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Pyrene is metabolized to bound residues by *Penicillium janthinellum* SFU403

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Abstract

We have previously shown that the filamentous fungus, *Penicillium janthinellum* SFU403 (SFU403) oxidizes pyrene to pyrene 1,6- and 1,8-quinones and that the level of pyrenequinones (PQs) subsequently declines suggesting that PQs are not terminal metabolites. The purpose of this study was to determine the fate of PQs in SFU403. First, we compared the fate of ^{14}C-pyrene in SFU403 and a non-pyrene-oxidizing fungus, a *Paecilomyces* sp.. After 7 days of incubation, more than 80% of the radioactivity was cell-associated in both fungi; however, while 90% of the ^{14}C could be extracted from the *Paecilomyces* sp. as unmetabolized pyrene, 65–80% of the bound radioactivity remained inextractable from SFU403. Further evidence that pyrene oxidation to PQs was required for irreversible binding was obtained by comparing the extent of ^{14}C bound to SFU403 when it was grown for 21 days under conditions that resulted in differing amounts of ^{14}C-pyrene oxidation. The results showed that ≈40% of the inextractable products were bound residues derived from pyrene metabolites. The balance (60%) could be attributed to strong sorption of unreacted pyrene. We used electron paramagnetic resonance spectroscopy and oxygen consumption studies to demonstrate that both NADPH and glutathione can reduce PQs by one electron to their corresponding semiquinone anion radicals *in vitro*. These studies demonstrate that PQs are metabolized by SFU403 to bound residues, possibly via semiquinone intermediates.

Abbreviations: EPR = electron paramagnetic resonance, GSH = glutathione (reduced form), NADPH = nicotinamide adenine dinucleotide (reduced form), PAH = polycyclic aromatic hydrocarbon, PQ = pyrenequinone, PSQ = pyrene semiquinone, RTLC = radio thin layer chromatography

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic, persistent byproducts of the incomplete combustion of fossil fuels (Keck et al. 1989). PAH biodegradation by filamentous fungi is mediated by either extracellular ligninolytic enzymes (basidiomycete (white-rot) fungi (Lamar 1992)), or by intracellular cytochrome P450 monooxygenases (van den Brink et al. 1998). Both pathways yield PAH quinones as major oxidation products (Cerniglia 1992; Field et al. 1992; Hammel et al. 1992; Launen et al. 1995; Wunder et al. 1994). Since many PAH quinones are mutagenic (Flowers et al. 1996; Miller et al. 1986; Okamoto and Yoshida 1980; Sbrana et al. 1995), it is important to understand the pathways by which these compounds can be further degraded.

In basidiomycetous fungi, PAH quinones are either accumulated as dead-end products (Andersson & Henrysson 1996) or are metabolized by one of several pathways catalyzed by extracellular, non-specific ligninolytic enzymes (Hammel et al. 1992). These reactions result in either complete degradation to CO_2 and H_2O, or to the formation of bound residues through oxidative crosslinking to the humic fractions of soil (Bogan et al. 1999; Burgos et al. 1996; May et al. 1997; McFarland et al. 1992; Qiu and Mc-

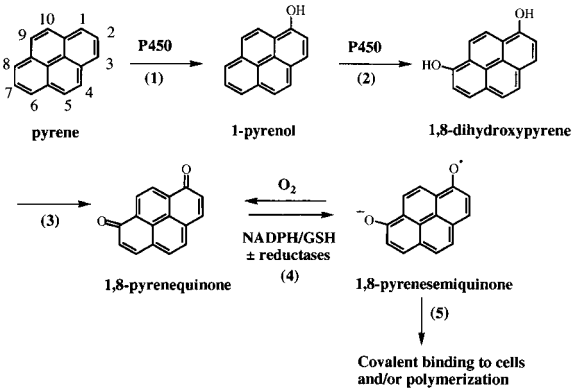

Figure 1. Pyrene metabolism by *Penicillium janthinellum* SFU403. Steps 1–3 were reported in Launen et al. (1995). *In vitro* evidence to support steps 4 and 5 are presented in this study. 1,6-pyrenequinone is expected to proceed through a similar pathway as is shown in this figure for 1,8-pyrenequinone. P450 refers to cytochrome P450 monooxygenase.

Farland 1991). The fate of PAH quinones in non-basidiomycete fungi is unknown.

The metabolism of pyrene to 1,6- and 1,8-pyrenequinones (PQs) by *Penicillium janthinellum* SFU403 (SFU403), a fungus previously isolated from petroleum-contaminated soils (Launen et al. 1995), proceeds as shown in Figure 1 (steps 1–3). Once produced, PQ levels peak by three days of culture (entrance to stationary phase), (Launen et al. 1999), and then decline to approximately 1/5 of their peak level by seven days (late stationary phase). SFU403 does not mineralize pyrene to CO_2 and H_2O (Launen et al. 1995) and converts only ~20% of ^{14}C-pyrene to transient water soluble products. Thus, neither mineralization nor conjugation with water-soluble compounds explains the decline of PQs in cultures of SFU403.

We have focussed on intracellular quinone reactions such as those that occur in higher eukaryotes, as models for PQ metabolism because PQs remain mostly cell-associated in SFU403, and this fungus does not secrete ligninolytic enzymes into the medium. In higher eukaryotes, aromatic quinone compounds, such as naphthoquinones, can undergo both one and two-electron reductions catalyzed by enzymes such as NADPH cytochrome P450 reductase, quinone oxidoreductase and DT diaphorase (Chesis et al. 1984; Joseph & Jaiswal 1994). The semiquinone anions that result from one electron reductions can redox cycle and generate reactive oxygen species, or bind to cellular macromolecules. Redox cycling of benzo[a]pyrene-7,8-dione and its corresponding semiquinone anion radical has been observed in rat hepatocytes (Flowers et al. 1996 and 1997), as has the direct binding of benzo[a]pyrene-7,8-dione and its semiquinone metabolite to cellular macromolecules (Flowers et al. 1996). Alternatively, semiquinones can initiate polymerization reactions (Vollmert 1973). To date there have been no studies on the potential for PQs to form pyrene semiquinone anion radicals (PSQs). Therefore, the objectives of this study were twofold: (1) to determine the extent to which pyrene oxidation to PQs is linked to the formation of bound residues, and (2) to determine whether biological reductants such as NADPH and glutathione could reduce 1,6- and 1,8-pyrenequinones to their corresponding PSQs.

Materials and methods

Fungal strains

All experiments employed SFU403 (ATCC # 201797) (Launen et al. 1995). To date, all of the *Penicillium* spp. we have tested showed significant pyrene-oxidizing activity. Therefore, a *Paecilomyces* sp. isolated from potatoes by Dr. Zamir Punja (Dept. of Biological Sciences, Simon Fraser University) was used as a non-pyrene oxidizing control; this *Paecilomyces* sp. oxidized only trace amounts of pyrene after seven days in liquid medium optimized for pyrene biotransformation.

Growth conditions

Ten mL cultures were grown in 50 mL Erlenmeyer flasks at 28 °C and 150 rpm. The three liquid growth media (OPT, MED, LOW) employed were chosen to control pyrene metabolism based on the results described in Launen et al. (1999). All were composed of a minimal salts medium supplemented with sodium nitrate, glucose and yeast extract as follows: optimal metabolism medium (OPT) was supplemented with 2.5% glucose, 1.5% sodium nitrate and 0.30% yeast extract; medial metabolism medium (MED) was supplemented with 10% glucose, 0.25% sodium nitrate and 0.15% yeast extract; and low metabolism medium (LOW) was supplemented with 2.5% glucose and 1.5% sodium nitrate but not yeast extract. All cultures were inoculated with freshly-harvested spore solutions to a final concentration of 2×10^6 spores/mL. Unlabelled pyrene (Aldrich) (0.025 mg/ml final concentration unless otherwise noted) and ^{14}C-pyrene (specific activity 32.2 mCi/mmol; Chemsyn

Laboratories, Lenexa, KS) were prepared and added concurrently as described previously (Launen et al.. 1995). All experiments included control flasks with cells or pyrene (separately) in each culture medium used. Triplicate flasks of each group were harvested and extracted after various periods of incubation at 28 °C and 150 rpm. Metabolism was monitored by thin layer chromatography of ethyl acetate extracts as described in Launen et al. (1995). Mass balance analyses were carried out as described below.

Extractions and radio-tracer methods

Unless otherwise noted, cultures were extracted with equal volumes of ethyl acetate (EtOAc) overnight and then two times further the following day. Complete mass balance analyses were conducted on triplicate cultures for all radiotracer experiments. ^{14}C-compounds in the cell and media EtOAc extracts and media after extraction were quantified using liquid scintillation counting (LSC) on a Beckman LS 8000 liquid scintillation counter with quench correction, using BCS LSC cocktail (Amersham). ^{14}C-pyrene and ^{14}C-pyrene metabolites (pooled 1-pyrenol and PQs) and 'polars' were quantified in the organic extracts using radio thin-layer chromatography (RTLC). All compounds more polar than 1,8PQ were considered as 'polars'. TLC conditions were as described in Launen et al. (1995). Using known standards for orientation, lanes were cut and assayed directly for ^{14}C (dpm) using LSC. After extraction, the remaining biomass was combusted for four min. using a Harvey Biological Oxidizer (Ox 300) and the resulting $^{14}CO_2$ was trapped in 15 mL of Harvey Biological Oxidizer ^{14}C-Cocktail and quantified for ^{14}C (dpm) using LSC. Inextractable cell-associated products (ICAP) were calculated as follows:

$$\text{ICAP}(\%) = \frac{\text{dpm remaining in cells after extraction (determined by combustion)}}{\text{total dpm associated with cells}} \times 100$$

Oxygen consumption

Oxygen consumption was quantified polarographically at 30 °C using a Clark oxygen electrode. Reagent concentrations and times of additions are described in the 'Results and discussion'.

Electron Paramagnetic Resonance (EPR) spectroscopy

EPR spectra were recorded on a Bruker ECS106 spectrometer operating in the X-band at a nominal frequency of 9.76 GHz. Samples of 1,6PQ (0.60 mM) or 1,8PQ (0.75 mM) were mixed with 20 mM NADPH in a 50 : 50 mixture of DMSO and Chelex-100 treated sodium phosphate buffer (5 mM; pH 7.2) containing 100 mM NaCl (Flowers et al. 1997). They were studied under anaerobic conditions at room temperature in a quartz flat cell mounted in a T_m mode microwave cavity. Typical operating conditions were 2.5 mW microwave power, 100 kHz field modulation and 0.14 G modulation amplitude. The program WINEPR SimFonia (Version 1.25 (Bruker Analytische Messtechnik GmbH)) was used to simulate EPR spectra.

Results and discussion

Pyrene metabolism to PQs is a requirement for the formation of inextractable cell-associated products (ICAP)

Table 1 shows that after one week of incubation, ≈80% of the added ^{14}C-pyrene became associated with fungal biomass in both SFU403 and the *Paecilomyces* sp.. However, only 10% of the total radioactivity was irreversibly bound to the *Paecilomyces* sp. whereas 65–80% of ^{14}C-pyrene in SFU403 was converted to inextractable cell-associated products (ICAP). The organic extract from SFU403 contained pyrenequinones and negligible amounts of pyrene. The high proportion of irreversibly-bound radioactivity in SFU403 was not a consequence of incomplete solvent extraction as similar levels of residual radioactivity were obtained with either passive extraction using ethyl acetate or toluene, or Soxhlet extraction for 24 hours in toluene/methanol (Table 1). Saponification of cells at 80 °C also did not release the bound radioactivity (data not shown). The lack of irreversible binding in the *Paecilomyces* sp., which does not oxidize pyrene, strongly implicates pyrene metabolites in the formation of ICAP.

To determine the relative proportion of ICAP derived from pyrene metabolism within SFU403, cultures were incubated with ^{14}C-pyrene in media resulting in low, medium and high (optimized) levels of pyrene oxidation (LOW, MED and OPT: see 'Materials and methods'). For pyrene oxidation, the three media can be ranked as expected: OPT ≫ MED >

Table 1. Formation of bound residues from ^{14}C-pyrene by *Penicillium janthinellum* SFU403 and a *Paecilomyces* sp.after 7 days of incubation. The effect of different extraction techniques on the recovery of ^{14}C from the cells and medium is also shown

Sample Extraction method[1]	Radioactivity recovered in each fraction (% initial ^{14}C)					
	Total recovery	Cell organic extract	Cell remainder[2]	ICAP	Medium organic extract	Aqueous remainder
P. janthinellum SFU403						
EtOAc	109 ± 11	31 ± 7	65 ± 21	68	3 ± 1	10 ± 1
Toluene	94 ± 14	11 ± 1	47 ± 14	81	1 ± 0	35 ± 0
Soxhlet	88 ± 8	12 ± 3	47 ± 4	80	7 ± 4	22 ± 3
Paecilomyces						
EtOAc	92 ± 5	80 ± 9	9 ± 5	10	1 ± 0	2 ± 0
No cells						
Toluene	108 ± 11	–	–	–	107 ± 11	1 ± 0
EtOac	86 ± 4	–	–	–	78 ± 3	8 ± 1

Cultures were grown in OPT medium plus unlabelled pyrene (added at $t = 0$) at a final concentration of 0.025 mg/ml along with 1×10^6 dpm/flask of ^{14}C-pyrene. Cultures were incubated for one week. All data shown are the mean ± S. D. of three experiments.
[1] EtOAc or Tol = overnight extraction with either ethyl acetate (EtOAc) or toluene (Tol) followed by two further extractions the following day. Soxhlet extraction was for 24 hours in toluene: methanol (10 : 1 v/v).
[2] Determined by combustion analysis (see 'Materials and methods').

LOW (Table 2). The levels of ICAP formed after 21 days followed the same rank order: 95%, 64% and 30% in OPT, MED and LOW, respectively (Table 2). Thus, the extent of pyrene oxidation to PQs paralleled the amount of ICAP formed. ICAP formation was not correlated to the amount of biomass as fungal biomass in MED cultures was greater than OPT after 21 days despite a lower ICAP level in the MED samples (Table 2).

Interestingly, we found that four-fold more biomass was formed in OPT and MED media when fungi were incubated with unlabelled pyrene alone rather than a ^{14}C-pyrene/unlabelled pyrene mixture (data not shown). This suggests that the levels of ^{14}C-radioactivity used in these experiments significantly inhibited fungal growth and metabolism. Such inhibition was further suggested by the finding that reduced levels of PQ were formed by SFU403 in OPT medium with ^{14}C-pyrene/unlabelled pyrene as a substrate (40% bioconversion) compared to unlabelled pyrene alone (100% bioconversion: Launen et al. (1999)).

To ensure that the metabolism of pyrene to PQs (which are mutagenic), did not result in decreased cell growth, cultures were grown in OPT and MED with and without pyrene for 10 days, and the biomass dry weights of triplicate samples were monitored. The same biomass was formed in the presence and absence of pyrene in either MED or OPT medium and in both cases MED medium supported a higher biomass yield (data not shown). Thus, exposure of SFU403 to 0.025 mg/mL pyrene for up to 10 days did not inhibit fungal growth.

Contribution of non-specific sorption to ICAP levels

Highly lipophilic chemicals such as PAHs are known to sorb to organic matter including fungal biomass (Barclay et al. 1995). To estimate the proportion of ICAP that was related to strong sorption of unreacted pyrene, we incubated ^{14}C-pyrene with heat-inactivated SFU403 and compared the results to live SFU403. After 21 days, all of the ^{14}C-radioactivity had partitioned into the biomass in both samples, but as before, none of the bound ^{14}C was extractable from biomass in live SFU403 (i.e., ICAP was 95%). In contrast, in the heat-killed SFU403, ICAP levels were 56%. RTLC analysis showed that only pyrene was present in the ethyl acetate extracts from the heat- killed cells. Thus, sorption of unreacted pyrene to SFU403 accounted for approximately 60% of the ICAP formed by SFU403 after 21 days. There-

Table 2. The effect of medium composition on the extent of ^{14}C-pyrene oxidation and the formation of ICAP by *Penicillium janthinellum* SFU403 after 3 and 21 days of incubation

Medium composition		Pyrene (%)	Metabolites (%)	Cell-associated (%)	ICAP	Total recovery[1] (%)	Dry Weight (mg)
LOW	3d	79 ± 8	0 ± 0	40 ± 3	3 ± 1	85 ± 8	27 ± 4
	21d	16 ± 11	20 ± 13	47 ± 7	30 ± 8	95 ± 18	19 ± 2
MED	3d	83 ± 1	1 ± 0	63 ± 3	1 ± 0	96 ± 2	66 ± 31
	21d	0 ± 0	33 ± 0	95 ± 6	64 ± 2	99 ± 8	262[2]
OPT	3d	2 ± 1	38 ± 5	30 ± 2	22 ± 3	107 ± 3	108 ± 7
	21d	0 ± 0	<1 ± 1	80 ± 11	95 ± 10	96 ± 14	76 ± 1

All values represent the mean ± S. D. for three cultures except where noted. All values are the percent of initial ^{14}C added except for ICAP. Unlabelled pyrene was added at $t = 0$ to a final concentration of 0.005 mg/ml to all cultures. The concentration of ^{14}C-pyrene was adjusted such that the LOW, MED and OPT cultures received 0.045, 0.182 and 0.227 μCi, respectively, thereby accounting for expected differences in culture wet weight.

[1]The percent ^{14}C-dpm values for the aqueous samples were not included for clarity but were included in the total recovery value.

[2]Average of two measurements.

fore, it can be deduced that the balance of ICAP, ≈40% is formed from pyrene metabolites, specifically PQs, and that this fraction represents the bound residues. This estimate of 40% bound residues fits the percentage bioconversion of ^{14}C-pyrene to ^{14}C-PQs: approximately 40% of ^{14}C-pyrene was converted to PQs by SFU403. Interestingly, a non-pyrene-oxidizing *Fusarium* sp. formed only 28% ICAP, presumed to be sorbed, but unreacted pyrene. The decreased sorption of pyrene to the *Fusarium* sp. relative to the heat-killed SFU403, suggests that there are differences in the uptake and sorption of unreacted pyrene between different fungi, possibly reflecting differences in fungal cell wall composition.

PQs can be reduced to their corresponding PSQs by intracellular reductants

The accumulation of pyrene metabolites in fungal biomass suggests that PQs are further metabolized to cell-bound products. Quinones are soft electrophiles and may bind directly to nucleophiles within the cell such as glutathione and proteins. Alternatively, in the presence of one-electron reducing agents, quinones can be reduced to semiquinone radicals, which may also react with cellular macromolecules or redox cycle with molecular oxygen, generating reactive oxygen species. For example, Flowers et al. (1997) showed that naphthoquinone and benzo[a]pyrene-7,8-dione can be reduced to their corresponding semiquinone radicals by NADPH and that the semiquinones can bind to DNA as well as redox cycle with oxygen. To determine whether PQs can undergo one-electron reduction yielding PSQs, EPR spectroscopy was used to detect the radical signal obtained from the incubation of 1,6PQ (0.60 mM) and 1,8PQ (0.75 mM) with NADPH (20 mM) under anaerobic conditions. The EPR spectra are shown in Figure 2, together with computer simulations using the following hyperfine constants: 2.27 G (2H), 1.79 G (2H), and 0.94 G (4H) for 1,6PSQ; and 1.90 G (2H), 1.72 G (2H), 1.52 G (2H), and 0.54 G (2H) for 1,8PSQ. These values are consistent with the expected radical structures, and similar spectra were obtained using a stronger reducing agent, sodium borohydride (data not shown). Although the radical concentration cannot be quantified using our apparatus, to observe the 1,8PSQ signal required a slightly greater concentration of PQ than for the 1,6 PSQ signal (0.75 mM and 0.60 mM, respectively). Furthermore, the 1,8PSQ signal was less stable than the 1,6PSQ signal. This suggests that the 1,6PQ may undergo one electron reduction more readily than 1,8PQ or that the 1,6PSQ radical is more stable under anaerobic conditions.

Oxygen consumption was then measured to assess the ability of the PSQs to redox cycle with oxygen. NADPH (0.13 mM) or GSH (0.03, 0.13 and 0.27 mM) was added to solutions of 1,6PQ and 1,8PQ (0.20mM) in OPT media and the increase in oxygen consumption after the addition of the reductant was monitored (Table 3). There was no significant increase in oxygen consumption upon addition of quinone to the media (data not shown); however, the addition of NADPH increased the O_2 consumption of 1,6PQ and 1,8PQ solutions by approximately 2.2-fold and 1.5-fold respectively. Similarly, GSH (0.13 mM) stimulated oxygen consumption by more than two-fold (Table 3). *Para*-methylbenzoquinone was used as a negative control that is unable to redox cycle (Rossi

Table 3. Stimulation of oxygen consumption by addition of NADPH or GSH to 1,6- or 1,8-pyrenequinone in OPT medium

Pyrenequinone Reductant		Oxygen consumption (mg O_2/L/h) Media + quinone	Media + quinone + reductant	Relative increase[1]
1,6-Pyrenequinone[2]				
NADPH	(0.13 mM)	0.7 ± 0.2	2.8 ± 0.3	2.2
GSH	(0.03 mM)	0.7 ± 0.8	1.20 ± 0.2	0.0
	(0.13 mM)	1.0 ± 0.0	2.4 ± 0.0	2.4
	(0.26 mM)	1.4 ± 0.6	3.8 ± 0.1	2.8
1,8-Pyrenequinone[2]				
NADPH	(0.13 mM)	1.0 ± 0.3	1.6 ± 0.9	1.5
GSH	(0.03 mM)	1.1 ± 0.3	1.0 ± 0.1	0.0
	(0.13 mM)	0.5 ± 0.2	2.2 ± 0.1	4.3
	(0.26 mM)	0.9 ± 0.1	2.9 ± 0.1	3.1

[1]Relative increase determined by dividing the oxygen consumption observed before addition of reductant with the oxygen consumption observed after addition.
[2]Both pyrenequinones were present at 0.20 mM dissolved in a 1 : 1 solution of DMSO and phosphate buffer (pH 7.0).
All data is the mean ± standard deviation of three separate experiments. An increase in oxygen consumption was observed regardless of the order of addition of quinone and reductants.

et al. 1986), and no stimulation of O_2 consumption was found when either NADPH or GSH (0.13 mM each) was added to *p*-methylbenzoquinone under our experimental conditions (data not shown). These results indicate that the intracellular reductants NADPH and GSH can reduce PQs to PSQs that can subsequently reduce molecular oxygen. The production of O_2 radicals by PQs may be responsible in part for their mutagenic properties in mammalian cells, as has been reported for benzo[a]pyrene-7,8-dione (Flowers et al. 1996; Flowers et al. 1997) and other aromatic quinones (Chesis et al. 1984). Whether significant redox cycling occurs in fungi exposed to PQs is presently unknown; however, in these experiments, the level of oxygen radicals generated was not sufficient to result in growth inhibition of SFU403. Direct binding of 1,6- and 1,8-PQ to cellular nucleophiles may also contribute to the formation of inextractable products and we are currently examining the extent to which the 1,6- and 1,8-PQ alkylate cellular nucleophiles.

Conclusions

PQs are oxidized metabolites of pyrene that are known to be mutagenic, particularly the 1,8PQ (Okamoto & Yoshida 1981; Okamoto & Yoshida 1980; Sbrana et al. 1995). In this report, we have shown that the major fate of PQs (or their derivatives) in SFU403 is to bind irreversibly to cells. Approximately half of ICAP formation was dependent upon pyrene metabolism whereas the balance was attributable to strongly sorbed, unreacted pyrene. Using oxygen consumption and EPR spectroscopy, we have clearly demonstrated that the intracellular reductants NADPH and glutathione (GSH) can directly reduce both 1,6- and 1,8-PQ to their corresponding semiquinone anion radicals. Although 3,6-pyrenequinone has been shown to redox cycle in the presence of carbonyl reductase from *Clostridium kluyverii* (Jarabak & Harvey 1993) to our knowledge, redox cycling by 1,6- and 1,8-pyrenequinones has not previously been reported. This is also the first report of the EPR spectra of these pyrene semiquinone anions. Thus, the intracellular one electron reduction of PQs to form PSQs, which could subsequently covalently bind with cell molecules forming ICAP, is a theoretically feasible mechanism for the formation of bound residues.

We have provided *in vitro* data that supports the involvement of a PSQ intermediate in the pathway of formation of bound residues. Further studies are required to confirm this *in vivo*. If bound residues are formed by fungi *in situ*, and remain stable over time in the presence of the soil microbial community, this process may be important in bioremediation, i.e.,

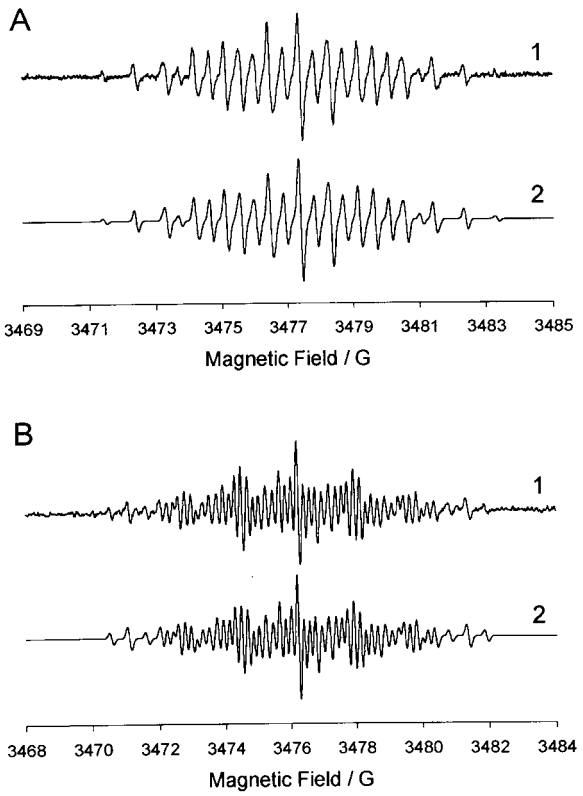

Figure 2. EPR spectra of the 1,6PQ semiquinone anion radical (A) and the 1,8PQ semiquinone anion radical (B) produced from the reaction of PQs with NADPH. (1) depicts one typical spectrum from three separate experiments and (2) depicts the computer simulation of each radical.

pyrene and other PAH may be detoxified by microbial metabolism that renders them non-bioavailable to other organisms. This would be similar to extracellular humification of PAH metabolites mediated by fungal extracellular enzymes and chemical reactions in the soil (Bogan et al. 1999; Kastner et al. 1995; May et al. 1997; McFarland et al. 1992; Qiu & McFarland 1991). Further research is ongoing to determine the chemical nature of the bound metabolites.

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