Non-K-Region *o*-Quinones as Enzyme-Generated Inactivators of Dihydrodiol Dehydrogenase†

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Received October 11, 1988; Revised Manuscript Received January 27, 1989

ABSTRACT: Homogeneous 3α-hydroxysteroid/dihydrodiol dehydrogenase from rat liver cytosol catalyzes the NAD(P)$^+$-dependent oxidation of non-K-region *trans*-dihydrodiols of polycyclic aromatic hydrocarbons, many of which are proximate carcinogens. These reactions proceed with K_m values in the millimolar range to yield highly reactive *o*-quinones that can be trapped as thioether adducts [Smithgall, T. E., Harvey, R. G., & Penning, T. M. (1988) *J. Biol. Chem. 263*, 1814–1820]. The enzymatically generated *o*-quinones, e.g., naphthalene-1,2-dione and benzo[*a*]pyrene-7,8-dione are potent inhibitors of the dehydrogenase, yielding IC_{50} values of 5.0 and 10.0 μM, respectively. Naphthalene-1,2-dione was found to be an efficient irreversible inhibitor of the enzyme and can inactivate equimolar concentrations of the dehydrogenase, yielding a $t_{1/2}$ for the enzyme of 10 s or less. By contrast (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene promotes a slower inactivation of the dehydrogenase, yielding a K_d of 70 μM and a limiting rate constant that corresponds to a $t_{1/2}$ at saturation of 23.2 min. Inactivation by this dihydrodiol has an obligatory requirement for NADP$^+$. Examination of the k_{cat} for the oxidation of (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene yields a partition ratio for the dihydrodiol of 200 000, suggesting that alkylation from the parent dihydrodiol is a rare occurrence. Benzo[*a*]pyrene-7,8-dione, which is the product of the enzymatic oxidation of (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, also promotes a time- and concentration-dependent inactivation of the dehydrogenase. By contrast with the naphthalene-1,2-dione, inactivation with the benzo[*a*]pyrene-7,8-dione is only achieved in the presence of a large molar excess of the quinone. In addition, it is not possible to observe time- or concentration-dependent inactivation of the dehydrogenase with the parent *trans*-dihydrodiol. Gel filtration failed to restore enzyme activity following inactivation with either naphthalene-1,2-dione or benzo[*a*]pyrene-7,8-dione, suggesting that these quinones covalently modify the enzyme. Micromolar concentrations of NADP$^+$ and 1 mM reduced glutathione (GSH) protect the enzyme from inactivation by either naphthalene-1,2-dione or benzo[*a*]pyrene-7,8-dione. These results suggest that the quinones preferentially alkylate free enzyme rather than the E·NAD(P)$^+$ complex. The ability of NADP$^+$ and glutathione to retard the inactivation event suggests that enzyme-generated *o*-quinones could inactivate dihydrodiol dehydrogenase in cells and tissues depleted of these cofactors.

Polycyclic aromatic hydrocarbons are environmental pollutants that require activation by host metabolism to exert their mutagenic and carcinogenic effects. In general, the route of activation results in the formation of bay-region diol epoxides (ultimate carcinogens) which covalently modify DNA (Conney, 1982; Gelboin, 1980; Koreeda et al., 1978). The immediate precursors of the diol epoxides are non-K-region *trans*-dihydrodiols (proximate carcinogens).

Dihydrodiol dehydrogenase[1] (EC 1.3.1.20) has been implicated in the detoxification of the non-K-region *trans*-dihydrodiols. Addition of the purified enzyme to the Ames test reduced the mutagenicity of benzo[*a*]pyrene, suggesting that the enzyme can suppress diol epoxide formation by oxidizing the precursor *trans*-7,8-dihydrodiol to the noncarcinogenic catechol (Glatt et al., 1979). Recently, purified dihydrodiol dehydrogenase has been shown to oxidize the *trans*-dihydrodiols of a number of polycyclic aromatic hydrocarbons including proximate carcinogens derived from benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, 5-methylchrysene, and 7,12-dimethylbenz[*a*]anthracene (Smithgall et al., 1986, 1988a). However, the products of these reactions are not catechols but highly reactive *o*-quinones that can be trapped as thioether adducts (Smithgall et al., 1988b). Preliminary observations indicate that these quinones rapidly form 1,4-addition products with simple nucleophiles such as 2-mercaptoethanol (Smithgall et al., 1988b), and similar reactions are possible with cysteine and glutathione. This has led to the postulate that once the *o*-quinones are formed, they are readily scavenged by cellular nucleophiles, e.g., glutathione, which may lead to their detoxification. Thus, dihydrodiol dehydrogenase may initiate a series of detoxification reactions provided that cellular nucleophiles are available for quinone adduct formation (Scheme I).

In addition to undergoing nonenzymatic conjugation reactions with cysteine and glutathione, the enzyme-generated *o*-quinones also have the potential to (a) redox cycle and hence

† This research was supported in part by National Cancer Institute Grant CA 39504 and National Cancer Institute RCDA K04 CA 01335 awarded to T.M.P.

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[1] Abbreviations: dihydrodiol dehydrogenase, *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.3.1.20); 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50); androsterone, 3α-hydroxy-5α-androstan-17-one; benzene dihydrodiol, *trans*-1,2-dihydroxy-3,5-cyclohexadiene; *trans*-1,2-dihydrodiol of naphthalene, (±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene; *trans*-7,8-dihydrodiol of benzo[*a*]pyrene, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

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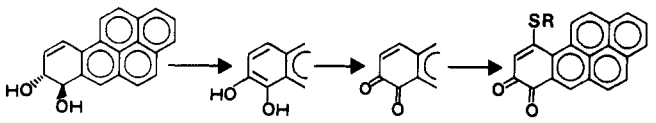
Scheme I: Reactions Initiated by Dihydrodiol Dehydrogenase



generate oxygen free radicals (superoxide anion), (b) act as substrates for glutathione *S*-transferase, (c) act as substrates for quinone reductase(s), and (d) directly alkylate macromolecules such as protein and DNA. The present study shows that under certain conditions the *o*-quinones generated by dihydrodiol dehydrogenase will covalently modify this enzyme.

Experimental Procedures

Materials. Benzene dihydrodiol was synthesized according to the procedure described by Smithgall and Penning (1986). The *trans*-1,2-dihydrodiol of naphthalene [(±)-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene] was prepared from naphthalene-1,2-dione (Platt & Oesch, 1983). Naphthalene-1,2-dione was synthesized from 2-naphthol (Aldrich) by the method of Fieser (1943). Benzo[*a*]pyrene-7,8-dione was provided by Ronald Harvey of the Ben May Cancer Institute of the University of Chicago. [1,3-^{3}H]-(±)-*trans*-7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene (364 mCi/mmol) was purchased from the National Cancer Institute Chemical Carcinogen Radiorepository, Midwest Research Institute (Kansas City, MO). Reduced glutathione was purchased from Sigma Chemical Co. (St. Louis, MO), and pyridine nucleotides were obtained from Pharmacia-LKB Biotechnology, Inc. (Piscataway, NJ).

Enzyme Purification. Dihydrodiol dehydrogenase is indistinguishable from 3α-hydroxysteroid dehydrogenase in Sprague-Dawley rat liver cytosol (Penning et al., 1984; Smithgall & Penning, 1988) and was purified from this tissue according to the published procedure (Penning et al., 1984). The purified enzyme had a specific activity of 2.0 μmol of androsterone oxidized min^{-1} mg^{-1} and 0.2 μmol of benzene dihydrodiol oxidized min^{-1} mg^{-1} under standard conditions (see below).

Standard Enzyme Assay. Enzymatic activity was measured by monitoring the oxidation of either a 3α-hydroxysteroid (androsterone) or a *trans*-dihydrodiol (benzene dihydrodiol). Androsterone assays were performed in a 1.0-mL system containing 75 μM androsterone, 2.3 mM NAD$^+$, and 4% acetonitrile in 100 mM potassium phosphate, pH 7.0. Benzene dihydrodiol assays were performed in 1.0-mL systems containing 1.0 mM benzene dihydrodiol, 2.3 mM NADP$^+$, and 4% methanol in 50 mM glycine, pH 9.0. Reactions were initiated by the addition of purified enzyme and monitored by following the increase in absorbance of the pyridine nucleotide at 340 nm; E = 6270 M^{-1} cm^{-1} for NADPH. No change in absorbance was observed in the absence of enzyme.

Inactivation Experiments. In a typical experiment, purified enzyme (250 μg) was dialyzed against 10 mM potassium phosphate, pH 7.0, plus 1 mM EDTA. The sample was then diluted to 1.3 mL, and aliquots (200 μL) were incubated in the presence of varying concentrations of either the inactivator or the inactivator plus protecting agent at 25 °C. Over time, portions (10 μL) were removed and diluted into 1-mL cuvettes that contained all the components of the 1-mL androsterone assay minus steroid. Once 12 time points had been taken, the samples were assayed for enzymatic activity by the addition of androsterone to the cuvettes. Since these assays are performed on samples that have been diluted 100-fold, the activity measured is the percent activity remaining and gives a measure of enzyme inactivation. Under these conditions the quinone is sufficiently diluted so that it is unable to affect enzyme measurements by acting as a reversible inhibitor.

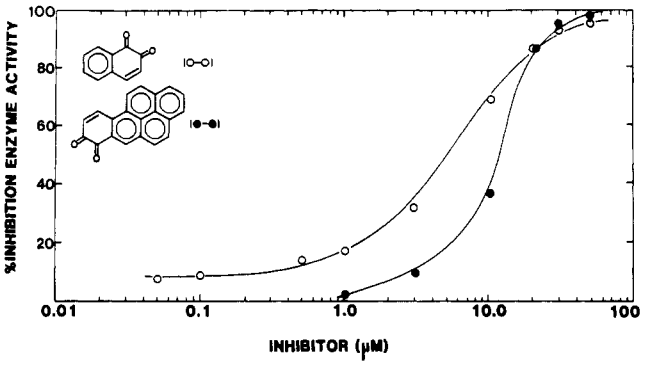


FIGURE 1: Inhibition of dihydrodiol dehydrogenase by naphthalene-1,2-dione and benzo[*a*]pyrene-7,8-dione. Dihydrodiol dehydrogenase activity was assayed in the presence of increasing concentrations of *o*-quinone. Since dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase reactions are catalyzed by the same enzyme, the more sensitive steroid assay was used to detect enzyme activity. The standard assay contained 75 μM androsterone (3α-hydroxysteroid), 2.3 mM NAD$^+$, 100 mM potassium phosphate, pH 7.0, and 4% DMSO. Reactions were initiated by the addition of the enzyme (1.86 μg), and the progress of the reaction was measured by monitoring the absorbance of the reduced pyridine nucleotide at 340 nm. The points shown represent single determinations.

Results

Polycyclic Aromatic o-Quinones as Inhibitors of Dihydrodiol Dehydrogenase. Non-K-region *trans*-dihydrodiols of polycyclic aromatic hydrocarbons are oxidized by dihydrodiol dehydrogenase with K_m values in the millimolar range (Smithgall et al., 1986). By contrast, *o*-quinones corresponding to the products of *trans*-dihydrodiol oxidation can inhibit the enzyme at concentrations at least 100-fold lower. Thus, naphthalene-1,2-dione and benzo[*a*]pyrene-7,8-dione yield IC_{50} values of 5.0 and 10.0 μM, respectively (Figure 1).

Enzymatic Oxidation of the trans-1,2-Dihydrodiol of Naphthalene in the Presence and Absence of the Quinone Scavenger 2-Mercaptoethanol. Comparison of the extent of enzymatic oxidation of the *trans*-1,2-dihydrodiol of naphthalene in the presence and absence of 2-mercaptoethanol shows that reactions devoid of thiol reached less than 20% of their theoretical end point (Figure 2A). This finding suggests that the *o*-quinone formed from the enzymatic oxidation of the diol inhibits the dehydrogenase, either by acting as a tight-binding inhibitor or by irreversible alkylation. 2-Mercaptoethanol presumably blocks inhibition of the enzyme by rapid addition to the reactive 4-position of the quinone (Smithgall et al., 1988b). Although the presence of the thiol reagent enhances the end point of the reaction, it has no effect on the initial velocity of *trans*-1,2-dihydrodiol oxidation (Figure 2B).

It should be emphasized that previous studies which examined the substrate and stereospecificity of purified dihydrodiol dehydrogenase for *trans*-dihydrodiols were not complicated by *o*-quinone-mediated enzyme inhibition (Smithgall et al., 1986). In these earlier studies *trans*-dihydrodiol oxidation was followed in the presence of either 50 mM glycine, pH 9.0, or 50 μM 2-mercaptoethanol, both of which can scavenge the quinones as they are formed.

Naphthalene-1,2-dione as an Irreversible Inhibitor of Dihydrodiol Dehydrogenase. In order to determine the mechanism of quinone-mediated inhibition of the dehydrogenase, the kinetics of this reaction were studied in more detail. Naphthalene-1,2-dione produces a rapid time- and concen-

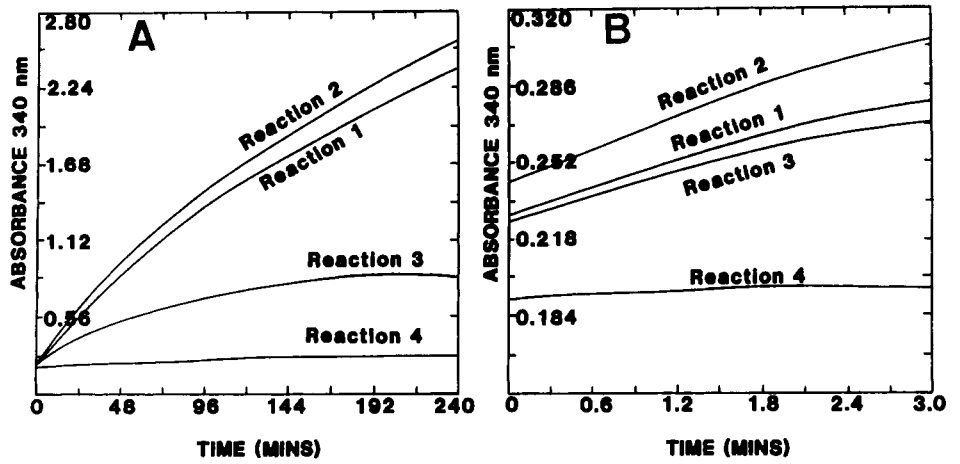


FIGURE 2: Enzymatic oxidation of the *trans*-1,2-dihydrodiol of naphthalene in the presence and absence of 2-mercaptoethanol. Reaction 1 contained 5.0 mM *trans*-1,2-dihydrodiol of naphthalene, 2.3 mM $NADP^+$, and 2.5 μM enzyme in potassium phosphate, pH 7.0, plus 2 mM 2-mercaptoethanol. Reaction 2 was the same as reaction 1 except the thiol concentration was 1 mM. Reaction 3 was the same as reaction 1 except no thiol was present. Reaction 4 was the same as reaction 1 except no enzyme was present. Panel A shows the entire time course; panel B shows the initial velocity of each reaction. The progress curves shown were obtained on a Beckman DU-7 spectrophotometer, which took data points every 15 s. A single progress curve may contain up to 960 data points. For clarity individual data points are not shown.

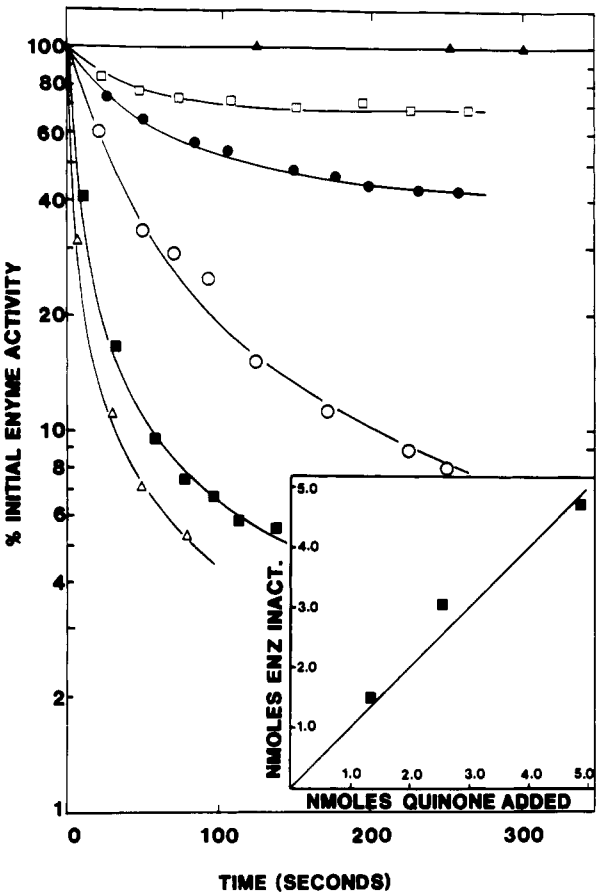


FIGURE 3: Time-dependent inactivation of dihydrodiol dehydrogenase by naphthalene-1,2-dione. Incubations contained 5.2 μM purified dihydrodiol dehydrogenase plus 1.25–10.0 μM naphthalene-1,2-dione in a final volume of 200 μL of 10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 8% DMSO. Time courses for enzyme inactivation by (▲) 0 μM quinone, (□) 1.25 μM quinone, (●) 2.5 μM quinone, (○) 5.0 μM quinone, (■) 7.5 μM quinone, and (△) 10.0 μM quinone are shown. At the times indicated, aliquots (10 μL) were diluted into 1-mL assay systems containing 100 mM potassium phosphate, pH 7.0, 2.3 mM NAD^+, and 4% acetonitrile. Reactions were initiated by the addition of 75 μM androsterone. Since the dilution factor for the quinone is 100-fold, the activity determined in these reactions is a measure of the amount of enzyme activity remaining. The inset shows a plot of the number of nanomoles of enzyme inactivated versus the number of nanomoles of quinone added and yields a slope of 1.0.

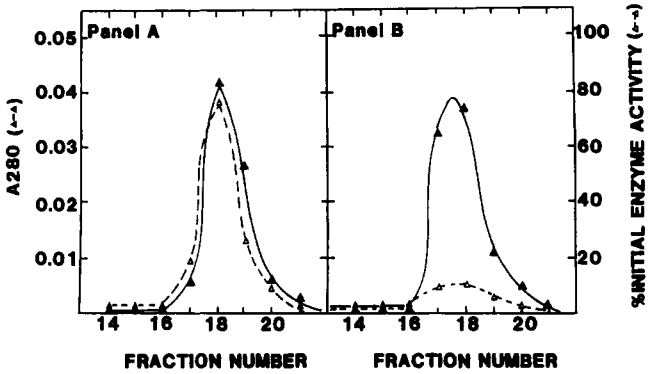


FIGURE 4: Gel-exclusion chromatography of native enzyme versus enzyme inactivated with naphthalene-1,2-dione. Control incubation (panel A): Purified dihydrodiol dehydrogenase (8.5 μM) was incubated in 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 8% DMSO for 30 min and then applied to a Sephadex G-25F column. The column was eluted in 10 mM potassium phosphate, pH 7.0, plus 1 mM EDTA. The A_{280} (▲) and enzyme activity (△) in each 1-mL fraction were measured. Experimental incubation (panel B): Purified dihydrodiol dehydrogenase (8.5 μM) was inactivated with a stoichiometric amount of naphthalene-1,2-dione. At the end of 30 min, 15% of the enzyme activity remained. The sample was applied and eluted from a Sephadex G-25F column as described above. The A_{280} (▲) and enzyme activity (△) in each 1-mL fraction were measured.

tration-dependent inactivation of dihydrodiol dehydrogenase (Figure 3). A number of unusual features are apparent. First, the $t_{1/2}$ of the enzyme in the presence of quinone is exceedingly short. Concentrations of the quinone that exceed the enzyme concentration by only 2-fold inactivate the enzyme with a $t_{1/2}$ of less than 10 s. Second, the degree of inactivation is stoichiometric. Incubations containing 1.25, 2.5, and 5.0 μM naphthalene-1,2-dione inactivate 25%, 50%, and 100% of a 5.0 μM solution of enzyme, respectively. Because of this kinetic behavior, the time-dependent inactivation observed cannot be analyzed by first-order kinetics. Further evidence for covalent bond formation between the quinone and the enzyme comes from gel-filtration experiments in which native and quinone-inactivated enzymes were applied to a Sephadex G-25F exclusion column. The quinone-inactivated enzyme showed no restoration of activity following chromatography, while the native enzyme retained full activity under these conditions (Figure 4).

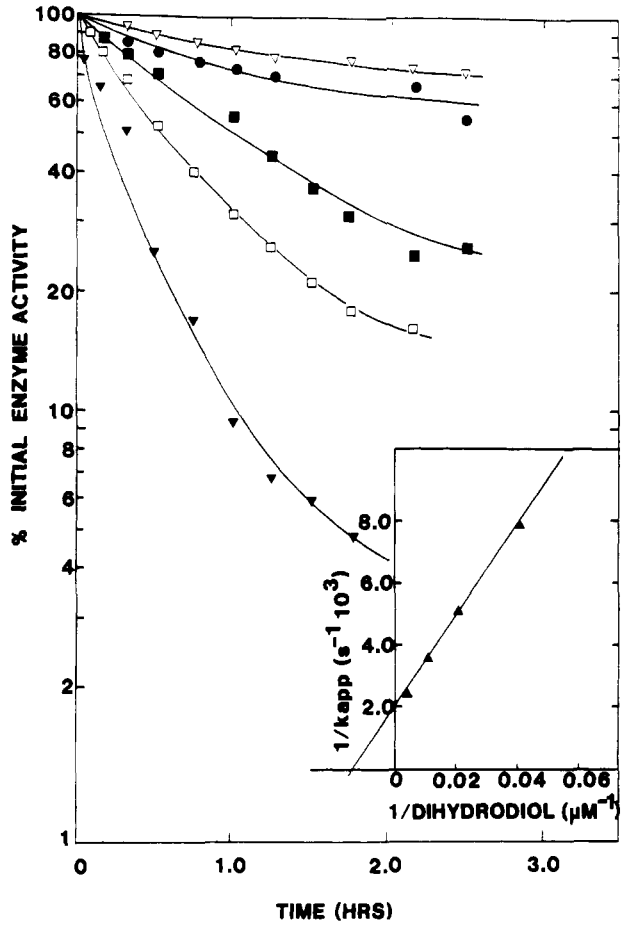


FIGURE 5: Time-dependent inactivation of dihydrodiol dehydrogenase by the *trans*-1,2-dihydrodiol of naphthalene. Incubations contained 5.0 μM purified dihydrodiol dehydrogenase, 10–250 μM *trans*-1,2-dihydrodiol of naphthalene (Npdiol), and 50 μM $NADP^+$ dissolved in 200 μL of potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 8% DMSO. Time courses for enzyme inactivation by (▽) 10 μM Npdiol, (●) 25 μM Npdiol, (■) 50 μM Npdiol, (□) 100 μM Npdiol, and (▼) 250 μM Npdiol are shown. At the times indicated, aliquots (10 μL) were removed and diluted into the standard androsterone assay as described. The inset shows a transformation of the data where $1/k_{app}$ (pseudo-first-order rate constant for inactivation) was plotted against 1/[inactivator] (Kitz & Wilson, 1962). This plot yields the $1/k + 2$ (limiting rate constant for inactivation) and the K_d (dissociation constant of the reversible enzyme–inhibitor complex).

Examination of the trans-1,2-Dihydrodiol of Naphthalene as a Suicide Substrate of Dihydrodiol Dehydrogenase. Since time-dependent inactivation of dihydrodiol dehydrogenase can be achieved with naphthalene-1,2-dione, the ability to observe inactivation in the presence of the *trans*-1,2-dihydrodiol of naphthalene and $NAD(P)^+$ was examined. Incubations that contained stoichiometric amounts of the *trans*-dihydrodiol of naphthalene and $NADP^+$ resulted in slow time-dependent inactivation of the enzyme (Figure 5). No inactivation could be observed in the absence of $NADP^+$, suggesting that enzymatic oxidation of the diol to the quinone is an obligatory step in the inactivation mechanism. Analysis of the inactivation data (Kitz & Wilson, 1962) gave a limiting rate constant that corresponded to a $t_{1/2}$ for the enzyme when saturated with quinone of 23.2 min. Knowing the k_{cat} for *trans*-dihydrodiol oxidation, a partition ratio for this suicide substrate of greater than 200 000 was calculated. This suggests that alkylation resulting from the oxidation of the *trans*-1,2-dihydrodiol of naphthalene is a rare event.

Enzymatic Oxidation of the trans-7,8-Dihydrodiol of Benzo[a]pyrene (trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]-pyrene) in the Presence and Absence of an o-Quinone Scavenger. Parallel studies to those described with the *trans*-1,2-dihydrodiol of naphthalene were conducted with the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene. When the enzymatic oxidation of the racemic (±)-[^{3}H]-7,8-*trans*-dihydrodiol of benzo[*a*]pyrene was followed in the presence or absence of thiol, there was no difference in the initial velocity or the end point of the reaction (Figure 6). This suggests that the enzyme-generated quinone, namely, benzo[*a*]pyrene-7,8-dione, is less effective as an enzyme inhibitor or inactivator than naphthalene-1,2-dione. This finding may be explained by the differences in reactivity of these two *o*-quinones. Previous studies indicate that naphthalene-1,2-dione is far more reactive than the benzo[*a*]pyrene-7,8-dione toward simple nucleophiles (Smithgall et al., 1988b). This difference in reactivity may be explained by the presence of a hindered C-10 position within the bay region of benzo[*a*]pyrene-7,8-dione.

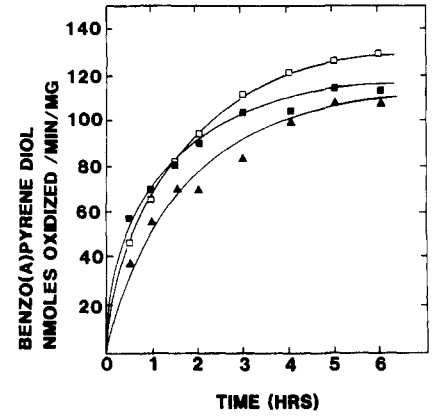


FIGURE 6: Enzymatic oxidation of the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene in the presence and absence of 2-mercaptoethanol. Incubations contained 20 μM [^{3}H]-*trans*-7,8-dihydrodiol of benzo[*a*]pyrene (200 000 cpm), 2.7 μM enzyme, 2.3 mM NAD^+, and 8% DMSO in 100 μL of 10 mM potassium phosphate buffer, pH 7.0, in the presence of 0 μM 2-mercaptoethanol (□), 20 μM 2-mercaptoethanol (■), or 1.0 mM 2-mercaptoethanol (▲). Reactions were initiated by the addition of enzyme and incubated at 37 °C. Reactions were quenched by the addition of ethyl acetate; the unreacted diol was extracted and applied to TLC plates (Smithgall & Penning, 1986). The amount of diol remaining was calculated from the specific radioactivity of the isotope after controlling for the recovery of the radionuclide and the counting efficiency. Subtraction of this value from the initial diol concentration gave estimates of the amount of diol oxidized. Each point represents the mean of triplicate determinations. The SD for each point was less than 10% and has been removed for clarity.

Benzo[a]pyrene-7,8-dione as an Irreversible Inhibitor of Dihydrodiol Dehydrogenase. Benzo[*a*]pyrene-7,8-dione promotes a time- and concentration-dependent inactivation of dihydrodiol dehydrogenase (Figure 7). However, the kinetics differ significantly from those observed with naphthalene-1,2-dione. First, a large excess of quinone over enzyme is necessary to achieve complete enzyme inactivation. Second, the rate of inactivation was considerably slower, yielding a $t_{1/2}$ on the order of minutes rather than seconds. In this instance, a first-order relationship between loss of enzyme activity and concentration of quinone can be assumed, allowing calculation of a limiting rate constant for inactivation that corresponds to a $t_{1/2}$ for the enzyme of 21 s and a K_d of 100 μM for the reversible enzyme–quinone complex. Gel-filtration experiments also establish that enzyme inactivated with benzo[*a*]-pyrene-7,8-dione is covalently modified by the quinone. Thus chromatography of the inactivated enzyme fails to restore enzyme activity while native enzyme retains full enzyme activity under these conditions (data not shown).

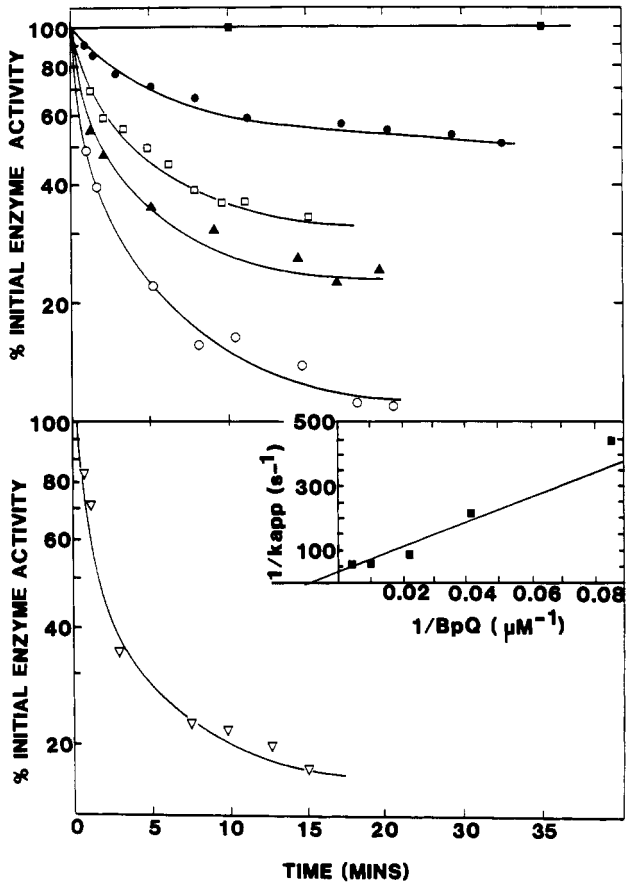


FIGURE 7: Time-dependent inactivation of the dihydrodiol dehydrogenase by benzo[*a*]pyrene-7,8-dione (BpQ). Incubations contained 4.0 μM purified dihydrodiol dehydrogenase plus 11.4–142.5 μM benzo[*a*]pyrene-7,8-dione in a final volume of 200 μL of 10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 8% DMSO. Time courses for enzyme inactivation by (■) 0 μM BpQ, (●) 11.4 μM BpQ, (□) 22.8 μM BpQ, (▲) 45.6 μM BpQ, (○) 91.2 μM BpQ, and (▽) 142.5 μM BpQ are shown. At the times indicated, aliquots (10 μL) were diluted into a 1-mL assay containing 100 mM potassium phosphate, pH 7.0, 2.3 mM NAD^+, and 4% acetonitrile. Reactions were initiated by the addition of 75 μM androsterone. Since the dilution factor for the quinone is 100-fold, the activity determined in these reactions is a measure of the amount of enzyme activity remaining. The inset shows a transformation of the data where $1/k_{app}$ is plotted against 1/[inactivator] (see legend to Figure 4).

Examination of the trans-7,8-Dihydrodiol of Benzo[a]pyrene as a Suicide Substrate of Dihydrodiol Dehydrogenase. Incubation of purified dihydrodiol dehydrogenase with the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene and pyridine nucleotide resulted in a small time-dependent loss of enzyme activity over 6 h (Figure 8). This loss of activity was only slightly greater than that observed in control incubations. Increasing the amount of $NADP^+$ to enhance the formation of benzo[*a*]pyrene-7,8-dione (inactivator) did not increase the extent of inactivation. It is concluded that the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene is essentially incapable of acting as a suicide substrate for dihydrodiol dehydrogenase.

$NADP^+$ and Glutathione Protect Dihydrodiol Dehydrogenase against Inactivation Mediated by either Naphthalene-1,2-dione or Benzo[a]pyrene-7,8-dione. Inactivation of purified dihydrodiol dehydrogenase by naphthalene-1,2-dione can be retarded by the presence of low micromolar concentrations of $NADP^+$ (10.0 μM) and millimolar concentrations of GSH (1 mM). Identical results can be obtained with benzo[*a*]pyrene-7,8-dione (Figure 9). These data suggest that (a) the enzyme-generated *o*-quinones prefer to alkylate free enzyme rather than the E·$NADP^+$ complex and (b) a

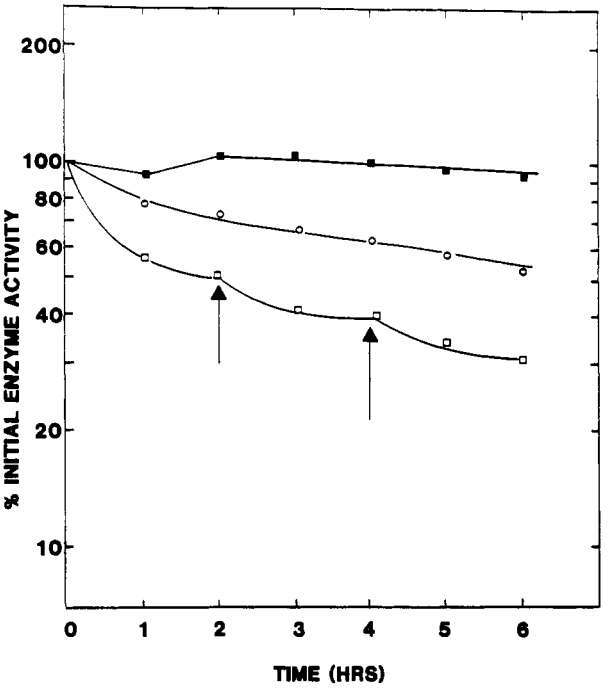


FIGURE 8: Inactivation of dihydrodiol dehydrogenase by the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene. Incubations contained 2.75 μM purified dihydrodiol dehydrogenase, 20 μM *trans*-7,8-dihydrodiol of benzo[*a*]pyrene, and 23 μM $NADP^+$ in 200 μL of potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 8% DMSO (□). At the times indicated by the arrows, an additional 23 μM $NADP^+$ was added. At various times, aliquots (10 μL) were removed and diluted into the standard androsterone assay, and the amount of enzyme activity remaining was determined. Time courses for control incubations that contained either enzyme alone (■) or enzyme plus *trans*-7,8-dihydrodiol of benzo[*a*]pyrene (○) are also shown.

cellular nucleophile such as glutathione, at physiological concentrations, can offer significant protection by scavenging the *o*-quinones. It should be emphasized that the protection afforded by $NAD(P)^+$ against inactivation with naphthalene-1,2-dione was only followed over 300 s. Over several hours $NADP^+$ is insufficient to offer complete protection since over this time course the *trans*-1,2-dihydrodiol of naphthalene inactivates the enzyme in the absence of a thiol scavenger (Figure 2A).

Discussion

Dihydrodiol dehydrogenase may initiate a series of detoxification reactions in which *trans*-dihydrodiols of polycyclic aromatic hydrocarbons, many of which are proximate carcinogens, are oxidized to *o*-quinones that can be scavenged by cellular nucleophiles (Smithgall et al., 1986). Since the discovery that *o*-quinones rather than catechols were products of this reaction, we have been examining the reactivity of the *o*-quinones in more detail.

The present paper establishes that in the absence of suitable protecting agents the enzymatically generated *o*-quinones can covalently modify dihydrodiol dehydrogenase to produce inactivated enzyme. Naphthalene-1,2-dione is a potent, rapid, and stoichiometric inactivator and can be used to titrate the dehydrogenase. By contrast, benzo[*a*]pyrene-7,8-dione is a less potent, slow enzyme inactivator, and a large molar excess is required to inactivate the dehydrogenase. Inactivation by the parent dihydrodiols can only be accomplished in the naphthalene series and appears to occur only once in several hundred thousand turnovers of substrate. Low micromolar concentrations of $NADP^+$ and millimolar concentrations of glutathione protect the enzyme from inactivation. This sug-

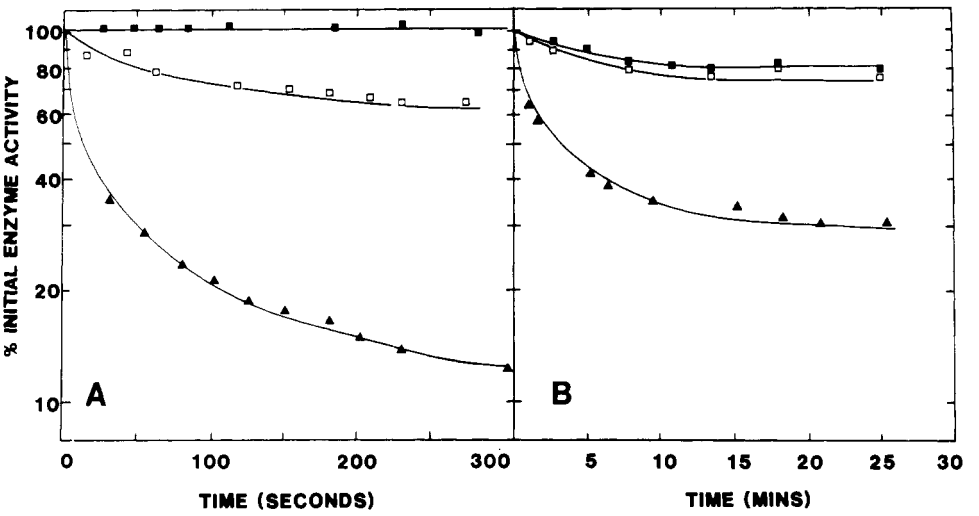
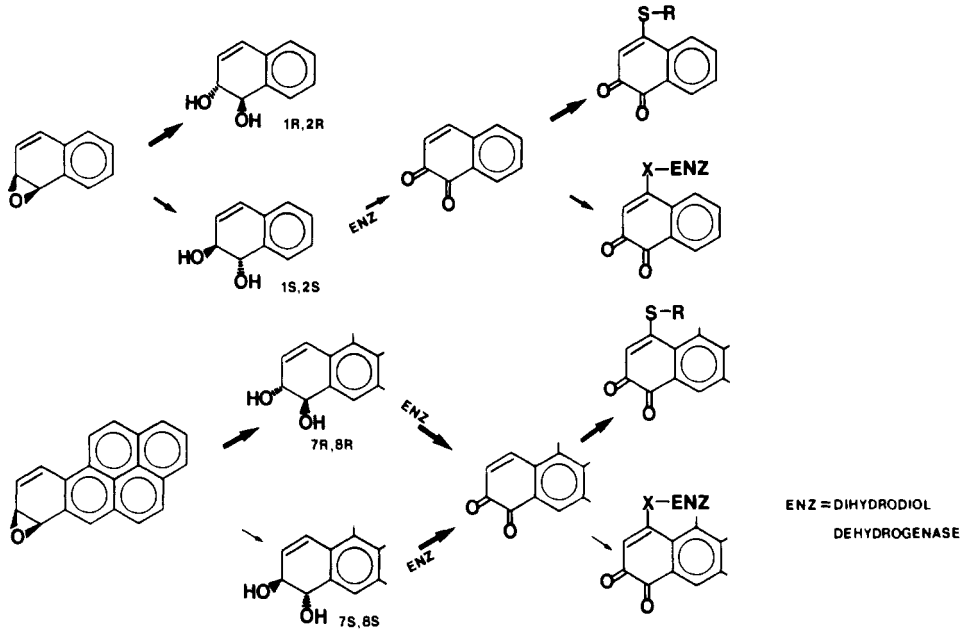


FIGURE 9: NAD(P)$^+$ and glutathione protect dihydrodiol dehydrogenase from inactivation by naphthalene-1,2-dione and benzo[*a*]pyrene-7,8-dione. Incubations (panel A) contained 6.4 μM purified dihydrodiol dehydrogenase and 10 μM naphthalene-1,2-dione either alone (▲) or with 10 μM NADP$^+$ (□) or with 1 mM GSH (■) in 200 μL of potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 8% DMSO. At the times indicated, aliquots (10 μL) were removed and diluted into the standard androsterone assay, and the amount of enzyme activity remaining was determined. Incubations (panel B) contained 6.4 μM purified dihydrodiol dehydrogenase and 40 μM benzo[*a*]pyrene-7,8-dione either alone (▲) or with 10 μM NADP$^+$ (□) or with 1 mM GSH (■) in 200 μL of potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 8% DMSO. At the times indicated, aliquots (10 μL) were removed and diluted into the standard androsterone assay, and the amount of enzyme activity remaining was determined.

Scheme II: Metabolism of Arene Oxides to *o*-Quinone Conjugates



gests that dihydrodiol dehydrogenase may play a sacrificial role when there is cellular depletion of these cofactors.

The significance of these findings is summarized in Scheme II. In the case of naphthalene, in vivo formation of the *trans*-1,2-dihydrodiol from the corresponding arene oxide most often leads to a racemic mixture (Jerina et al., 1970). Dihydrodiol dehydrogenase will then oxidize only the (+)-*S*,*S* isomer to yield the naphthalene-1,2-dione which in the presence of thiol and low micromolar concentrations of NADP$^+$ will be driven to adduct formation. Thus inactivation of the dehydrogenase will be an unusual occurrence.

In the case of the benzo[*a*]pyrene series, the non-K-region 7,8-epoxide is predominantly transformed to the (−)-7*R*,8*R*-*trans*-dihydrodiol (Yang et al., 1985). However, dihydrodiol dehydrogenase can convert both the (−)-7*R*,8*R* and (+)-7*S*,8*S* isomers to the corresponding 7,8-dione (Smithgall et al., 1986). Since this compound is less reactive than naphthalene-1,2-dione with simple nucleophiles, slow inactivation of the dehydrogenase may proceed under conditions of glutathione and NADP$^+$ depletion.

It has been reported that dihydrodiol dehydrogenase can also function as a quinone reductase. Indeed, 9,10-phenanthrenequinone is the substrate with the highest turnover number (Penning et al., 1984). This observation suggests that the quinone inactivators of the dehydrogenase could, under appropriate conditions, act as substrates in the reverse direction. A study on the quinone substrate specificity of the dehydrogenase shows that the K-region *o*-quinones of polycyclic aromatic hydrocarbons are much preferred as substrates over the non-K-region *o*-quinones (Oesch et al., 1984). For example, benzo[*a*]pyrene-7,8-dione was reduced at a rate that was only $^1/_{40}$th of that observed for benzo[*a*]pyrene-4,5-dione.

These observations are supported by our own findings, which indicate that non-K-region *o*-quinones are not efficiently reduced by the enzyme (Penning, unpublished results).

Although it has been our working hypothesis that dihydrodiol dehydrogenase plays essentially a detoxification role in polycyclic aromatic hydrocarbon metabolism, the data presented here provide the first direct evidence that *o*-quinones generated by dihydrodiol dehydrogenase can cause damage to macromolecules. In this regard it is important to note that inactivation can be acheived with the quinone (fully oxidized) in the absence of NADPH, suggesting that covalent modification of the enzyme occurs independently of any damage that may arise from the generation of oxygen free radicals.

The formation of reactive radicals by redox cycling (e.g., semiquinones, superoxide anion) is known to be responsible for many of the toxic effects of quinones at the cellular level. Recently, adducts have been well characterized between deoxyguanosine and either benzoquinone or hydroquinone plus ferric chloride (Jowa et al., 1986). Additional ESR data have provided evidence that semiquinone anion radicals result from the addition of amino acids, peptides, and proteins to quinones derived from catechols and catecholamines (Kalyanaraman et al., 1987). Conceivably, covalent bond formation between the *o*-quinones and dihydrodiol dehydrogenase could occur by a free radical mechanism resulting in the formation of a semiquinone radical attached to the enzyme. The formation of such a semiquinone could then act catalytically to promote the inactivation of other dihydrodiol dehydrogenase molecules. However, the stoichiometric titration of enzyme activity with naphthalene-1,2-dione argues against this possibility.

We have also indicated that once the *o*-quinones are scavenged as thioether adducts of glutathione, this could lead to their rapid excretion as water-soluble metabolites. This hypothesis may require some reassessment in light of the recent findings that glutathione adducts of menadione and toluquinone can redox cycle as their glutathione conjugates (Wefers & Sies, 1983). Thus whether or not adduct formation represents a detoxification route will depend upon the relative rates of redox cycling and excretion of *o*-quinone glutathione conjugates.

It is clear from the foregoing discussion that the *o*-quinones generated by dihydrodiol dehydrogenase have the potential to alkylate protein and form semiquinone radicals following simple addition reactions. Additionally, cysteinyl and glutathionyl adducts of the *o*-quinones may redox cycle to generate oxygen free radicals. A complete assessment of the reactivity of these *o*-quinones is therefore necessary to address the potential role of dihydrodiol dehydrogenase in polycyclic aromatic hydrocarbon metabolism.

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