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Electroanalytical approaches to understanding benzene metabolism*

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Abstract: Electrochemical techniques are ideally suited to the study of the metabolism of aromatic xenobiotics because the metabolites are frequently easier to oxidize than the parent compounds. In many cases, the trace metabolites have the lowest oxidation potentials and hence electrochemical methods have the greatest selectivity for these compounds. The sensitivity of dual-electrode liquid chromatography–electrochemistry for the detection and identification of trace metabolites was demonstrated by the detection of the secondary metabolite, hydroquinone, in a microsomal incubation containing benzene and ascorbic acid. The use of an electrochemical detector in a series configuration provides increased selectivity for chemically reversible metabolites such as hydroquinone. Electrochemical methods can also be used to generate metabolites. The products of the electrochemical oxidation of phenol and biphenol are compared with those generated in a peroxidase incubation.

Keywords: *Benzene metabolism; liquid chromatography–electrochemistry; cyclic voltammetry; peroxidase; cytochrome P-450.*

Introduction

Many aromatic compounds are known to be metabolized to reactive quinone or quinone-like intermediates. Examples include acetaminophen [1, 2], benzidine [3, 4], aniline [5] and benzene [6]. These reactive intermediates can bind to cellular macromolecules and produce tissue necrosis or cancer. Because the metabolism of these compounds frequently involves one or more oxidation (or reduction) reactions, electrochemical methods are ideal for their study. In many cases, the biological oxidation can be mimicked at an electrode surface. As an example of the utility of electrochemical techniques for the study of aromatic metabolism, their use in the investigation of benzene metabolism and toxicity will be discussed.

Benzene is an important industrial solvent and a component of unleaded gasoline. Chronic exposure to benzene is associated with acute myelogenous leukaemia. Benzene is also a myleotoxin and has been shown to produce blood dyscrasias including lymphocytopenia, thrombocytopenia, pancytopenia and aplastic anaemia. Sammett *et al.* demonstrated that metabolism of benzene by the liver is a prerequisite for toxicity [7]. Phenol, the major metabolite of benzene, can be further metabolized to hydroquinone and catechol. These compounds can be further oxidized to electrophilic quinones. The quinones can then undergo 1,4-Michael addition reactions with cellular macromolecules, causing toxic effects. A diagram showing the possible metabolic pathways of benzene is shown in Fig. 1.

Although many of the studies involving benzene metabolism have concentrated on the liver, there is considerable interest in the metabolism of benzene in bone marrow, where the toxicity actually occurs. Bone marrow contains very little cytochrome P-450, but does contain a significant amount of peroxidase activity [8]. Therefore, most studies of bone marrow metabolism have been concerned with the peroxidase-mediated metabolism of benzene and its metabolites. Examples of the use of electrochemical techniques in the study of the metabolism of benzene and phenol by both microsomal and peroxidase enzymes will be presented.

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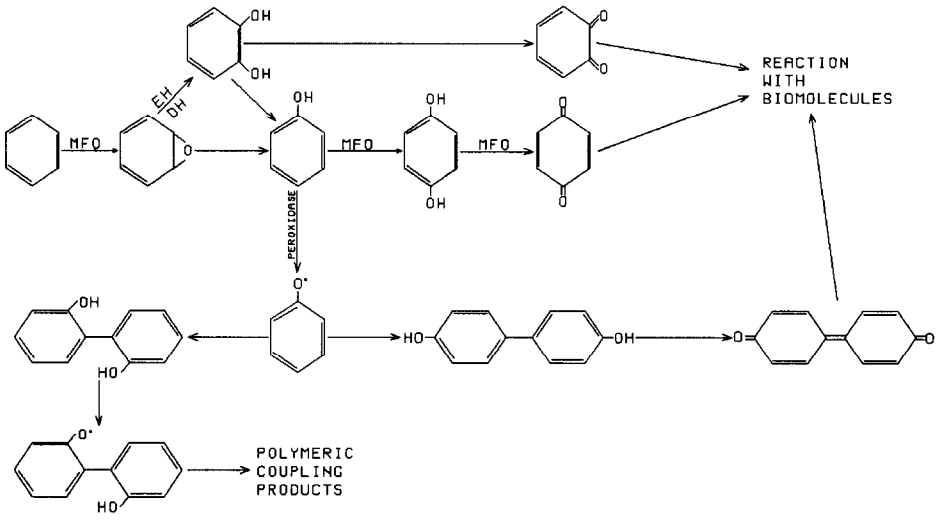

Figure 1
General pathway for benzene metabolism. MFO, mixed function oxidase; EH, epoxide hydrolase; DH, dehydrogenase.

Experimental

Chemicals

Chemicals were purchased from the following sources: benzene and hydroquinone, J.T. Baker; glutathione, β-nicotinamide adenine dinucleotide (NADPH), horseradish peroxidase type VI, catalase, hydrogen peroxide and ascorbic acid, Sigma; phenol, Mallinckrodt; catechol, Kodak; 4,4′-biphenol and 2,2′-biphenol, Aldrich; and ammonium acetate, Fisher. All chemicals were reagent grade or better and were used as received.

Cyclic voltammetry

Cyclic voltammetry experiments were performed using a BAS-100 Electrochemical Analyzer (Bioanalytical Systems, West Lafayette, IN, USA). Solutions were approximately 1 mM in ammonium acetate buffer, pH 4. A glassy carbon working electrode, platinum auxiliary and Ag/AgCl reference were employed for all studies. The scan rate was 100 mV s^{-1}.

Chromatographic systems

Two separate chromatographic systems were utilized in these experiments. For the microsomal experiments, an LC-154D liquid chromatographic system equipped with a Biophase C-18, (5 μm, 25 cm × 4.6 mm; Bioanalytical Systems) was employed. The mobile phase consisted of ammonium acetate (0.01 M, pH 4).

The second system was devoted to the determination of the peroxidase metabolites of phenol and biphenol. In this case, an LKB model 2150 pump was used (Bromma, Sweden) with a Rheodyne model 7125 injector. The column was a Supelco C_8 column (3 μm, 7.5 cm × 4.6 mm). For the determination of the peroxidase metabolites of phenol, a mobile phase of ammonium acetate (0.01 M, pH 4) ≃ acetonitrile (4:1, v/v) was employed. For the detection of the glutathione conjugates of 4,4′-biphenol, the buffer contained only 15% acetonitrile.

The electrochemical detector used for both studies was an LC-4B dual-electrode detector equipped with a glassy carbon working electrode and a Ag/AgCl reference (Bioanalytical Systems). A 20-μl injection loop was used in all studies.

Microsomal incubations

Microsomes were prepared from the livers of Swiss male mice (Laboratory Supply, Indianapolis, IN, USA) as previously described [1]. Protein concentrations were determined by the method of Lowry *et al.* using bovine serum albumin as the standard [9]. Benzene incubations with microsomal protein contained the following: 1 ml of microsomal

protein (3 mg protein ml^{-1}) suspended in 0.028 M KCl–phosphate buffer, pH 7.4; 2 mM benzene (final concentration) dissolved in 10 μl of acetone; 1.4 mM NADPH and 15 mM $MgCl_2$. The total volume of the incubation mixture was 1210 μl. The mixture was incubated at 37°C for 60 min. The reaction was terminated by the addition of 200 μl of 1 M $HClO_4$ and the mixture was centrifuged for 20 min at 14,000 rpm. The supernatant was injected directly into the liquid chromatograph.

Peroxidase incubations

Incubations of phenol and biphenol were performed under conditions similar to those reported by Eastmond and co-workers [10]. For the biphenol incubations, 500 μM of biphenol was incubated with 5 mM glutathione, 10 μg ml^{-1} of horseradish peroxidase and 0.03% hydrogen peroxide for 2 min. The reaction was terminated by the addition of either 650 units of catalase or 0.1 M phosphoric acid.

Coulometric flow cell

In order to compare the products of electrochemical oxidation of phenol with those produced in a peroxidase incubation, a coulometric flow cell was employed to generate the 4,4′-diphenylquinone species. Figure 2 shows the cell design, which has been described previously in more detail [1]. To generate the glutathione conjugates of 4,4′-biphenol, a 0.1 mM solution of 4,4′-biphenol in pH 7.4 phosphate buffer was pumped through the flow cell at approximately 200 μl min^{-1}. The potential of the cell was set at 1.00 V versus Ag/AgCl. The resulting quinone was dripped into an equimolar solution of glutathione and stirred continuously. The products of the reaction were injected directly into the liquid chromatograph.

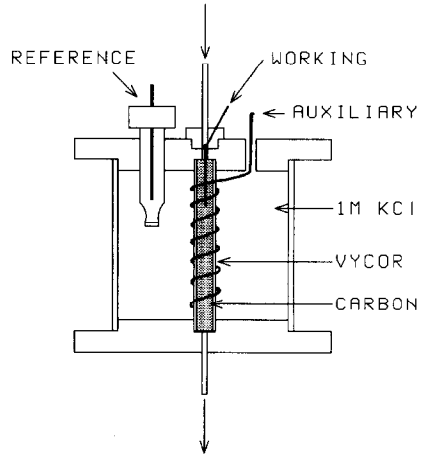

Figure 2
Coulometric flow cell design: cell is made of Plexiglas and contains supporting electrolyte. Arrow indicates direction of flow.

Results and Discussion

Cyclic voltammetry of benzene metabolites

Figure 3 shows the relative redox potentials of a number of benzene metabolites, including hydroquinone, catechol and 4,4′-biphenol. Several of the secondary metabolites of benzene have very low oxidation potentials. In addition, five of these metabolites — hydroquinone, catechol, 4,4′-biphenol, the glutathione conjugate of hydroquinone (HQ–SG) and trihydroxybenzene — are chemically reversible, i.e. they produce quinones which are stable over the time period of the cyclic voltammetry experiment (about 5 s).

Microsomal metabolism of benzene

As benzene becomes increasingly metabolized, the selectivity of liquid chromatography–electrochemistry (LCEC) for the metabolites increases. The major microsomal metabolite of benzene is phenol, which can easily be detected at +1.0 V versus Ag/AgCl reference electrode. Hydroquinone is present at much lower levels in the microsomal incubations, but it is oxidized at a lower redox potential (+0.7 V). It has been detected previously by LCEC, but only following extraction with ethyl acetate and concentration of the organic layer under nitrogen [11]. It is not detectable at these levels by UV methods [11].

The fact that hydroquinone was so difficult to detect in the microsomal incubation implies that it was either (a) generated at very low levels or (b) oxidized rapidly to benzoquinone, which can then react with sulphhydryl and amine sites of the microsomal protein. Attempts to determine benzoquinone directly in microsomal incubations of benzene have failed, presumably due to the short half-life of that species in the presence of endogenous nucleophiles. Tunek *et al.* have reported that the addition of glutathione substantially reduces the amount of covalent binding of benzene metabolites to microsomal protein [12]. In order to determine if benzoquinone was being generated, glutathione was added to

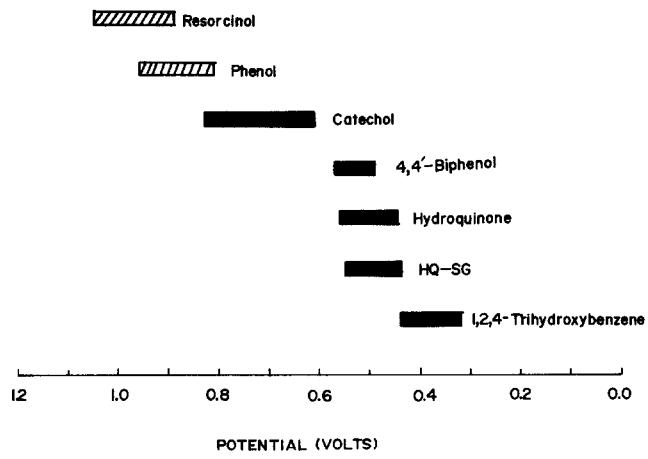

Figure 3
Cyclic voltammetry data for hydroxylated metabolites of benzene: scan rate, 100 mV s^{-1}; glassy carbon electrode, pH 4. HQ–SG, glutathione conjugate of hydroquinone. The potentials range from the half-wave ($E_{1/2}$) to the peak potential (E_p). The solid and hatched blocks indicate chemically reversible and chemically irreversible oxidations, respectively.

a microsomal incubation of benzene to trap the quinone as the glutathione conjugate [13, 14]. When this was done, the glutathione conjugate was easily detected by LCEC without any preconcentration steps [14].

Smart and Zannoni have reported that the addition of 5 mM ascorbic acid reduces the amount of covalent binding of benzene metabolites to microsomal protein by 75% [15]. Since ascorbic acid is a good reducing agent, it is likely that it reduces the reactive *p*-benzoquinone back to the less reactive hydroquinone. To verify this, a microsomal incubation of benzene containing 5 mM ascorbic acid was prepared. Hydroquinone was detected (at the low picomole injected level) under these conditions. Therefore, ascorbic acid either prevents the oxidation of the hydroquinone or rapidly reduces the benzoquinone back to hydroquinone before it can undergo addition reactions. Figure 4 shows the resulting hydroquinone peak, which was detected directly in the microsomal incubation supernatant without any preconcentration steps.

To verify that the peak in the microsomal incubation was hydroquinone, the voltammetry of the unknown peak was compared with that of authentic hydroquinone. This was done by measuring the current response at points along the current–potential curve and determining the ratio of the current at various potentials to the current on the diffusion-limited plateau (where the current value is independent of potential). Table 1 shows that the retention times and the voltammetry of the two compounds are virtually identical. The voltammetric information and the fact that the peak is not in the microsomal blank further confirms the identity of the LCEC peak as hydroquinone.

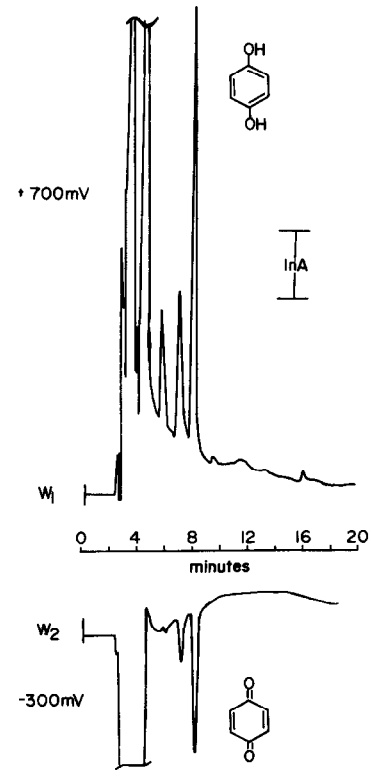

Figure 4
Detection of hydroquinone in a microsomal incubation containing NADPH, benzene and ascorbic acid. W_1 = +700 mV; W_2 = −300 mV (W = working electrode).

Table 1
Voltammetric characterization of hydroquinone in a microsomal incubation of benzene and ascorbic acid

	k'†	+600/+700 mV*	+500/+700 mV*
Standard hydroquinone	2.3	0.77	0.41
Microsomal incubation unknown	2.3	0.78	0.40

*Ratio of peak currents at indicated potentials.
†k' = Capacity factor.

Dual-electrode LCEC can be used to increase the selectivity of the electrochemical detector for metabolites, which can produce "quinone-type" reactive intermediates. By setting two electrodes in series, one at an oxidizing potential (in this case, +700 mV) and the other at a reducing potential (in this case, −300 mV), compounds exhibiting chemical reversibility can be selectively detected [16]. Figure 4 is an example of this in which benzoquinone is detected selectively at the downstream electrode. The oxidation of NADPH and ascorbic acid are not reversible and can be discriminated against in this configuration. Both the voltammetric identification and series configuration provide information regarding the identity of the metabolites without the extensive sample clean-up required for other techniques such as mass spectrometry or NMR [13].

Peroxidase metabolism of phenol

Since phenol is the major metabolite of benzene, it has been postulated that phenol or one of its metabolites is oxidized further by peroxidase present in bone marrow to quinone-type intermediates. Figure 1 shows the pathway for this metabolism. To generate the glutathione conjugate of 4,4′-biphenol, a coulometric flow cell was employed. Biphenol is oxidized to 4,4′-biphenyl quinone by a 2-electron, 2-proton oxidation. Reaction with glutathione yielded one chromatographic peak with a retention time of approximately 6 min.

The products of the flow cell were then compared with those of a peroxidase incubation of 4,4′-biphenol containing glutathione (Fig. 5). Again, glutathione was used to trap the quinone as the glutathione conjugate. In this case, a large peak with the same retention time as the product generated by the flow cell was obtained. A small secondary product was

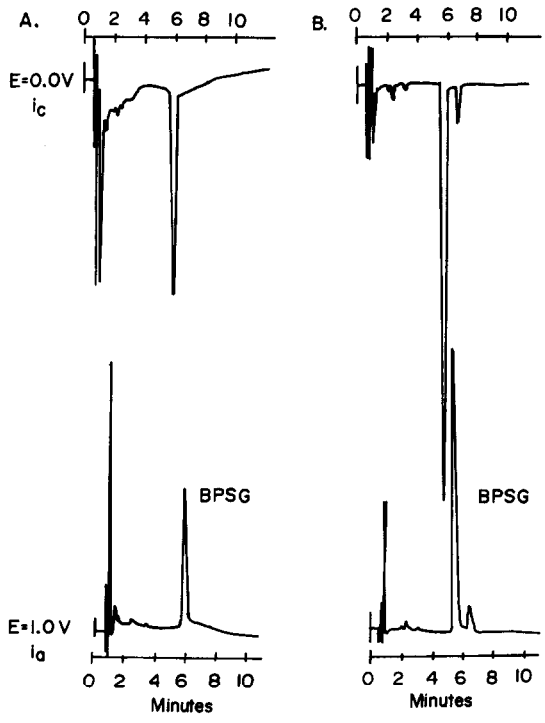

Figure 5
Comparison of metabolites of biphenol generated by (A) flow cell and (B) peroxidase incubation of biphenol with glutathione. LCEC series configuration. E_1 = +1.0 V; E_2 = +0.0 V. BPSG = glutathione conjugate of biphenol. i_c = cathodic current; i_a = anodic current.

also detected. This could be a second glutathione conjugate [10]. The peaks present in the flow cell and incubation mixture exhibited identical voltammetry. In addition, both peaks were chemically reversible. The fact that the chemical reversibility was maintained indicates that the biphenol ring system remained intact. The oxidation of biphenol by peroxidase is similar to that of benzidine and it appears that both a conjugation reaction and the reduction of the quinone by glutathione are occurring [3].

A peroxidase incubation of phenol was also performed and the products were compared with those from the flow cell. Figure 6 shows a chromatogram of the products of the peroxidase incubation; 4,4′-biphenol, some 2,2′-biphenol and an unknown product were detected. The coulometric flow cell produced only 4,4′-biphenol and the unknown product. The reasons for the absence of 2,2′-biphenol may be that it forms polymeric products which are absorbed to the carbon electrode. Further experiments comparing the electrochemically generated products with the peroxidase metabolites of phenol are underway.

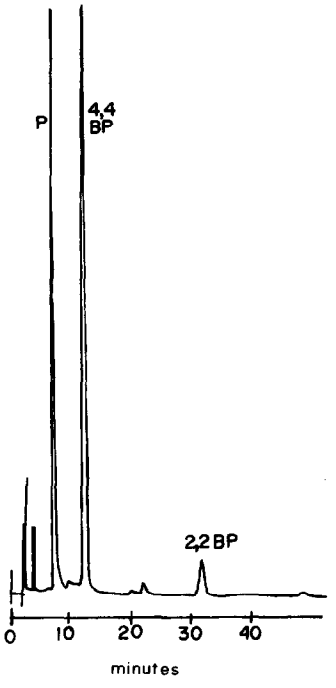

Figure 6
Peroxidase metabolism of phenol. Detection of 4,4'-biphenol (4,4-BP) and 2,2'-biphenol (2,2-BP). E = +1.00 V. Chromatographic conditions given in text. P = phenol.

Electrochemical methods are useful for the detection, identification and generation of aromatic metabolites. Frequently, the metabolites of aromatic compounds are more easily oxidized than the parent compound. In these cases LCEC can be used to detect trace metabolites selectively in complex samples without preconcentration or extensive sample clean-up. A coulometric flow cell can be used to generate compounds which may be metabolites. These can then be compared to metabolites produced by a biological system. Finally, the voltammetry of the metabolites can be used to identify compounds even when only picomole amounts of material are available. Additional examples of the use of LCEC for the study of xenobiotic metabolism have been reviewed recently [17].

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