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THE USE OF LIQUID CHROMATOGRAPHY WITH DUAL-ELECTRODE ELECTROCHEMICAL DETECTION IN THE INVESTIGATION OF GLUTATHIONE OXIDATION DURING BENZENE METABOLISM

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SUMMARY

Liquid chromatography with electrochemical detection provides a powerful tool for the study of glutathione oxidation during benzene metabolism. The use of two dual-electrode detectors allows for the detection of oxidized and reduced glutathione and of phenol and quinone metabolites of benzene. The role of glutathione as a reductant is explored in this paper. Results indicate that hydrogen peroxide is the oxidizing agent primarily responsible for glutathione exidation during benzene, phenol and hydroquinone metabolism.

INTRODUCTION

Long term exposure to benzene has been associated with a number of cancerous blood disorders, including aplastic anemia and leukemia[1–4]. The use of benzene as an industrial solvent and antiknock additive in gasoline increases the concern of the public for the mechanism by which this compound can elicit a carcinogenic response[5]. Previous studies of benzene metabolism have indicated that it must be metabolized by the liver before it can exert its toxic effects[6]. Benzene has been shown by us and others to be metabolized to both phenol and hydroquinone by liver microsomal protein[7,8]. Catechol, although an *in vivo* metabolite of benzene, has not yet been detected in the *in vitro* studies with cytochrome P-450. A pathway of the *in vitro* metabolism of benzene by microsomal protein is shown in Fig. 1.

Hydroquinone can be further oxidized to *p*-benzoquinone by microsomal protein in the presence of NADPH and O_2[9,10]. The resulting *p*-benzoquinone reacts quickly with thiols and other nucleophiles, making it an attractive candidate for the reactive intermediate in benzene metabolism. Several other xenobiotics are proposed to be metabolized to reactive quinone type intermediates. Among these are acetaminophen[11], benzidine[12], and 6-hydroxydopamine[13]. Since benzene is the simplest aromatic compound that is also carcinogenic, it can provide a model for these other compounds in the investigation of the mode of detoxification of quinone type intermediates.

One issue of current interest, especially in acetaminophen metabolism, is the role of glutathione in the detoxification of quinone intermediates[14–16]. Glutathione

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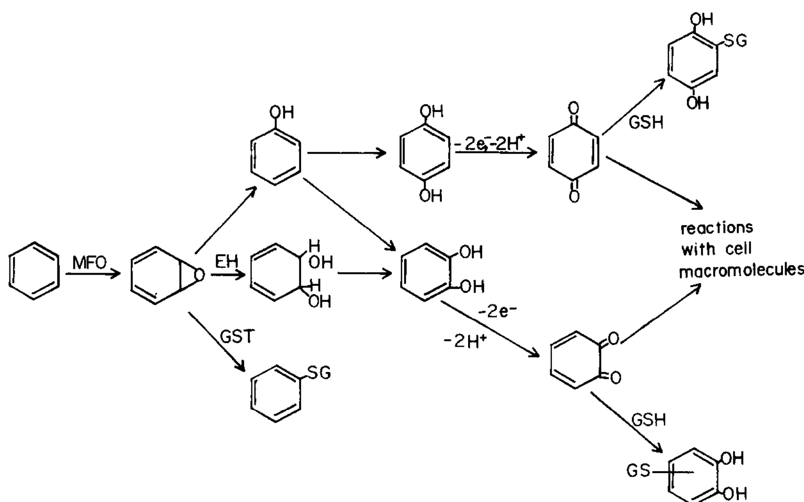


Fig. 1. Pathway of benzene metabolism by microsomal protein *in vitro*.

can act either as a nucleophile, a one-electron reductant or as a cofactor for glutathione peridase[16]. Michael addition to a quinone ring is the most obvious method of quinone conjugation by glutathione. It has been shown previously that the addition of a thiol to *p*-benzoquinone is spontaneous and does not need to be catalyzed by a glutathione-s-transferase[9]. The role of glutathione as a reducing agent, however, has not been as extensively studied. This is primarily due to a lack of good methods for measuring the thiol and disulfide concentrations.

Liquid chromatography with electrochemical detection (LC–ED) supplies the methodology not only for measuring the phenolic benzene metabolites, but also for quantitating glutathione and the disulfide. Using a dual-electrode detector, consisting of two gold/mercury electrodes, the concentrations of both glutathione and the disulfide can be measured simultaneously[17]. The phenolic metabolites of benzene are most easily measured with the dual glassy carbon electrode in parallel, as illustrated in Fig. 2. The parallel configuration allows the detection of both phenol and quinone metabolites of benzene in the same sample with only one injection. Using these two methods of electrochemical detection, the role of glutathione in the detoxification of a quinone intermediate can be easily studied. Since *p*-benzoquinone is commercially available, it provides an ideal candidate for an investigation of this type. Presented here is the use of LC–ED in the study of glutathione oxidation during benzene metabolism.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from the following sources: benzene, J. T. Baker; phenol, monochloroacetic acid and acetonitrile, Mallinkrodt; disodium ethylenediamine tetraacetate (EDTA), 30% hydrogen peroxide, hydroquinone and ammonium acetate, Fisher Scientific; *p*-benzoquinone, Matheson, Coleman & Bell; sodium octyl

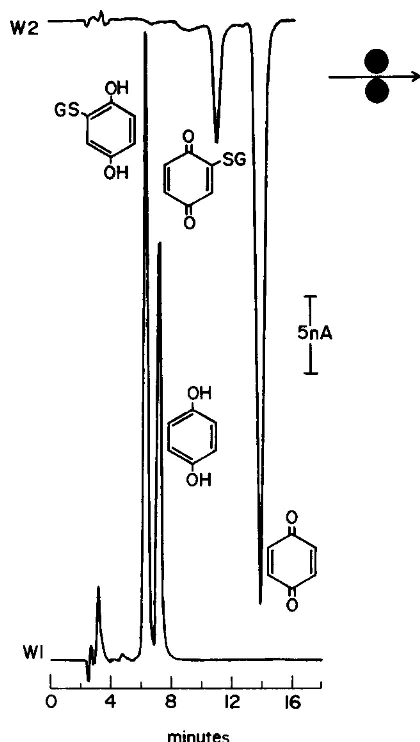


Fig. 2. Chromatogram of the oxidized and reduced forms of hydroquinone and the glutathione conjugate with a dual glassy carbon electrode in the parallel configuration. The mobile phase 0.1 *M* ammonium acetate buffer pH 4 with 5% acetonitrile. The flow-rate was 1 ml/min.

sulfate, Eastman-Kodak; oxidized and reduced glutathione, catalase, diaphorase and NADPH, Sigma. All compounds were of analytical or better grade and were used as received. Methanol was distilled in glass prior to use.

Equipment

Two Bioanalytical Systems LC-154 liquid chromatographic systems were employed for these studies. Both systems used tandem LC-4B amperometric controllers for the dual electrode studies and were modified so as to exclude oxygen. The apparatus used to deoxygenate the mobile phase and exclude oxygen from the system has been described previously[18].

The detector used for the determination of hydroquinone, the glutathione conjugate of *p*-benzoquinone, *p*-benzoquinone and the oxidized form of the glutathione conjugate consisted of a dual glassy carbon electrode in the parallel configuration. The mobile phase employed was acetonitrile–0.1 *M* ammonium acetate buffer pH 4.0 (3:97). Flow-rate was 1 ml/min. The detectors were set at +700 mV and −300 mV *vs.* a Ag/AgCl reference electrode.

A second system was set up for the determination of glutathione and glutathione disulfide. A dual gold/mercury cell in series was used in these studies. This configuration is illustrated in Fig. 3. The first electrode acts as a "post-column re-

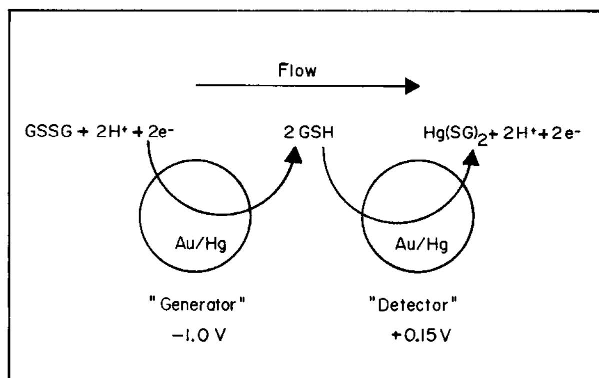


Fig. 3. Series dual gold/mercury cell for the detection of oxidized and reduced glutathione.

actor" and reduces the disulfide to the corresponding thiol, which can then be detected by the catalytic oxidation of mercury on the second electrode. The preparation of the dual gold/mercury cell has been previously described[17]. The mobile phase used qas 0.1 *M* monochloracetic acid, adjusted to pH 3, containing 4% methanol and 1 m*M* sodium octyl sulfate. The flow-rate was 1 ml/min. The first electrode was set at a potential of -1.00 V *vs.* Ag/AgCl. The downstream electrode was set at $+150$ mV *vs.* Ag/AgCl.

The samples discussed in this paper contained a large quantity of glutathione. This created a problem in the detection of small quantities of disulfide. Mercury is easily oxidized in the presence of thiols and this is the basis of the detector. However,

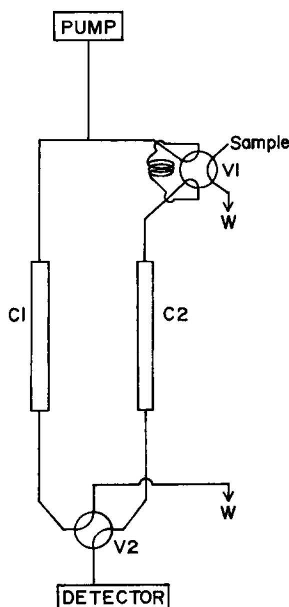


Fig. 4. valve set-up for the determination of small quantities of disulfide in the presence of large quantity of thiols (>1.0 m*M*).

when a large quantity of thiol passes over the electrode there can be a dramatic decrease in surface mercury concentration. This decrease in surface mercury leads to a reduced response and problems in the reproducibility of peak heights. The use of a low-pressure valve after the column eliminates this problem. Fig. 4 illustrates the apparatus which was utilized for the determination of the disulfide in these experiments. A second column was added to the system in order to keep the pressure difference equal within the valve. In determinations of the disulfide, glutathione can be shunted to waste so as not to ruin the gold–mercury surface with a high concentration of thiol. The valve was then switched back to the detector in order to detect the disulfide. This arrangement increased the electrode lifetime and gave more reproducible results.

The column utilized in both systems was a Biophase C_{18} 5 μm column (25 cm × 4.6 mm), purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A 20-μl injection loop was used in all studies. A Biophase 5 μm C_{18} precolumn was also placed before the analytical column in both liquid chromatographic systems. This protected the two analytical columns from the build-up of protein on top of the column. The precolumns were replaced when the back pressure for the system became too high.

Hydrodynamic voltammograms

In order to perform studies of the benzene metabolites, their electrochemical behavior on the glassy carbon electrode must be investigated. Fig. 5 shows the hydrodynamic voltammograms (HDV's) for all the metabolites of interest. These were produced by making repetitive injections of the same analyte in the liquid chromatograph and decreasing the potential by 50 mV between injections. The current response is then normalized to the current response at the limiting current plateau. This is the point on the HDV where the current does not change with potential. The HDV's for glutathione on both glassy carbon and the gold/mercury electrode have been published previously[17]. The potentials of the detectors were normally set in the range giving the maximum current response for a given quantity of compound.

Microsomal incubations

Microsomes were prepared from the livers of Swiss male mice, as described previously[11]. Protein concentrations were determined by the method of Lowry *et al.*[19] using bovine serum albumin as a standard.

Microsomal incubation mixtures of benzene, phenol, and glutathione (GSH) consisted of the following: 1 ml of microsomal protein (2 mg/ml), 3 m*M* substrate, 0.7 m*M* NADPH and 5 m*M* GSH. Incubation mixtures of hydroquinone with glutathione consisted of 500 μl of microsomal protein (3 mg/ml), 0.7 m*M* NADPH, 1 m*M* GSH and 0.1 m*M* hydroquinone. Incubations with benzene and phenol were allowed to proceed for 45 min. Hydroquinone was incubated with microsomal protein for only half an hour at 37°C. All incubations stopped with 1 *M* perchloric acid (200 μl/ml of incubation mixture) and centrifuged to remove the protein. After centrifugation, the samples were injected directly into the liquid chromatographs. All quantitative analyses were performed by LC–ED.

The extent of nonenzymatic oxidation of glutathione by *p*-benzoquinone was determined in the following manner: 0.1 m*M* *p*-benzoquinone was added to 1 m*M*

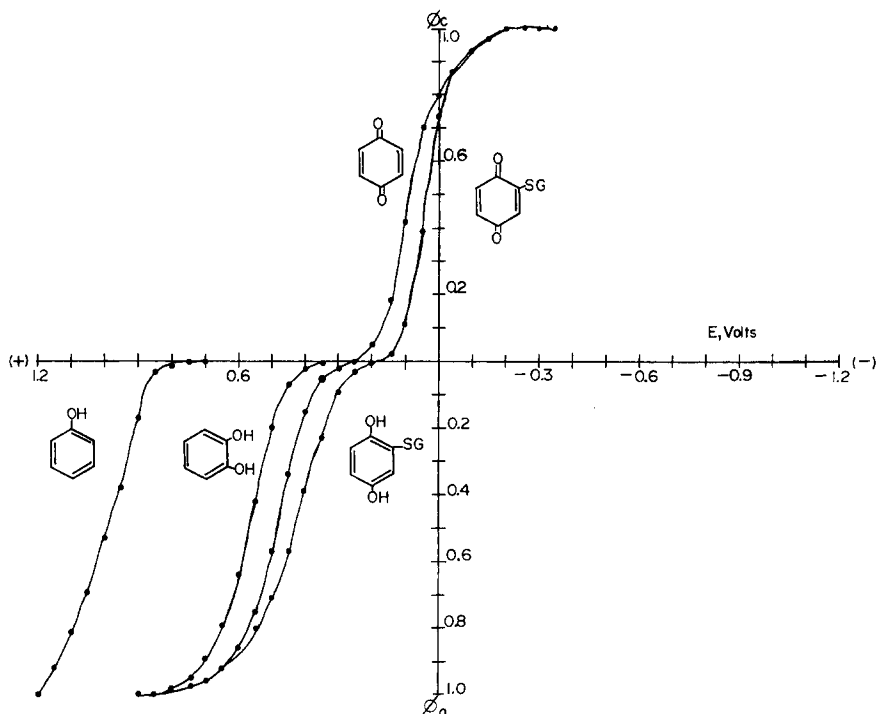


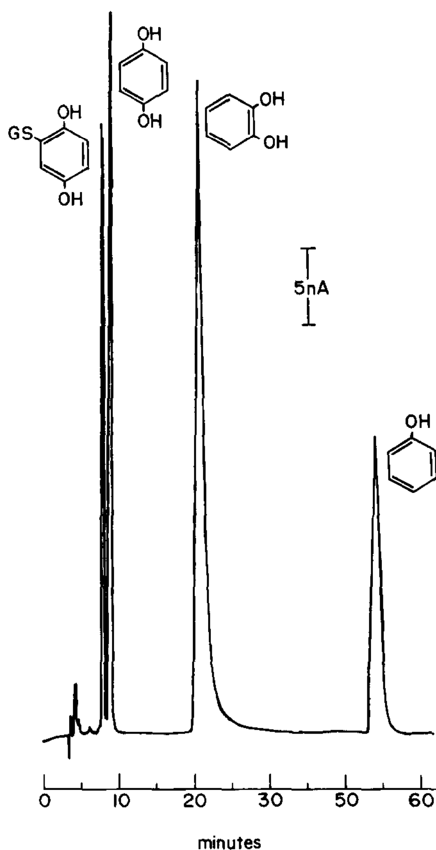
Fig. 5. Hydrodynamic voltammograms for the benzene metabolites of interest.

glutathione in potassium chloride–phosphate buffer pH 7.4. The amount of conjugate and oxidized glutathione was then determined. Oxidation of glutathione by hydrogen peroxide was also investigated by adding 4.0 m*M* hydrogen peroxide to a 1 m*M* solution of glutathione and measuring the disulfide production. The effects of EDTA on this process were also determined by repeating the experiment in 0.1% EDTA solution.

RESULTS AND DISCUSSION

A standard chromatogram of the phenolic metabolites of benzene is shown in Fig. 6. The chromatogram resulting from the use of the dual gold/mercury detector for the determination of oxidized and reduced glutathione is shown in Fig. 7. With the use of the valve switching apparatus, the electrode lifetime is considerably lengthened. The valve is normally switched at approximately 8 min. Use of this valve switching technique as well as other methods of determining small concentrations of disulfides in the presence of high concentration of thiols will be discussed elsewhere[20].

Table I shows the results of the nonenzymatic oxidation of glutathione by *p*-benzoquinone. There is little or no chemical oxidation of glutathione by *p*-benzoquinone. The completeness of the reaction can be verified by testing for unreacted *p*-benzoquinone with the dual-electrode detector. No *p*-benzoquinone was detected in the reaction mixture. The primary reaction of glutathione with *p*-benzoquinone appears to be a 1,4-Michael addition to the quinone ring.



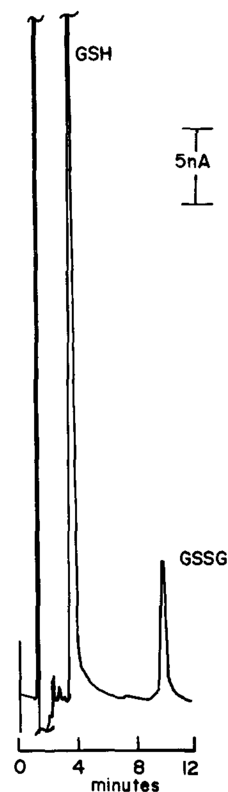


Fig. 6. Chromatogram of the easily oxidized phenolic metabolites of benzene under the same conditions as Fig. 2 except that a single electrode is set at 1.0 V *vs.* Ag/AgCl.

Fig. 7. Chromatogram of glutathione and the disulfide with dual electrode detection. The mobile phase was 0.1 *M* monochloroacetic acid pH 3 with 4% methanol. The flow-rate was 1 ml/min. The first electrode was set at -1000 mV and the second electrode at $+150$ mV *vs.* Ag/AgCl reference electrode.

TABLE I

NONENZYMATIC OXIDATION OF GLUTATHIONE AT pH 7.4

All reactions were performed in potassium chloride-phosphate buffer (pH 7.4). The total volume of reaction mixture was 10 ml. Concentrations of reactants are given in the Materials and Methods section.

Conditions	*GSSG (nmoles/ml)*
GSH	0.25
GSH + *p*-benzoquinone	0.29
GSH + hydrogen peroxide	169
GSH + hydrogen peroxide + EDTA	0.25

TABLE II

OXIDATION OF GSH DURING BENZENE AND PHENOL METABOLISM

All incubation mixtures consisted of approximately 2 mg of microsomal protein plus the indicated substrates. The data Shown are the result of three trials. Final volume was 1.260 ml.

Conditions	*GSSG (nmol/ml incubation)*
GSH	168 ± 4
GSH + NADPH	188 ± 31
GSH + benzene	103 ± 13
GSH + benzene + NADPH	197 ± 13
GSH + phenol	130 ± 4
GSH + phenol + NADPH	238 ± 7

In contrast to the nonenzymatic reaction, there is significant oxidation of glutathione in the presence of microsomal protein and NADPH (Table II). This oxidation of glutathione is independent of the substrate for cytochrome P-450 and occurs simply in the presence of microsomal protein and NADPH. The addition of diaphorase, a quinone reductase, does not inhibit the oxidation of glutathione, but does decrease the conjugate formation (Table III). However, the decrease in disulfide formation in the presence of catalase leads to the conclusion that hydrogen peroxide produced by the microsomes is the oxidizing agent responsible for the glutathione oxidation. To verify this, glutathione and hydrogen peroxide were mixed in potassium chloride–phosphate buffer and allowed to react for approximately 20 min. The oxidation of thiols by hydrogen peroxide has been previously reported[21,22]. It is also known to be further catalyzed by the presence of Fe^{3+} and Cu^{2+}. The enzyme glutathione peroxidase also catalyzes this reaction *in vivo*. Experiments with glutathione and hydrogen peroxide presented here also indicate that glutathione can be directly oxidized by hydrogen peroxide as indicated in Table I. The addition of EDTA to the reaction mixture slows down the reaction rate considerably.

On the basis of the microsomal studies it appears that the primary role of glutathione in the detoxification of quinones is 1,4-Michael addition. Glutathione also affords protection against hydrogen peroxide, produced during microsomal oxidation or mitochondrial respiration. Hydrogen peroxide could be a dismutation

TABLE III

GSSG PRODUCTION DURING HYDROQUINONE METABOLISM *IN VITRO*

All incubation mixtures contained 1.5 mg of microsomal protein, suspended in potassium chloride–phosphate buffer (pH 7.4). The total volume of incubation mixture was 0.800 ml. HQ = hydroquinone, GSH = glutathione, GSSG = oxidized glutathione, HQ–SG = glutathione conjugate of *p*-benzoquinone. The results shown above are the average and standard deviation of three trials.

Conditions	*HQ (nmol)*	*HQ–SG (nmol)*	*GSSG (nmol)*	*HQ oxidized (%)*
HQ + GSH	62 ± 11	1.0 ± 0	5.4 ± 4	77
HQ + GSH + NADPH	10 ± 5	38 ± 2	113 ± 16	12
HQ + GSH + NADPH	20 ± 6	50 ± 1	6.7 ± 4	86
HQ + GSH + NADPH	69 ± 3	5.5 ± 2	90 ± 17	24

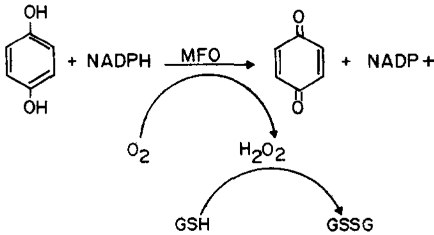


Fig. 8. Mechanism of glutathione oxidation by mouse liver microsomes during benzene and hydroquinone metabolism.

product of the superoxide ion, produced during the activation of oxygen by cytochrome P-450[23,24]. Fig. 8 shows the mechanism of oxidation, as indicated by the results presented here.

Although oxidation by hydrogen peroxide appears to be the primary source of disulfide *in vitro*, this is an artificial system which allows several parts of the cell to interact which may not normally be in the vicinity of one another. This experiment does, however, indicate that microsomal studies involving glutathione oxidation are not necessarily dependent on the substrate concentration but on the amount of hydrogen peroxide or other activated oxygen species produced by the microsomes.

The use of LC-ED for studies of benzene metabolism and glutathione oxidation clearly has several advantages. The detector has a fairly large linear range and is sensitive to picomole amounts of product. No chemical steps must be performed in order to separate the oxidized and reduced forms of the thiols. The two forms can be resolved by the column and detected independently. Samples may be analyzed immediately after incubation without the need for multiple extractions, cleanup or preconcentration. The use of the dual gold/mercury electrode can simplify toxicological studies involving glutathione depletion or disulfide formation by allowing the direct determination of oxidized glutathione (GSSG) and GSH with minimal sample preparation and virtually no endogenous interferences. Likewise, benzene metabolites can be quantified in the low picomole range in samples such as microsomal incubations without the use of radiolabelled compounds or extensive preconcentration.

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