Radiochemical Detection of Dihydrodiol Dehydrogenase: Distribution of the Enzyme in Male Sprague-Dawley Rat Tissues and Its Sensitivity to Inhibition by Indomethacin and 6-Medroxyprogesterone Acetate

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ABSTRACT

Dihydrodiol dehydrogenase (EC 1.1.1.10) catalyzes the NADP+-dependent oxidation of (−)7-R,8R-dihydroxy-dihydro-benz(a)pyrene and (+)-7S,8S-dihydroxy-dihydro-benz(a)pyrene, which are potent proximate carcinogens (Smithgall, Harvey, and Penning, J. Biol. Chem., 261: 6184–6191, 1986). Using benzenedihydrodiol [(+)-trans-1,2-dihydroxy-3,5-cyclohexadiene] as a model substrate for these reactions, dihydrodiol dehydrogenase can be assayed in rat liver cytosol by measuring the change in absorbance of the pyridine nucleoide. This method lacks the sensitivity to detect the enzyme in extrahepatic tissues. Here we describe a sensitive radiochemical assay for dihydrodiol dehydrogenase in which the oxidation of benzenedihydrodiol to pyrocatechol is coupled to O-methylation catalyzed by catechol-O-methyltransferase (EC 2.1.1.6). In this oxidized pyrocatechol formed in the oxidation step can be radio-labeled using [3H]S-adenosyl-[methyl-3H]methionine as methyl donor. The resulting tritiated product, guaiacol, is readily extracted into toluene and quantified by scintillation counting. Using [3H]S-adenosyl-[methyl-3H]methionine at a specific activity of 0.1 μCi/nmol, the assay provides a 5000-fold increase in sensitivity over the existing spectrophotometric method. The radiochemical assay was validated by comparing the K₅₀ and V₅₀ values obtained for the 40–75% (NH₄)₂SO₄ fraction of rat liver cytosol with those measured spectrophotometrically. There was close agreement between values determined radiochemically (K₅₀ = 0.77 ± 0.11 mM, V₅₀ = 2.14 ± 0.13 nmol/min/mg protein) and values determined spectrophotometrically (K₅₀ = 0.96 ± 0.10 mM, V₅₀ = 6.31 ± 0.50 nmol/min/mg protein). Using the radiochemical method, dihydrodiol dehydrogenase activity was detected in extrahepatic sites of polycyclic aromatic hydrocarbon metabolism: lung > small intestine > testis > bladder > prostate. Specific activities varied over 50-fold (0.866–0.007 nmol/min/mg protein) and did not show a strict inverse correlation with organ susceptibility to chemical carcinogens. Such a study has been hampered by the absence of a sensitive enzyme assay. This paper describes a highly sensitive radiochemical assay for the detection of dihydrodiol dehydrogenase in which the oxidation of benzenedihydrodiol to pyrocatechol is coupled to O-methylation catalyzed by catechol-O-methyltransferase (EC 2.1.1.6) in which S-adenosyl[methyl-3H]methionine is used as methyl donor (Fig. 1). This method provides a 5000-fold increase in sensitivity over the existing spectrophotometric method. We have used this assay to detect dihydrodiol dehydrogenase in extrahepatic rat tissue cytosols for the first time, and have determined the sensitivity of these activities to inhibition by indomethacin and 6-medroxyprogesterone acetate.

INTRODUCTION

Dihydrodiol dehydrogenase catalyzes the NADP+-dependent oxidation of benzenedihydrodiol to form catechol, a reaction that was first described in acetone powders of rabbit liver by Ayengar et al. (1). This reaction is believed to be a model for the oxidation of trans-dihydriodols of polycyclic aromatic hydrocarbons which are proximate carcinogens formed in vivo. Using benzenedihydrodiol as substrate, the dehydrogenase has been purified to homogeneity from rat liver cytosol by two independent methods (2, 3). Mutagenicity studies have shown that when the purified enzyme is added to the Ames test, in the presence of mouse liver microsomes and NADPH, the number of revertants normally observed with benzo(a)pyrene is significantly reduced (4). These indirect observations support the view that the enzyme may detoxify the trans-dihydrodiol proximate carcinogens formed in situ by oxidizing them to less reactive intermediates. Most recently, studies from this laboratory (5) have shown that the homogeneous enzyme can catalyze the NADP+-dependent oxidation of (−)7-R,8R-dihydroxy-dihydro-benz(a)pyrene and (+)-7S,8S-dihydroxy-dihydro-benz(a)pyrene which are the major and minor trans-dihydrodiol proximate carcinogens formed during the activation of benzo(a)pyrene. By catalyzing these reactions, the enzyme can suppress the formation of the ultimate carcinogens, the anti- and syn-diol-epoxides of benzo(a)pyrene.

The oxidation of trans-dihydriodols of benzo(a)pyrene catalyzed by the purified dehydrogenase is potentially inhibited in a persistent manner by therapeutic concentrations of indomethacin (IC₅₀ = 10 μM) (6). Others have shown that the oxidation of benzenedihydrodiol catalyzed by the homogeneous enzyme is potently inhibited by submicromolar concentrations of 6-medroxyprogesterone acetate, a synthetic progesterin (7). It is conceivable, then, that the nonsteroidal antiinflammatory drugs and synthetic progestins may modulate the metabolism of PAH by inhibiting the flux of trans-dihydriodols through this potentially important detoxification pathway and, therefore, may have important implications for the initiation phase of chemical carcinogenesis.

A knowledge of the distribution of dihydrodiol dehydrogenase in rat tissues would greatly enhance our understanding of the significance of trans-dihydrodiol oxidation in the detoxification of chemical carcinogens. Such a study has been hampered by the absence of a sensitive enzyme assay. This paper describes a highly sensitive radiochemical assay for the detection of dihydrodiol dehydrogenase in which the oxidation of benzene-dihydriodiol to catechol is coupled to O-methylation catalyzed by catechol-O-methyltransferase (EC 2.1.1.6) in which S-adenosyl[methyl-3H]methionine is used as methyl donor (Fig. 1). This method provides a 5000-fold increase in sensitivity over the existing spectrophotometric method. We have used this assay to detect dihydrodiol dehydrogenase in extrahepatic rat tissue cytosols for the first time, and have determined the sensitivity of these activities to inhibition by indomethacin and 6-medroxyprogesterone acetate.

MATERIALS AND METHODS

Chemicals. S-Adenosyl-l-methionine (p-toluene sulfonate salt) was a product of the Sigma Chemical Co. (St. Louis, MO). S-Adenosyl-l-
The oxidation of benzenedihydrodiol in air to catechol. The amount of nonenzymatic rates appear to remain linear for as long as 4 h. These counter with a machine efficiency for tritium of 53%. Using the specific radioactivity of $[^3\text{H}]\text{SAM}$ in the assay, the number of nmol of guaiacol containing 6.67 g 2,5-diphenyloxazole and 83.3 mg p-Ms-2-(5-phenyloxazolyl)benzene per liter. The amount of $[^3\text{H}]\text{guaiacol}$ formed was quantified by counting samples in a Tracer model 43 scintillation counter.

Radiochemical Assay for Dihydrodiol Dehydrogenase. Assays were conducted in 1.5-m1 microcentrifuge tubes containing 50 mM KPO$_4$ buffer of pH 7.8, 1.0 mM dithiothreitol, 1.0 mM MgCl$_2$, 2.3 mM NADP$^+$, 50 mM $[^3\text{H}]\text{SAM}$ (0.1 $\mu$Ci/nmol), 10 units of COMT, and 1.0 mM benzenedihydrodiol in a final volume of 0.1 ml. The composition of this assay requires further comment. The concentration of SAM represents a compromise between that necessary for maximal COMT activity and that required to maintain a high specific activity of the radioisotope. In this connection, porcine liver COMT is reported to have $K_m$ of 56 $\mu$M for SAM (9). COMT used in the reactions was a product of Pharmacia PL-Biochemicals (Piscataway, NJ). Benzenedihydrodiol was synthesized from 1,4-cyclohexadiene (Aldrich Chemical Co., Milwaukee, WI) as previously described (8).

RESULTS

Radiochemical Assay for Dihydrodiol Dehydrogenase Activity. The basis of the radiochemical assay for dihydrodiol dehydrogenase is to couple the formation of catechol, derived from the oxidation of benzenedihydrodiol, to O-methylation catalyzed by COMT using $[^3\text{H}]\text{SAM}$ as methyl donor. In establishing this assay we originally used the 40-75% ammonium sulfate fraction of rat liver cytosol. Assays were run in duplicate, and sets containing no enzyme were used to determine the nonenzymatic rate. Initial velocities for each substrate concentration were calculated from the appropriate progress curves and transformed by the Wilkinson hyperbolic method (10) or the Esensthal Cornish-Bowden direct linear method (11) to yield $K_m$ and $V_{max}$ values. Similar kinetic constants were determined spectrophotometrically. In these experiments, 1.0 ml assay systems containing benzenedihydrodiol (0.25-2.00 mM) plus 2.3 mM NADP$^+$ in 50 mM KPO$_4$ of pH 7.8 were incubated in the presence of enzyme and the change in the absorbance of the pyridine nucleotide was measured over 5 min. In both sets of assays, the final concentration of methanol was 4%.

Inhibition of Guaiacol Formation by Indomethacin and 6-Medroxyprogesterone Acetate. The ability of 30 $\mu$M indomethacin and 10 $\mu$M 6-medroxyprogesterone acetate to inhibit the dehydrogenase was examined using the standard radiochemical assay. Indomethacin was dissolved in methanol and 6-medroxyprogesterone acetate was dissolved in acetone. The final organic solvent concentration was kept constant at 4%. Reactions were initiated with the addition of cytosol and incubated at 37°C for 1 h. In every tissue examined, preliminary experiments established that formation of $[^3\text{H}]\text{guaiacol}$ in the coupled assay was linear for at least 1 h.

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Protein Determinations. Protein determinations were made using the method of Lowry et al. (12) with crystalline bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) as standard.

Fig. 2. Linearity of the radiochemical assay with protein and time. In A, varying amounts of the 40-75% ammonium sulfate fraction of rat liver cytosol were incubated with 1.0 mM benzenedihydrodiol, 2.3 mM NADP$^+$, 50 $\mu$M $[^3\text{H}]\text{SAM}$ (0.1 $\mu$Ci/nmol), and 10 units of COMT. The titrated product was extracted into toluene and quantified by scintillation counting.
comigrate with an authentic guaiacol standard ($R_s = 0.66$) using chloroform:ethyl acetate (1:1) as the running solvent. $[^3]$H$]$SAM has an $R_s$ value of 0.0 in this solvent system. It is estimated that using $[^3]$H$]$SAM at a specific activity of 0.1 μCi/nmol, the radiochemical assay is at least 5000-fold more sensitive than the existing spectrophotometric method. Thus based on the extinction coefficient of NADP$^+$ ($E = 6,270 \text{M}^{-1}\text{cm}^{-1}$) the limit of detection of the spectrophotometric assay is 1 nmol of product formed per minute. However, using 100-fold less tissue the radiochemical procedure can detect 5 pmol of product formed per hour.

As a further means of validating the radiochemical assay, we compared the $K_m$ and $V_{max}$ values for the 40–75% ammonium sulfate fraction of rat liver cytosol derived by both the radiochemical and spectrophotometric methods (Fig. 3). The two methods gave comparable results. The kinetic constants determined radiochemically were found to be $K_m = 0.77 \pm 0.11$ mM and $V_{max} = 2.14 \pm 0.13$ nmol/min/mg protein. While those determined by the spectrophotometric method were found to be $K_m = 0.96 \pm 0.10$ mM and $V_{max} = 6.31 \pm 0.50$ nmol/min/mg protein. The 3-fold difference in the $V_{max}$ values estimated by the two methods may be due to the significant dilution (100-fold) of the 40–75% ammonium sulfate rat liver cytosol fraction which is necessary prior to conducting the radiochemical assay.

Distribution of Dihydrodiol Dehydrogenase in Rat Tissues. In male Sprague-Dawley rats a number of extrahepatic tissues have been implicated in polycyclic aromatic hydrocarbon metabolism. These include the lung (13–16), small intestine (17, 18), testis (19), and prostate (20). To determine whether the cytosols from these and other tissues can catalyze the NADP+-dependent oxidation of trans-dihydriodols, the radiochemical assay was used. Specific activities of dihydrodiol dehydrogenase in male extrahepatic tissues varied by almost 60-fold and were highest in the liver and lung followed by the heart, small intestine, testis, seminal vesicle, bladder, prostate, and spleen (Fig. 4). Before the specific activity of benzene dihydrodiol oxidation was determined in any cytosol, linearity with both protein and time was demonstrated.

Inhibition by Indomethacin and 6-Medroxyprogesterone Acetate. Studies in both crude and highly purified preparations of rat liver dihydrodiol dehydrogenase have shown this enzyme to be inhibited to 90% by either 30 μM indomethacin or 10 μM 6-medroxyprogesterone acetate (6–8). Similar results can be obtained with the radiochemical assay when rat liver cytosol is used as a source of enzyme (Fig. 5). To investigate whether extrahepatic tissues that play a predominant role in polycyclic aromatic hydrocarbon metabolism also contain a drug-sensitive dihydrodiol dehydrogenase, the inhibition profile of benzene-dihydriodil oxidation was determined. In male rats dihydrodiol dehydrogenase activity in lung, testis, and small intestine was inhibited by 50–75% at concentrations of 30 μM indomethacin. Low micromolar concentrations of 6-medroxyprogesterone acetate (10 μM), however, provided significant inhibition only in the small intestine. Cytosols prepared from the other tissues showed no significant inhibition with either drug (data not shown).

DISCUSSION

Dihydrodiol dehydrogenase is a relatively little studied enzyme which may play an important role in the detoxification of metabolically activated PAH. Indirect evidence suggests that the purified enzyme can detoxify a diol-epoxide generated from benzo(a) anthracene (21) while studies on the purified enzyme
in this laboratory that it will oxidize the proximate carcinogens derived from benzo(a)pyrene to as yet unidentified products (5). To establish a role for this enzyme in the detoxification of PAH it is important to show that it is widely distributed and that it has a high specific activity in tissues responsible for PAH metabolism. It is also conceivable that levels of the enzyme may show an inverse correlation with organ susceptibility to PAH-induced chemical carcinogenesis. The lack of a sensitive assay for dihydrodiol dehydrogenase has prevented such measurements. Thus at the present time the existence of this enzyme has only been documented in livers of rabbits (1), rats (2, 3), guinea-pigs (22), and mice (23). The radiochemical assay described here takes advantage of the enzymatic oxidation of benzenedihydrodiol which results in the formation of catechol (1). The commercial availability of COMT and [3H]SAM of high specific radioactivity has now permitted the detection of dihydrodiol dehydrogenase in extrahepatic tissues. Such measurements have allowed us to draw conclusions about the role of the dehydrogenase in PAH detoxification.

Dihydrodiol dehydrogenase appears to be widely distributed in male rat tissues; the enzyme was detected in nine organs and its specific activity varied by 60-fold. The enzyme was present in sites known to be involved in the extrahepatic metabolism of polycyclic aromatic hydrocarbons in Sprague-Dawley rats. These tissues include the lung (13–16), small intestine (17, 18), testis (19), and bladder (16). In each of these tissues the 7,8-trans-dihydrodiol and the 9,10-trans-dihydrodiol of benzo(a)pyrene are formed following metabolism of the parent hydrocarbon (13, 16, 17, 19). These trans-dihydrodiols are presumably substrates for the various dihydrodiol dehydrogenases.

The tissue distribution of dihydrodiol dehydrogenase (liver = lung > heart > small intestine > testis > seminal vesicle > bladder > prostate > spleen) does not parallel that observed for other enzymes involved in PAH detoxification and/or activation. For example cyt-1-P450 (that subtype of cyt-P450 responsible for making diol epoxides) is of much higher activity in the liver than the lung (24) while glutathione-S-transferase (that enzyme levels but could reflect preferential formation of mutagenic PAH-DNA adducts or the absence of DNA repair mechanisms. It is important to realize that the distribution of other enzymes involved in the detoxification of PAH (e.g., glutathione-S-transferase) also fail to yield good inverse correlations with organ susceptibility to PAH-induced carcinogenesis. Thus in male Sprague-Dawley rats the small intestine has the third highest level of transferase but is susceptible to dihydrodiol dehydrogenase. In addition, 6-medroxyprogesterone acetate and 7,12-dimethylbenzo(a)anthracene-induced tumor formation (27). These findings suggest that a multitude of factors are involved in the determination of tissue susceptibility to chemical carcinogenesis and include levels of both activation and detoxification enzymes, nature of PAH-DNA adduct formation, and repair thereof.

We have recently shown that the nonsteroidal antiinflammatory drug indomethacin is a potent inhibitor of the oxidation of the trans-7,8-dihydrodiol of benzo(a)pyrene catalyzed by the homogeneous dihydrodiol of rat liver cytosol (6). In the current study several major tissues involved in the extrahepatic metabolism of polycyclic aromatic hydrocarbons (lung, small intestine, and testis) contain indomethacin-sensitive dihydrodiol dehydrogenase. In addition, 6-medroxyprogesterone acetate inhibits the dihydrodiol dehydrogenase present in rat liver and small intestine cytosols. Such studies suggest that nonsteroidal antiinflammatory drugs and synthetic progestins may prevent the oxidation of trans-dihydrodiols and influence tissue susceptibility to chemical carcinogenesis. With this view in mind, a recent report which shows that the coadminstration of 6-medroxyprogesterone acetate and 7,12-dimethylbenzo(a)-anthracene significantly increases the incidence of multiple intestinal adenomas and carcinomas (27) becomes most inter-
est ing. It is conceivable that 6-medroxyprogesterone acetate may be acting as a cocarcinogen by preventing the oxidation of trans-dihydrodiol proximate carcinogens at the level of dihydridiol dehydrogenase.

The dihydrodiol dehydrogenase of rat liver cytosol is known to be indistinguishable from 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) (3, 30). Previous studies in male rat tissues have shown that the 3α-hydroxysteroid dehydrogenase of liver, lung, testis, seminal vesicles, prostate, heart, and spleen to be sensitive to inhibition by low micromolar concentrations of indomethacin (31). Since our present findings indicate that only the liver, lung, and testicular dihydrodiol dehydrogenases are indomethacin-sensitive, it may be concluded that the 3α-hydroxysteroid dehydrogenase and dihydrodiol dehydrogenase activities cannot be mediated by the same protein in the remaining tissues (heart, spleen, prostate, bladder, and seminal vesicle). A further complication in male rat tissues is that although 3α-hydroxysteroid dehydrogenase and dihydrodiol dehydrogenase of liver, lung, small intestine, and testis are inhibited by indomethacin, only the enzyme in the liver and small intestine is affected by 6-medroxyprogesterone acetate. Such data suggest that the coexistence of 3α-hydroxysteroid dehydrogenase and dihydrodiol dehydrogenase on the same protein may be a property shared only by the enzyme in the male rat liver and small intestine.

In summary, several major conclusions arise from this work. Dihydridiol dehydrogenase is present in extrareplicative tissue involved in PAH metabolism. The distribution of the enzyme does not parallel that observed for other enzymes involved in PAH activation or detoxification. Levels of dihydridiol dehydrogenase do not show an inverse correlation to organ susceptibility to chemical carcinogenesis. In some tissues concerned with the further metabolism of PAH, trans-dihydridiol oxidation catalyzed by the enzyme is potently inhibited by indomethacin and 6-medroxyprogesterone acetate.

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