solubilized coal macromolecule was used.

The relationship describing the initial velocity of an enzyme reaction in the presence of an inhibitor is as follows:

$$v_o = \frac{[V_{max}][S]}{K_m[1 + I/K_I] + [S]}$$

**(Equation 1)**

Where:

Vo = initial velocity.

Vmax = maximum velocity.

Km = the Michaelis-Menton Constant for the enzyme.

I= the concentration of the enzyme inhibitor.

$K_I$ = the Inhibitor constant.

This equation predicts that in the presence of a very potent competitive inhibitor (*i.e.,* one with a very small $K_I$) that is also a substrate, the enzyme's affinity for the inhibitor is such that the inhibitor is preferentially attacked and substantial catalysis of the substrate occurs only when the inhibitor concentration reaches very low levels. This phenomenon is illustrated in Figure 29 for a hypothetical enzyme reaction and inhibitors having $K_I$ values of $1 \times 10^{-3}$ mM, $1 \times 10^{-5}$ mM and $1 \times 10^{-7}$ mM. As expected, as the enzyme catalyzes a decrease in inhibitor concentration, the initial velocity of the enzyme reaction increases. However, in the presence of the most potent inhibitors (those having $K_I$ values of $1 \times 10^{-5}$ mM and $1 \times 10^{-7}$ mM) it appears that the substrate is converted to product only after a substantial lag period during which time the inhibitor is reduced to very low concentrations·

This is consistent with the reaction mechanism (Scheme 1) we have proposed for oxidation of soluble coal macromolecule by lignin peroxidase. In this mechanism, hydrogen peroxide first oxidizes the enzyme to the reactive peroxidase intermediate known as Compound I which mediates the one electron oxidation of veratryl alcohol to veratryl alcohol cation radical. Normally, this radical undergoes another one electron oxidation by lignin peroxidase to form veratryl aldehyde, the product whose absorbance at 310 nm is measured in this assay. However, in the presence of soluble coal macromolecule, the veratryl alcohol cation radical functions as a secondary oxidant by oxidizing the soluble coal macromolecule. In this process, the coal

macromolecule is decolorized and the veratryl alcohol radical is converted back to veratryl alcohol.

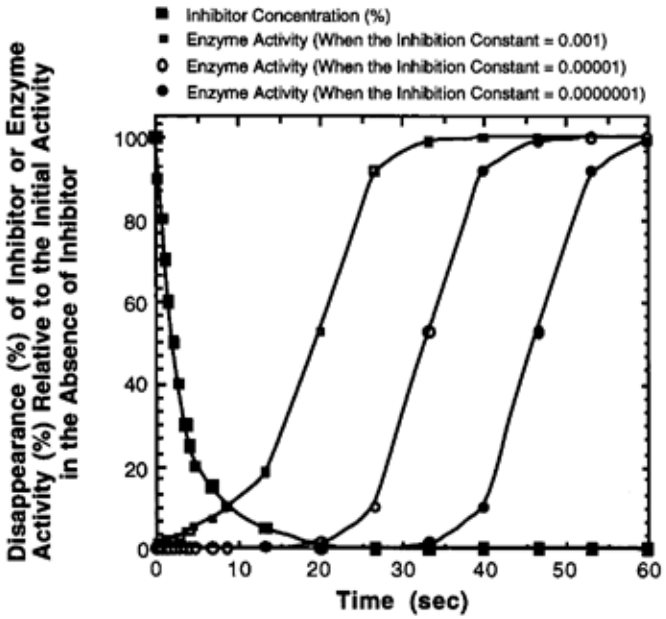



Figure 29. Effect of potent enzyme inhibitors on the initial activity of an enzyme as a function of time. Theoretical enzyme activity data was calculated using equation 1, assuming that the Km of the enzyme for the substrate was 0.089 mM and that the initial concentration of the the substrate was 2.0 mM. The rate equation for a first order reaction was used to calculate enzyme mediated disappearance of the inhibitor. A $t_{1/2}$ of 2 sec and a first order rate constant (k) of 0.35 $sec^{-1}$ was used in these calculations.

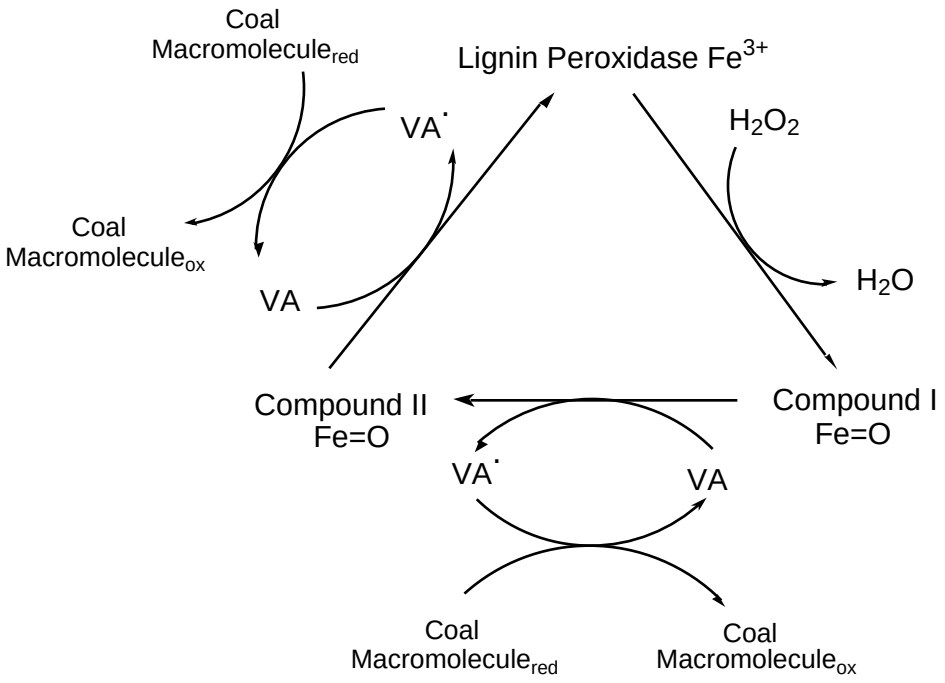
It should be noted that equation 1 predicts that catalysis of substrate does occur, but at very low levels. For example, although the velocities of the reactions occurring in the presence of the inhibitors having $K_I$ values of 1 x $10^{-5}$ mM and 1 x $10^{-7}$ mM appear to be zero in Figure 29, they actually increase from 0 to 1.1% and from 0 to 0.11%, respectively, during the first 20 seconds of the apparent lag periods.

It was, therefore, of interest for us to determine if very low rates of reaction were occurring in incubations containing coal macromolecule, veratryl alcohol and lignin

peroxidase. In most experiments in which coal macromolecule was present, measurable rates during the first part (*i.e*., the first 10 seconds) of the lag periods were not found (Figure 27). However, this too is consistent with and, indeed, predicted, by our proposed reaction mechanism (Scheme 1) in which the veratryl alcohol cation radical is reduced back to veratryl alcohol thus preventing accumulation (and measurement) of veratryl aldehyde, the product of this reaction.

Veratryl aldehyde would be expected to accumulate only when the concentration of oxidizable coal macromolecule becomes limiting. Figure 28 shows that when the concentrations of lignin peroxidase and coal macromolecule are increased at the same ratio, the lag period disappears. This too supports our proposed reaction mechanism because increasing enzyme concentration would be expected to increase the rate of veratryl alcohol cation radical formation which, in turn, would cause an increase in the rate of coal macromolecule oxidation resulting in the disappearance of the lag when higher enzyme concentrations are used.

In addition to oxidizing the veratryl alcohol cation radical back to veratryl alcohol, it appears that at higher concentrations of soluble coal macromolecule some functional groups in the macromolecule may cause it to behave as a reversible competitive inhibitor (*i.e.,* as an inhibitor that reversibly binds the active site but is not oxidized by the enzyme) Indeed, results using Lineweaver-Burke analysis are consistent with the conclusion that coal macromolecule is a competitive inhibitor of veratryl alcohol oxidase activity. Further complicating the kinetics of inhibition is the observation that sufficiently high concentrations of coal macromolecule causes formation of precipitable inactive enzyme-coal macromolecule complexes.




Scheme 1. Proposed reaction mechanism for oxidation of soluble coal macromolecule by lignin peroxidase from *Phanerochaete chrysosporium*.

A major interest of our research centers on the role of fungal oxidative enzymes in the biodegradation of low rank coal. Research to date suggests that such enzymes have little or no role in solubilization of low rank coal. There is, however, some evidence that indicates that soluble coal macromolecules are substrates for lignin peroxidases and that these enzymes may function to depolymerize soluble coal macromolecules (Wondrak *et al.* 1989). We showed that oxalate solubilized coal macromolecule is an excellent competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidation and that this coal macromolecule was probably a good inhibitor because it was a good substrate. However, we did not find any evidence that would suggest that substantial depolymerization of the macromolecule occurred. To further investigate the role of lignin peroxidase in solubilization of low rank coal macromolecule we obtained a sample of the nitric acid oxidized North Dakota Lignite used by Wondrak *et al.* (1989).

This material was a generous gift from Dr. Willis Wood. Soluble coal macromolecule designated as fraction B-III by these authors was prepared as described (Wondrack *et al*. 1989). This material was confirmed to be a good inhibitor of lignin peroxidase. As noted previously in this report, the concentration dependent lag seen using oxalate solubilized coal macromolecule was also observed when B-III was present in reaction mixtures. This is illustrated in Figure 30.

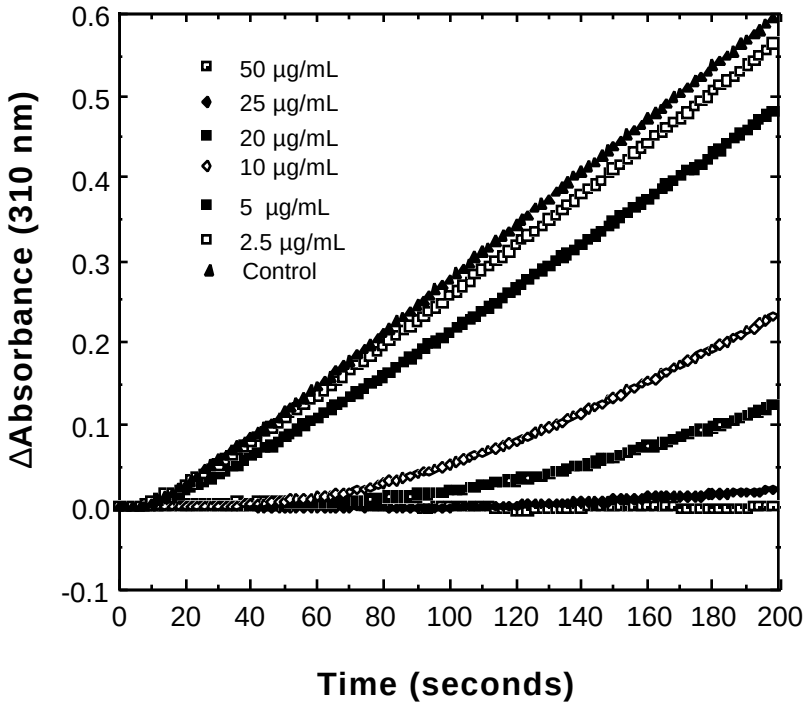



Figure 30. Effect of increasing concentration of coal macromolecule B-III on lignin peroxidase activity. Reaction mixtures contained the indicated amounts of coal macromolecule B-III prepared according to the procedures described by Wondrack *et al.* (1989), 116 nM lignin peroxidase H8, 1.5 mM veratryl alcohol and 0.5 mM hydrogen peroxide in 220 mM sodium tartrate buffer, pH 4.5. Reactions were initiated by the addition of hydrogen peroxide and monitored at 310 nm at room temperature.

The ability of this material to serve as a substrate for lignin peroxidase was assessed used two different enzyme assays. In the first, we showed that lignin peroxidase H8

was able to mediate partial decolorization of this material (Figure 31). In the second assay, depolymerization was monitored by gel permeation high performance liquid chromatography (Data not shown). Although disappearance of the parent material was

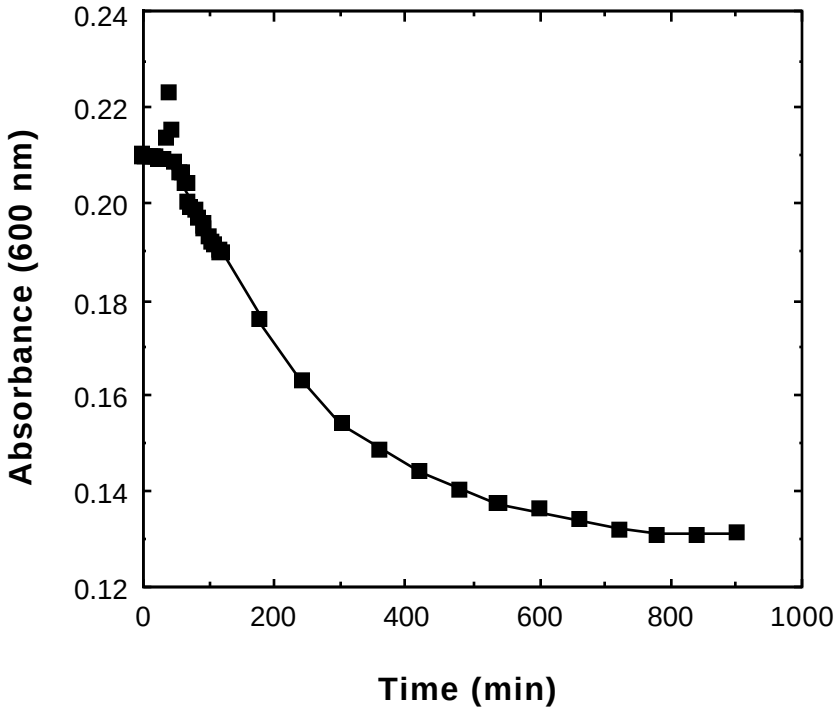



Figure 31. Decolorization of coal macromolecule B-III by lignin peroxidase H8 from *Phanerochaete chrysosporium* . The reaction mixture contained 116 nM lignin peroxidase H8, coal macromolecule B-III, 1.5 mM veratryl alcohol and 1.0 mM hydrogen peroxide. All components of the reaction mixture except for coal macromolecule were dissolved in 220 mM sodium tartrate buffer, pH 4.5. Coal macromolecule was added in 50 µL of water. The total reaction volume was 1.0 mL. Reactions were initiated by the addition of hydrogen peroxide and monitored at 600 nm at room temperature. In control incubations in which hydrogen peroxide and/or enzyme was omitted no decolorization was observed.

observed, we could not detect the formation of lower molecular weight products. Furthermore, we showed that, as with biomimetically solubilized coal macromolecules, lignin peroxidase formed a precipitate with part of the B-III mixture. This is important because such a precipitate might be retained on a GPC column or on filters during GPC-HPLC and could lead one to incorrectly conclude that substantial depolymerization had occurred.

Our results suggest that lignin peroxidases oxidize but do not mediate substantial depolymerization of soluble coal macromolecules. However, the question of depolymerization still remains open as it is possible that lignin peroxidases mediate cleavage of covalent bonds but that the cleavage products remain tightly associated with each other.

### iii. On The Role of Lignin Peroxidase, Mn Peroxidase and Laccase on the Depolymerization of Soluble Coal Macromolecule.

Wood rotting fungi secrete a number of extracellular enzymes that participate in the biodegradation of lignin and other compounds. These enzymes also appear to have a role in the biodegradation of soluble coal macromolecule. We have shown that *in vitro* lignin peroxidases mediate decolorization of coal macromolecule. Interestingly, decolorization was far from complete suggesting that there are many chromophoric groups in this material that are not accessible to oxidation by this enzyme. Of interest is the observation that decolorization was dependent on the presence of veratryl alcohol which we propose is oxidized to veratryl alcohol radical an intermediate that, in turn, oxidizes soluble coal macromolecule.

We have not yet been able to establish a direct role for laccases or Mn peroxidases in coal macromolecule decolorization or depolymerization. However, it is likely that these enzymes are involved. Hofrichter and Fritsche (1997) have shown that the Mn peroxidase from *Nematoloma frowardii b19* was able to mediate substantial decolorization and depolymerization of base solubilized coal macromolecule from a German lignite. Of interest is the fact that decolorization took place over the course of several days. It should also be noted that decolorization and depolymerization is

probably due to the ability of Mn peroxidases to mediate oxidation of $Mn^{2+}$ complexes to $Mn^{3+}$ complexes which, unlike veratryl alcohol radicals, appear to be sufficiently stable enough to diffuse from the active site of the enzyme and mediate oxidation of coal macromolecule at sites that are remote from the active site of the enzyme.

Lastly, it should be noted fungal peroxidases also mediate formation of hydroxyl radical, an active oxygen species that appears to have an important role in lignin degradation and in the biodegradation by white rot fungi of a number of environmentally persist compounds (Barr and Aust, 1994). It is likely that this highly reactive species will also have a role in degradation of soluble coal macromolecule.

In summary, it thus appears that like lignin degradation, degradation of coal macromolecule is a very complex process, involving participation of a variety of hydrogen peroxide generating enzymes and several extracellular oxidases. Furthermore, free radicals and other reactive species generated by these extracellular oxidases appear to have an important role in this process. A complete understanding of this process will require continued scrutiny.

## Conclusions

The following are the major conclusions of this study. Conclusions 1 to 5 address directly our five specific objectives. Conclusions 6 to 8 address other significant findings of this research.

1) Solubilization and depolymerization of coal macromolecule from low rank coal are clearly distinct events in lignin degrading fungi. Fungi secrete oxalate ions which chelate metal ions in low rank coal thus breaking ionic linkages between coal macromolecules. The relatively polar coal macromolecules are then soluble in water. A variety of other metal chelators, including several Krebs Cycle intermediates are able to mediate solubilization of low rank coal. The fact that a variety of fungi that are not noted for their ability to degrade lignin but are able to produce large amounts of oxalate or certain Krebs Cycle intermediates accounts, at least in part, for the fact that nonlignin degrading fungi are also able to mediate substantial solubilization of low rank coal.

Subsequent biodegradation of soluble coal macromolecule by lignin degrading fungi appears to be dependent on oxidation by extracellular oxidases that are secreted. Generation of reactive species such as veratryl alcohol cation radical and $Mn^{3+}$complexes by extracellular oxidases are clearly important for biodegradation of soluble coal macromolecule.

2) Solubilization and subsequent metabolism of coal macromolecule are under nutritional control. Solubilization occurs in nutrient nitrogen sufficient media. Under these conditions, expression of the lignin degrading system of *P. chrysosporium* is suppressed. In nutrient nitrogen limited media the lignin degrading system is

expressed and substantial biodegradation was shown to occur.

3) Lignin peroxidases mediate decolorization of soluble coal macromolecule. The mechanism by which this occurs is summarized below. We have not yet shown a direct role for fungal Mn peroxidases or laccasses in coal macromolecule biodegradation. However, it is likely that they are, indeed, involved in this process as others (Hofrichter and Fritsche, 1997) have presented convincing evidence that Mn peroxidases from *Nematoloma frowardii* are, indeed, involved in depolymerization of base solubilized coal macromolecule from a German lignite.

4) Kinetic evidence suggests that lignin peroxidases in the presence of veratryl alcohol oxidize soluble coal macromolecule by a mechanism in which veratryl alcohol is first oxidized to veratryl alcohol cation ion radical. This radical then oxidizes the coal macromolecule and is, in turn, reduced back to veratryl alcohol. From a purely academic perspective, soluble coal macromolecule is an interesting inhibitor of lignin peroxidase mediated veratryl alcohol oxidase activity as it functions as an inhibitor by three distinct mechanisms: 1) by reducing the veratryl alcohol radical back to veratryl alcohol; 2) by functioning as a competitive inhibitor and; 3) by physically binding to the enzyme thereby causing its precipitation and inactivation.

As noted above, others (Hofrichter and Fritsche, 1997) have shown that Mn peroxidases are also involved in depolymerization of soluble coal macromolecule. In this case, $Mn^{2+}$ complexes are oxidized to $Mn^{3+}$ complexes which are able to mediate oxidation of soluble coal macromolecule.

We have also shown that hydrogen peroxide is able to mediate decolorization of soluble coal macromolecule. However, substantial decolorization only occurs at

hydrogen peroxide concentrations that are nonphysiological.

A thorough understanding of the role in coal macromolecule degradation by fungal extracellular oxidases and the role of reactive species generated by these enzymes will require further research.

5) Cohen *et al.* (1990) showed that oxalate ions secreted by *T. versicolor* was responsible for the ability of this fungus to solubilize low rank coal and subsequent studies by a number of laboratories have shown that this metal chelator is secreted by other wood rotting fungi, including *P. chrysosporium*. Thus, it is highly likely oxalate mediated solubilization of low rank coal is common among those fungi that possess coal solubilization ability. A substantial amount of our research effort focused on characterizing low rank coal macromolecule solubilized using sodium oxalate. Because oxalate mediates low rank coal solubilization *in vivo*, we regarded solubilization *in vitro* as a biomimetic process. Spectrally (IR, $^{1}$H-NMR and UV-visible) the biomimetically solubilized product (Bumpus *et al.* 1998) resembled coal macromolecule solubilized biologically by *T. versicolor* (Wilson *et al*. 1987).

6) Although lignin peroxidases are able to oxidize (*i.e*., decolorize) biomimetically, solubilized low rank coal macromolecule, we were not able to unequivocally show that depolymerization had occurred. In part this is due to our observation that not only is coal macromolecule a mixture of macromolecules of differing molecular weights, individual macromolecules within this mixture apparently are able to form aggregates and these aggregates do not necessarily dissociate when oxidized by lignin peroxidases.

7) In large measure, oxalate is effective for solubilization of only highly oxidized low

rank coals (lignites and leonardites). It was therefore of interest that *P. chrysosporium* and *T. versicolor* were able to solubilize substantially preoxidized (150$^{o}$C for 7 days) Wyodak-Anderson subbituminous coal and preoxidized Lewiston-Stockton bituminous coal as these coals are only marginally solubilized by oxalate. Solubilization of a preoxidized bituminous coal is a relatively uncommon observation for a member of the Basidiomycetes. However, certain Ascomycetes (*Penicillium sp*. and *Cunninghamella sp.*) have been reported to solubilized Illinois # 6, Pittsburgh # 8 and Pennsylvania Upper Freeport bituminous coals all of which are resistant to oxalate mediated solubilization. Taken together, these results suggest that for subbituminous coals and bituminous coals, a process other than oxalate mediated metal chelation and subsequent cleavage of ionic linkages is required as an initial event in the solubilization process.

8) Possibly our most important observation is that common Lewis bases such as phosphate/hydrogen phosphate/dihydrogen phosphate and bicarbonate/carbonate ions are able to mediate extensive solubilization of leonardite at physiological pH values. Thus, if it becomes economical to use solubilized leonardite for methane production by anaerobic fermentation, it is likely that solubilization using Lewis bases will be more cost competitive that fungal solubilization procedures.

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