Published in final edited form as:

Curr Protoc Toxicol. 2011 May ; CHAPTER: Unit4.37. doi:10.1002/0471140856.tx0437s48.

Aldehyde Reduction by Cytochrome P450

Immaculate Amunom¹, Sanjay Srivastava², and Russell A. Prough³

Immaculate Amunom: iamunom@xenotechllc.com; Sanjay Srivastava: s0sriv01@gwise.louisville.edu

¹ Xenotech LLC, Lenexa, KS 66219

² Department of Medicine/Cardiovascular Medicine, University of Louisville School of Medicine, Louisville, KY 40292

³ Department of Biochemistry & Molecular Biology, University of Louisville School of Medicine, Louisville, KY 40292

Abstract

This protocol describes the procedure for measuring the relative rates of metabolism of the α,β -unsaturated aldehydes, 9-anthracene aldehyde (9-AA) and 4-hydroxy-*trans*-2-nonenal (4-HNE); specifically the aldehyde reduction reactions of cytochrome P450s (CYPs). These assays can be performed using either liver microsomal or other tissue fractions, spherosome preparations of recombinant CYPs, or recombinant CYPs from other sources. The method used here to study the reduction of a model α,β -unsaturated aldehyde, 9-AA, by CYPs was adapted from the assay used to investigate 9-anthracene oxidation as reported by Marini et al. (Marini et al., 2003). For experiments measuring reduction of the endogenous aldehyde, 4-HNE, the substrate was incubated with CYP in the presence of oxygen and NADPH and the metabolites were separated by High Pressure Liquid Chromatography (HPLC), using an adaptation of the method of Srivastava *et al.* (Srivastava et al., 2010). For study of 9-AA and 4-HNE reduction, the first step involves incubation of the substrate with the CYP in appropriate media, followed by quantification of metabolites through either spectrofluorimetry or analysis by HPLC coupled with a radiometric assay, respectively. Metabolite identification can be achieved by HPLC GC-mass spectrometric analysis. Inhibitors of cytochrome P450 function can be utilized to show the role of the hemoprotein or other enzymes in these reduction reactions. The reduction reactions for CYP's were not inhibited by either anaerobiosis or inclusion of CO in the gaseous phase of the reaction mixture. These character of these reactions are similar to those reported for some cytochrome P450-catalyzed azo reduction reactions.

Keywords

Cytochrome P450; reduction reactions; α,β -unsaturated aldehydes; inhibitors; 4-hydroxy-2-nonenal; 9-anthracene aldehyde

Introduction

It is well established that the principal reaction of cytochromes P450 is to catalyze the heterolytic cleavage of molecular oxygen allowing the insertion of atomic oxygen into an organic molecule. This allows formation of more polar hydroxylated or oxygenated

R. Prough: Phone: (502) 852-7249 Fax: (502) 852-2660 raprou01@gwise.louisville.edu (to whom correspondence should be addressed).

I. Amunom: Phone: (913) 438-7450 Fax: (913) 227-7100

S. Srivastava: Phone (502) 852-5834 Fax: (502) 852-3663

compounds with the stoichiometry shown in equation 1. The catalytic mechanism has been extensively reviewed by Guengerich (Guengerich, 2001;Guengerich, 2007).

However, a more rare reaction catalyzed by cytochromes P450 are reduction reactions for compounds that contain quinones, azo-, halogenated, nitro-, N-hydroxy-, and hydroperoxide functional groups (Guengerich, 2001;Guengerich, 2007;Guengerich, 2008) as shown in equation 2. Although many of these reactions are sensitive to molecular oxygen, tissues display lower oxygen tensions than ambient solution oxygen tension (heart 16 μ M and liver 30–40 μ M) and therefore, these reduction reactions may be more favorable *in vivo* than *in vitro* in the presence of ambient oxygen concentrations of approximately 240 μ M (Guengerich, 1983).

We have studied the metabolism of α,β -unsaturated aldehydes derived from lipid peroxidation, such as 4-hydroxy-*trans*-2-nonenal (4-HNE) and acrolein, using primary hepatocytes and expressed cytochromes P450. We have shown that many cytochromes P450 catalyze the oxidative metabolism of these aldehydes to the carboxylic acid in an NADPH- and O_2 -dependent manner (Amunom et al., 2007). Specific cytochromes P450 of the CYP1A, 2B, 2C, and 3A families were effective in oxidizing aldehydes to their carboxylic acid product. In recent studies (Prough et al., 2009), we also have detected a reaction in which 4-HNE is reduced to 1,4-dihydroxynonene (DHN) in either the presence or absence of oxygen. The reduction reaction was not affected by replacing the normal air atmosphere with either argon (anaerobiosis) or carbon monoxide (inhibitor of P450). Addition of other inhibitors, such as metyrapone, troleanomycin or phenytoin significantly inhibited both the oxidative and reduction reactions. We also found that an aromatic aldehyde, 9-anthracene aldehyde, is reduced by a limited number of P450s, CYP3A4, CYP2B1, and CYP1A2. The α,β -unsaturated bond in aldehydes may allow reduction of these aldehydes to their alcohols, much like the oxygen-insensitive azo reduction reactions. This precedent suggests that there may be other reduction reactions for olefinic carbonyl compounds by the CYPs and the tools for study described by Levine (Levine, 1991) for azo reduction may be important approaches to characterize these reactions. The protocols shown below provide methods to study a model aromatic aldehyde 9-AA or the endogenous aldehyde, 4-HNE, in the relatively rare reduction reactions leading to formation of alcohols by some P450s. We also provide methods to characterize these reactions with regard to their sensitivity to oxygen, carbon monoxide, and specific P450 inhibitors.

For routine assay, the assay for oxidation/reduction of 9-anthracene aldehyde is the easier assay, since it does not require synthesis of radiolabeled 4-HNE or uses a relatively unstable α,β -unsaturated aldehyde. One can utilize a time stop assay and process many samples for spectrofluorometric analysis within a day, while the HPLC analysis of 4-HNE metabolism requires approx. 90 minutes per HPLC analysis of each sample. For enzyme samples containing high activity of either P450, aldehyde dehydrogenase or aldo-keto reductase, the assay may be measured kinetically to obtain a rate of fluorescence change per minute. However, the limits of this direct fluorometric assay may be limited to relative pure enzymes and the reduction reaction product which has a relatively high quantum yield.

Metabolism of 9-anthracene aldehyde, a model α,β -unsaturated aldehyde

This protocol is used for investigating the oxidative and reductive metabolism of 9-anthracene aldehyde to 9-anthracene carboxylic acid and 9-hydroxymethyl-anthracene (9-A-MeOH), respectively, by cytochrome P450. From our studies (Amunom et al., 2007; Prough et al., 2009), we know that these two reactions occur simultaneously during metabolism by some P450s, such as human P4503A4 and murine P4502c29. In the first extraction step of the procedure, sodium hydroxide is added to the reaction mixture and the substrate and extraction with ethyl acetate separates 9-A-MeOH (organic phase) from the water soluble carboxylic acid (water phase). 9-A-MeOH in the organic phase can be directly quantified using a spectrofluorometer. Subsequently, the carboxylic acid can be extracted into the organic phase under acid conditions and quantified due to its fluorescence as will be described below.

Materials

Microsomal fractions, expressed P450 fractions, or purified P450

9-anthracene aldehyde (9-AA or 9-anthraldehyde) (Fluka 10603, Sigma-Aldrich, St. Louis, MO)

anthracene-9-carboxylic acid (9-ACA) (Aldrich S59065, Sigma-Aldrich, St. Louis, MO)

9-(hydroxymethyl)anthracene (9-A-MeOH) or 9-anthracene methanol (Aldrich 187240, Sigma-Aldrich, St. Louis, MO)

DL-isocitric acid trisodium salt hydrate (I1252, Sigma-Aldrich, St. Louis, MO)

isocitrate dehydrogenase (I2516, 20–60 U/mg; Sigma-Aldrich, St. Louis, MO)

NADPH (N5130, Sigma-Aldrich, St. Louis, MO) or NADP⁺ (N5755, Sigma-Aldrich, St. Louis, MO)

10 mM NADPH (or NADP⁺) stock (see recipe)

0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM ethylene diamine tetraacetic acid (EDTA) (see recipe)

NADPH-regenerating system consisting of 4.25 mM isocitric acid, 50 mM MgCl₂, and 1.3 Units/mL isocitrate dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.4 (see recipe)

2.5 mM 9-anthracene aldehyde (see recipe)

1 mM 9-ACA (see recipe)

1 mM 9-A-MeOH (see recipe)

0.5 N sodium hydroxide (221465, Sigma-Aldrich, St. Louis, MO, see recipe,)

0.5 N hydrochloric acid (320331, Sigma-Aldrich, St. Louis, MO, see recipe)

ethyl acetate (E145, Thermo Fisher Scientific, Waltham, MA)

15 mL conical tubes (e.g., 15 mL polypropylene conical tubes or borosilicate tubes with screw caps, Thermo Fisher Scientific, Waltham, MA)

quartz fluorometer cuvette, 3 mL volume (several vendors)

spectrofluorimeter (e.g., Model LS50B, Perkin-Elmer, Boston, MA)

Oxidation of 9-anthracene aldehyde by cytochrome P450

1. Dilute sample of interest to a final protein concentration of 0.2 to 1 mg/mL using 0.1 M potassium phosphate, pH 7.4 containing 1 mM EDTA to prepare the reaction mixtures. If using liver microsomes, dilute to final concentration in the reaction mixture of 0.25 mg microsomal protein/mL. If using recombinant P450 of known P450 content, dilute to a final concentration of 50 nM CYP in the reaction mixture of 2 mL final volume.
2. Prepare a stock solution of NADPH (10 mM stock allows addition of 20 μ L NADPH solution to achieve 100 μ M concentration).
3. Prepare a stock solution of 9-AA (2.5 mM stock allows addition of 20 μ L of 9-AA solution to achieve a 25 μ M)
4. Prepare the NADPH-regenerating system by combining isocitric acid, 4.25 mM; 50 mM MgCl_2 , 50mM; and isocitrate dehydrogenase, 1.3 Units/mL in 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA (see recipe).

Note: Either NADPH or NADP^+ can be utilized with this regenerating system, but if NADP^+ is used, one should make the regenerating system with the NADP^+ included so that it is fully reduced by isocitrate dehydrogenase before one adds the reaction mixtures to the incubation tubes. One should compare the two sources of NADPH to ensure that the same reaction rate is obtained, if one plans on using NADP^+ .

5. Set water bath to 37 $^{\circ}\text{C}$ and place the incubation mixtures in 15 mL conical tubes. Transfer enough volume of each component (25 μ M 9-AA, the NADPH regenerating system and either diluted microsomes or recombinant CYP) into the tube; use the NADPH-regenerating system to achieve a final volume of 2 mL.
6. Place the tubes in a shaking water bath and incubate for 5 minutes to allow the temperature of the reaction mixture to come to 37 $^{\circ}\text{C}$.
7. Initiate the reaction by adding NADPH to a final concentration of 100 μ M and continue to incubate in the shaking water bath for 10–20 minutes.

Note: Most reactions with most microsomal protein fraction or recombinant P450s we have tested are linear to 15–20 minutes. If you utilize NADP^+ for the reaction, initiate the reaction by addition of the enzyme source.

8. After selecting the reaction time for the assay, terminate the reaction by adding 1 mL of 0.5 N NaOH.
9. Add 4 mL of ethyl acetate and mix by shaking vigorously for 30–60 seconds. This step separates any residual substrate and non-oxidized product from the oxidized product in the organic phase. To separate the phases, briefly centrifuge the reaction in a low speed benchtop centrifuge or allow the tubes to stand in a rack on the bench top for 5–10 minutes.

Note: A rotary extraction wheel may also be used for 5 minutes for the extraction procedure. Alternatively, the extraction can be performed by shaking the sealed tube by hand for 30–60 seconds.

10. Using a pipette, carefully transfer 1 mL of the aqueous phase into a new 15 mL conical tube.

Note: The aqueous phase contains the oxidized product, 9-anthracene carboxylic acid and will be the lower phase since the density of ethyl acetate is 0.897 g/mL.

The organic phase will contain the substrate and any 9-hydroxymethyl-anthracene, the reduced product of 9-anthracene aldehyde. One can remove the ethyl acetate phase for ease of recovery of the aqueous phase for the re-extraction under acid conditions.

11. Acidify the aqueous phase by adding 1 mL of 0.5 N HCl. Then add 4 mL of ethyl acetate and mix vigorously for 30–60 seconds. Either mixing by hand or use of a rotary extraction wheel is sufficient to extract 9-ACA into the organic phase.

Note: Acidification allows protonation of the carboxylic acid product, so that it can be extracted into the organic phase for quantification.

12. Using a pipette, transfer 2 mL of the organic phase into a quartz fluorometer cuvette and quantify the amount of 9-anthracene carboxylic acid in the second organic phase by measuring fluorescence with a spectrofluorometer; the two wavelengths of importance are 255 nm excitation and 458 nm emission.
13. A standard curve can be constructed using 9-anthracene carboxylic acid (9-ACA) in the low μM concentration range (0, 2, 4, 8 μM 9-ACA final). This standard curve can be used to develop a constant of fluorescence/ μM 9-ACA. (see recipe).
14. Calculation: Determine the rate of formation of 9-ACA from the fluorescence measured (ΔF) using the following calculation, where the dilution factor includes the factor 1.5 due to addition of NaOH, 4 due to the fraction of aqueous phase taken for extraction, and the reciprocal of the final protein concentration.

$$\text{nmol 9-ACA formed/min/mg protein} = \Delta F / \text{time (min)} / (\text{fluorescence} / \mu\text{M}) * \text{dilution factor}$$

Reduction of 9-anthracene aldehyde by cytochrome P450

1. Using a pipette, transfer 2 mL of the organic phase from the alkaline extraction procedure (**step 9 above**) into a cuvette and quantify the amount of 9-hydroxymethyl-anthracene (reduced product) in the organic phase by measuring the fluorescence with a spectrofluorometer. The wavelengths of importance for 9-anthracene methanol are 255 nm excitation and 411 nm emission (see Figure 1 for spectra).
2. A standard curve can be constructed using 9-anthracene methanol in the low μM concentration range (0, 2, 4, 8 μM 9-A-MeOH final). This standard curve can be used to develop a constant of fluorescence/ μM 9-A-MeOH. (see recipe).
3. Calculation: Determine the rate of formation of 9-A-MeOH formation from the fluorescence measured (ΔF) using the following calculation, where the dilution factor includes the factor 2 due to the 4 mL for the ethyl acetate step and the reciprocal of the final protein concentration.

$$\text{nmol 9-A-MeOH/min/mg protein} = \Delta F / \text{time (min)} / (\text{fluorescence} / \mu\text{M}) * \text{dilution factor}$$

4. The fluorescence excitation and emission spectra for 9-A-MeOH and 9-ACA can be measured independently as seen in Figure 1.

Metabolism of 4-HNE by P450s

Materials

2 mM stock solution of ^3H -4-HNE (approx. 0.5–1.0 $\mu\text{Ci}/\mu\text{mol}$, see recipe) microsomal protein or recombinant CYP P450

10 mM NADPH stock solution (see recipe)

0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA (see recipe)

15% trichloroacetic acid (see recipe)

0.1 % trifluoroacetic acid (see recipe)

HPLC quality water (high quality water from local source or purchased from Thermo Fisher Scientific, Waltham, MA or Burdick & Jackson solvents from VWR International, Westchester, PA)

HPLC acrylonitrile (Thermo Fisher Scientific, Waltham, MA or Burdick & Jackson solvents from VWR International, Westchester, PA)

Radiometric Flo-one s model A-515 detector, Packard Instrument Co., Downers Grove, IL)

Ultimagold scintillation fluid (Packard Instrument Co., Downers Grove, IL)

Note: Unlike the 9-AA incubation reaction, we do not include the NADPH-regenerating system in the 4-HNE metabolism incubation reaction. We observed that the presence of the isocitrate/isocitrate dehydrogenase mixture and glycerol in this incubation mixture interferes with the HPLC separation of 4-HNE metabolites.

1. First determine the volume of diluted microsomes (or CYP) that will be required for your incubation reaction and dilute the microsomes to 0.25 mg/mL protein with the reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA to attain a 2 mL reaction mixture. If using recombinant CYP of known P450 content, dilute to 50 nM in a 2 mL reaction mixture.
2. Pipette sufficient 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA into 15 mL conical tubes to achieve a 2 mL volume after taking into account the volume of protein, HNE and NADPH to be added.
3. Add the substrate, 4-HNE, to obtain a final concentration of 25 μ M in a reaction volume of 1 mL (see recipe).

Note: When using radioisotopes the procedures and training of each organization's Radiation Safety Program must be followed. Disposable paper should be used to cover the work bench and the water bath and the work benches should be monitored for radioactive spills using liquid scintillation counting. The reaction mixtures, HPLC effluent and derivatized samples should be discarded as radioactive waste at the conclusion of the experiment.

4. Add the required volume of microsomal fractions or recombinant P450 to the buffer solution.
5. Place the tubes in a shaking 37 °C water bath for 5 minutes to allow the mixture to come to 37 °C.
6. Initiate the reaction by adding 20 μ L of NADPH, the final concentration of NADPH in the reaction should be 100 μ M.
7. Incubate the reaction in the 37 °C shaking water bath for 10–20 minutes.
8. Terminate the reaction by snap freezing in dry ice or liquid nitrogen.
9. Thaw the frozen samples and then add 2 mL trichloroacetic acid (TCA) to achieve 7.5% TCA concentration. This step denatures the protein in the samples.

10. Separate the denatured protein from the sample mixtures by centrifugation at $13,000 \times g$ for 5 min at 4°C in a bench top or high speed centrifuge.
11. Carefully collect the supernatant into clean tubes; remove an aliquot to measure radioactivity in a scintillation counter (e.g., Packard Tricarb 2100TR scintillation counter, Packard Instrument Co., Downers Grove, IL) with Ultimagold scintillation fluid (Packard Instrument Co.)

Note: Since one requires knowledge of the mass balance of the total radioactivity to ascertain metabolic rates, the efficiency of transfer and HPLC steps requires this information.

12. The next step is to separate the metabolites utilizing high performance liquid chromatography (HPLC).

Separation of 4-HNE metabolites by HPLC

Equipment—The HPLC system used by Amunom *et al.* (2007) consisted of a Waters automated gradient controller with dual model 510 pumps (Milford, MA), a Waters manual injector (2 mL loop) and a C18 reversed phase column (5 μ m, 250 mm $\times$ 4.6 mm Varian, Walnut Creek, CA) connected to a radioactivity detector (Radiometric Flo-one s model A-515 detector, Packard Instrument Co., Downers Grove, IL) or to a fraction collector (Biorad Model 2210 Biorad, Hercules, CA).

1. The mobile phase is essentially gradient ranging from 100% solvent A (0.1% (v/v) TFA in water) to 100% solvent B (40% solvent A, 60% acetonitrile).
2. Deliver the solvent into the HPLC column at a flow rate of 1 mL/min for 75 min occurred as follows: linear gradients from 100% to 76% solvent A (0–15 min), from 76% A to 74% A (15–40 min), from 74% to 40% A (40–45 min), from 40% to 0% A (45–60 min), and then elute the metabolites with 100% B for (60–75 min).

Note: These conditions achieve resolution of the GSH conjugates, 1,4-dihydroxy-trans-2-nonene (DHN), 4-hydroxy-trans-2-nonenoic acid (HNA), and 4-HNE (Amunom et al., 2007; Srivastava et al., 2010).

3. At the end of the elution process, the HPLC conditions are changed to 100% A over 10 minutes prior to starting the next run and left at 100% A for 10 minutes before the next run. This equilibrates the column to the original solvent conditions and allows the operator to flush the injector at these conditions as well..
4. Under these conditions, the approximate retention times for the 1,4-dihydroxy-trans-2-nonene (DHN) (reduced metabolite of 4-HNE) was 31 minutes.

Note: The incubation reaction and HPLC analysis are performed in triplicate. In addition to the 4-hydroxy-trans-2-nonenal, the other metabolites, 1,4-dihydroxy-trans-2-nonene and 4-hydroxy-trans-2-nonenoic acid, can be collected and quantified by radiometric analysis.

5. If an online radiometric detector is not available, one can collect fractions with a fraction collector and take aliquots for liquid scintillation counting or use the collected fractions for GC-Mass spectral analysis to confirm identity and quantification.

Note: Measure the radioactivity of the total HPLC effluent by integrating the data from the radiometric detector or summing the counts obtained by liquid scintillation counting.

Identification of metabolites by GC-mass spectral analysis

Equipment—For the analyses performed in Amunom *et al.* (2007), the gas chromatography system was an Agilent 6890/5973 GC/MS system (Agilent Technologies) under 70 eV electron ionization conditions.

Materials

N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Fluka 15222, Sigma-Aldrich, St. Louis, MO)

carrier gas for gas chromatograph

Gas chromatography-electrospray ionization/mass spectrometry (GC-EI/MS)

identification of 4-HNE metabolites—The first step for GC-mass spectral analysis is to derivatize the carboxyl and hydroxyl groups on the metabolites with BSTFA to increase the volatility and stability of the metabolites. This improves the GC separation of the metabolites.

1. For identification and quantification of HNE, DHN, and HNA, HPLC fractions corresponding to the retention time of reagent HNE or the various standards were dried under vacuum, resuspended in 0.5 mL water and incubated with 5 mg pentafluoro-benzyl hydroxylamine (PFBHA) for 30 minutes at room temperature. Five hundred μ L methanol was added and the samples extracted with 2 mL hexane. The hexane layer (upper layer) was removed, dried under a stream of nitrogen and then derivatized with 20 μ L of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) for 1 hour at 60°C as described previously (Srivastava et al., 2010).
2. Cool the derivatization reaction mixture to room temperature and use 1 μ L aliquots for analysis.
3. Separate the metabolites using a bonded phase capillary column (DB-5MS, 30 m $\times$ 0.25 mm ID $\times$ 0.25 μ m film thickness from J7W Scientific Folsom, CA, USA).
4. Set the GC injection port and interface temperature to 280°C, with helium gas (carrier) maintained at a pressure of 14 psi.
5. Inject the samples for 1 minute in the splitless mode with the inlet port purged.
6. Maintain the GC oven temperature at 100 °C for 1 min, and then increase the temperature at a rate of 10°C per minute to 280 °C, then hold at this temperature 5 minutes.
7. An internal standard of deuterated 4-HNE, DHN, or HNA allows quantification of the metabolites if desired. The mass spectra obtained can also verify the identity of the metabolite based up its retention time and mass spectrum.

The effect of anerobiosis or carbon monoxide on the metabolism of 9-AA and 4-HNE by cytochrome P450s

Materials

- 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA (see recipe)
- 74 mM glucose (Aldrich 158968, Sigma-Aldrich Chemical Company, St. Louis, MO) (see recipes)
- 0.33 mg/mL glucose oxidase (G2133, Sigma Chemical Company, St. Louis, MO) (see recipes)

- 1400 U/mL catalase (C30, Sigma-Aldrich Chemical Company, St. Louis, MO) (see recipes)
- Source of argon or carbon monoxide (commercial gas cylinder with gas regulator)
- 50 mL Erlenmeyer flask fitted with a serum stopper fitted with 1 mL tuberculin syringes
- Serum stopper (either 14-126BB (13.88 mm ID Erlenmeyer flask opening) or 14-126DD (15.85 mm I.D. Erlenmeyer flask opening) from Thermo Fisher Scientific, Waltham, MA)
- 1 mL tuberculin syringe (Thermo Fisher Scientific, Waltham, MA)
- Tygon tubing (Thermo Fisher Scientific, Waltham, MA)

1. Prepare the incubation reaction as described above for either 9-anthracene aldehyde or 4-HNE as described above (see recipe).
2. Fit a 50 mL Erlenmeyer flask with a serum stopper and syringes to allow for the circulation of carbon monoxide, one for gas influx and one for gas efflux (Figure 2).

Note: Since carbon monoxide is highly toxic, these reactions mixtures and incubations should be performed in a well vented chemical hood to protect the researchers.

3. Add the appropriate volume of buffer; 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA or the NADPH-regenerating solution into the Erlenmeyer flask to achieve a final volume of 2 mL, as described above.
4. Connect the N₂ or CO regulator to Tygon tubing and insert a 1 mL Tuberculin syringe trimmed (remove the flange near opening where syringe is inserted) into the other end of the tubing (Figure 2). Insert the modified syringe attached to the Tygon tubing from gas cylinder to a short 18 gauge needle and insert it into the serum stopper. Attach a short piece of small I.D. Tygon microbore tubing to the syringe needle attached through the serum stopper to the modified outer sleeve of the syringe to allow the gas to bubble through the solutions. Insert a second 18 gauge needle through the serum stopper to allow efflux of the gas from the sealed flask and preventing over-pressurization. Gently bubble the buffer solution for 5 minutes with either air, argon or carbon monoxide (see Figure 2).
5. Add the appropriate amount of the buffer solution containing a mixture of 74 mM glucose, 0.33 mg/mL glucose oxidase, and 1400 U/mL catalase (1400 U/mL) through the serum stopper with a 1 mL syringe and allow to sit with gas exchange for 5 minutes.

Note: The glucose oxidase reaction consumes molecular oxygen to catalyze glucose oxidation to glucuronic acid, a reaction that generates hydrogen peroxide. The hydrogen peroxide is removed by its conversion to 1/2O₂ and water by added catalase. If the hydrogen peroxide is not removed from the reaction, it can potentially inactivate the P450.

6. Add the appropriate concentration of P450 (as described above) using a 1 mL syringe to add the solution through the serum stopper, while allowing gas flow through the headspace of the sealed Erlenmeyer flask.
7. Continue to flush the headspace of the reaction mixture with either carbon monoxide or argon for 3 minutes.

8. Initiate the incubation reaction by adding 20 μ L NADPH and incubate in a shaking water bath at 37°C for 10–20 minutes. During this time, the headspace of the reaction should be continuously flushed with argon or carbon monoxide.
9. Terminate the reaction by adding NaOH (for the 9-AA incubation) or snap freeze in liquid nitrogen (the 4-HNE incubation).
10. Extract and analyze the products as described above.

Recipes

10 mM NADPH stock (MW 1142.12; add 11.4 mg/mL water; can keep for 1–2 weeks at 4° C)

10 mM NADP⁺ stock (MW 743.41; add 7.44 mg/mL water; can keep for 1–2 weeks at 4° C)

100 mM ethylene diamine tetraacetic acid (EDTA, 431788, Sigma-Aldrich, St. Louis, MO) MW 292.24; Add 29.2 g EDTA into 100 mL water)

0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. Prepare the buffer by adding 2.6 g/L of KH₂PO₄ and 19.48 g/L of K₂HPO₄ to 800 mL distilled water or equivalent. Add 10 mL of 100 mM EDTA and after ensuring the pH is 7.4, bring the solution to 1 L.

0.5 M MgCl₂ stock (M35, Thermo Fisher Scientific, Waltham, MA) MW 95.21; add 11.9 g MgCl₂ to a final volume of 250 mL with water

NADPH-regenerating system consisting of 4.25 mM isocitric acid (MW 258.07), 50 mM MgCl₂, and 1.3 Units/mL isocitrate dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA: Add 10 mL 0.5 M MgCl₂ to approx. 80 mL of 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA. Add 25.8 mg sodium isocitrate and subsequently add sufficient isocitrate dehydrogenase to attain 130 U/100 mL. Adjust volume to 100 mL and store on ice until ready to prepare incubation tubes. Add NADP⁺ at this step if using this pyridine nucleotide so that it will be fully reduced.

2.5 mM 9-anthracene aldehyde, 9-AA (MW 206.24, 5.15 mg/10 mL dissolved in DMSO or alcohol)

Note: this reagent is light sensitive; store in brown bottle with a tight cap or in a bottle wrapped in aluminum foil.

1 mM 9-anthracene carboxylic acid, 9-ACA (MW 222.3; 22.23 mg/10 mL DMSO)

1 mM 9-(Hydroxymethyl)anthracene, 9-A-MeOH (MW 208.3; 20.8 mg/10 mL DMSO)

0.5 N sodium hydroxide (MW 40; add 10 g NaOH pellets to 400 mL water until dissolved and adjust volume to 500 mL)

0.5 N hydrochloric acid (Concentrated HCl is 12.39 N; add 10 mL HCl to 240 mL water; adjust volume to 250 mL with water)

15% trichloroacetic acid (Add 15 g TCA to a final volume of 100 L with water)

0.1 % trifluoroacetic acid (Add 1 mL TFA to a final volume of 1 L water)

Oxygen scavenging solution: The following three materials should be added to form a solution in 0.1 M potassium phosphate, pH 7.4 containing 1 mM EDTA.

- A. 74 mM glucose (Aldrich 158968, Sigma-Aldrich Chemical Company, St. Louis, MO; MW 180.16) Add 3.33 g α -D-glucose to 250 mL of 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA
- B. 0.33 mg/mL glucose oxidase (G2133, Sigma Chemical Company, St. Louis, MO) Add 82.5 mg glucose oxidase to the 250 mL solution of 0.1 M potassium phosphate buffer containing glucose and 1 mM EDTA)
- C. 1400 U/mL catalase (C30, Sigma-Aldrich Chemical Company, St. Louis, MO) Add 1400 U of catalase per mL of 0.1 M potassium phosphate buffer containing 1 mM EDTA, glucose and glucose oxidase

Radiolabelled HNE is not commercially available and must be synthesized from fumaraldehyde dimethyl acetal to form its dimethyl acetal as described by Chandra and Srivastava (Chandra and Srivastava, 1997). The method involves oxidation to form the 4-oxo-nonenal dimethylacetal, which was subsequently reduced with sodium borotritide. Before use, [4-³H] HNE was obtained by acid hydrolysis of dimethyl acetal and purified by HPLC. The [4-³H] HNE thus synthesized had a specific activity of approximately 90 mCi/mmol. A recent *Methods in Enzymology* providing details for the synthesis should be consulted (Srivastava et al., 2010) to obtain details.

[³H]-4-HNE (Item 32100, Cayman Chemical, Ann Arbor, MI). Radiolabelled 4-HNE described above is mixed with unlabeled 4-HNE to obtain a stock of around 0.5–1.0 Ci/mmol substrate with a chemical concentration of approximately 2 mM.

Commentary

Background Information

Historically, the unique observations of the laboratories of Fuller and Bovet (Trefouele J et al., 1935; Fuller, 1937) provided a clear understanding of the fact that reductive bioactivation of the antibacterial azo dye protosil is critical in production of the active principle, sulfanilamide, in rabbits. Their observations of a reduction reaction *in vivo* was not verified biochemically for several decades. This is a classic pro-drug reductive activation process demonstrating the potential of enzyme systems like cytochrome P450 and aldo-keto reductases in foreign and endogenous compound disposition or bioactivation.

As discussed above, the azo, nitro and other reduction reactions have not been as well characterized as other carbon-oxidation reactions catalyzed by the cytochromes P450 (Guengerich, 2001; Guengerich, 2007). Pharmacologists and toxicologists have considered these reduction reactions for some time since these reactions are critical for production of pharmaceutical agents against bacterial infection and impacts the use of dyes for food and clothing coloring. Levine (Levine, 1991) described the role of azo dyes in carcinogenesis and mutagenesis, and also addressed reductive metabolism in bacterial and mammalian cells. While the oxidative metabolism of azo dyes through N-dealkylation, aromatic ring hydroxylation and N-hydroxylation reactions are well studied, the mechanistic basis for azo reduction was poorly understood. Azo functional group reduction is a facile reaction in the absence of molecular oxygen, reduction in the presence of molecular oxygen depends on the chemistry of the azo compound in question. Like the nitro compounds, redox cycling of free radical intermediates with molecular oxygen can preclude formation of a stable reduced product. Unproductive reduction results in release of reactive oxygen species, which may account for some of the toxic properties of some azo compounds.

The oxidation-reduction potential for ferric cytochromes P450 is ~ -300 mV. Due to the high reactivity of this hemoprotein in its ferrous form with molecular oxygen, it is surprising that it catalyzes reduction reactions in the presence of 240 μ M oxygen. The scientific

community assumes that all ferric cytochromes P450 bind oxygen and subsequently molecular oxygen is rapidly reductively cleaved. In tissues with low oxygen tension, there is a high possibility that the reduction reactions would be favored since its oxidation-reduction potential should allow ferric cytochrome P450 to be an excellent 1-electron reducing catalyst (Guengerich, 1983).

Except for our report of 4-HNE reduction (Prough et al., 2009), no other cytochrome P450-dependent reduction reactions for endogenous compounds are known, other than the reduction of organic hydroperoxides, such as lipid hydroperoxides. However, the literature demonstrates that azo, nitro, N-hydroxy amines, quinones, halogenated, and hydroperoxy compounds can be reduced *in vitro*, suggesting that other functional groups which undergo reduction may exist. Indeed, Levine and coworkers (Levine and Zbaida, 1991) studied the redox potential of azo compounds in an attempt to elucidate a chemical feature which allows their reduction in the absence or presence of molecular oxygen. They demonstrated azo compounds whose reduction was sensitive to O₂ and CO and those that were insensitive. A common chemical feature of the two classes were that the insensitive class tended to have electron-donating substituents on the aromatic ring, such as -OH, -NH₂, -NH-CH₃, or -N(CH₃)₂ groups. The sensitive class of substrates were those that contained electron-withdrawing groups, such as -SO₃H, -COOH, -COOCH₃, and -AsO₃H₂. Cyanide ion was shown to potentially inhibit reduction of the O₂-insensitive azo dye substrates, suggesting that the insensitivity/sensitivity may be a specific property of the ferrous cytochrome P450 (Levine and Zbaida, 1991). Azide did not differentially affect azo reduction of the compounds studied. One explanation for the differences in O₂/CO sensitivity is that the hydrazine intermediates formed by azo group reduction may have differential sensitivity to reoxidation by molecular oxygen to the azo intermediate or alternatively, they are not sensitive and are reduced to primary amines. We have shown that hydrazines are easily oxidized by cytochromes P450 and are sensitive to metal-catalyzed oxidation (Prough et al., 1981; Tweedie et al., 1987). The mechanism accounting for the oxygen sensitivity or insensitivity remains unsolved.

Milton et al. (Milton et al., 1990) demonstrated that clofibrate was a potent inducer of cytochrome P450_{4A1} in rat liver and defined several inhibitors of the enzyme, including saturated fatty acids and clofibrate itself. Levine demonstrated that animals treated with various chemicals resulted in induction of liver microsomal enzymes involved in azo dye reduction and each condition displayed a different substrate specificity for azo dyes (Raza and Levine, 1986b; Raza and Levine, 1986a). Induction by clofibrate increased azo dye reductase activity for nearly all dyes, suggesting a role for the CYP4 family of cytochrome P450s in azo dye reduction. Phenobarbital, pregnenolone-16 α -carbonitrile, and isosafrole were also inducers for reduction reactions for azo dyes, such as O-methyl-Red (Raza and Levine, 1986a). Members of the CYP1, CYP2, CYP3, and CYP4 families all reduce azo dyes under anaerobic conditions and depending on the structure of the azo dye, possibly under aerobic conditions. Mallett et al. (Mallett et al., 1982; Mallett et al., 1985) utilized purified NADPH:cytochrome P450 oxidoreductase and CYP2B1 to reconstitute the anaerobic reduction of the azo dye amaranth, an oxygen-sensitive reaction. Amaranth reduction reactions were dependent upon the oxidoreductase, CYP2B1 and dilauroyl phosphatidylcholine. In the presence of added FMN, the oxidoreductase appeared to be the source of reduction. They proposed two mechanisms of reduction for amaranth; either direct reduction by oxidoreductase that was stimulated by addition of FAD, and cytochrome P450-mediated reduction in the absence of FAD.

Raza and Levine (Raza and Levine, 1986b; Raza and Levine, 1986a) demonstrated that azo dyes were most effectively reduced in liver microsomes from ciprofibrate-treated rats. In addition, they demonstrated that addition of N,N-dimethyl-4-aminoazobenzene to reaction

mixtures containing liver microsomes from clofibril acid-treated rats inhibited lauric acid hydroxylation and that lauric acid inhibited *N,N*-dimethyl-4-aminoazobenzene reduction with these same microsomal fractions. Their work also demonstrated that a CYP4A-specific inhibitor, 10-undecynoic acid, inactivates laurate hydroxylation, but not azo reductase activity, suggesting that the active site for oxidative metabolism is differentially affected by the inhibitor for laurate oxidation, but not for the site for azo dye reduction. They also reconstituted NADPH:cytochrome P450 oxidoreductase with purified CYP4A1 to demonstrate that *N,N*-dimethyl-4-aminoazobenzene azoreduction is catalyzed by this enzyme in the absence or presence of oxygen, but in the presence of oxidoreductase alone, the flavoprotein could only reduce it under anaerobic conditions. Finally, they noted that in the reconstituted system, inclusion of FAD greatly enhanced azo reduction; the oxidoreductase alone was nearly as effective in reducing azo dyes in the presence of FAD than the reconstituted system. Levine and Lu (Levine and Lu, 1982) performed a more detailed study that demonstrated that azo reduction by cytochrome P450 is a 2-electron process yielding the stable hydrazine (2-electron) or amine (4-electron) reduction product, while the oxidoreductase apparently only catalyzes 1-electron reduction to an intermediate that is rapidly oxidized to the azo derivative.

Other Reductive Enzymes—Other enzyme systems capable of reducing azo dyes, quinones, hydroxylamines, and hydroperoxides have been described in the literature. It is well known that many flavoproteins reduce quinones in particular and azo dyes, including NADPH:cytochrome P450 oxidoreductase (Masters et al., 1965), NAD(P)H:quinone oxidoreductase (Lind et al., 1982), and various NADH dehydrogenases. Quinones have been used as artificial substrates for the study of these enzymes when the endogenous reactant is not known or is difficult to isolate for study (i.e., cytochrome P450 or cytochrome *b*₅). Their reduction can be followed by loss of absorbance of the quinone or loss of NAD(P)H. When either FAD or FMN are added to preparations containing flavoproteins, the rates of azo reduction are greatly increased since reduced flavin in high concentration reduce directly these chemicals. Our group (Prough et al., 2009) noted low levels of 4-HNE reduction by NADPH:cytochrome P450. Mallet et al. (Mallett et al., 1985) noted that addition of flavin greatly stimulated reduction of the azo dye amaranth, representing the enzyme-mediated reduction of free flavin which subsequently results in chemical reduction of azo dyes by reduced flavin. Several distinct N-hydroxy-amine reductases or sulfonamides have been described by Havemeyer et al. (Havemeyer et al., 2010); the molybdenum-containing enzyme, named, mitochondrial amidoxime reducing component. Recently the molybdo-protein has been expressed in a recombinant system and characterized. It requires NADH:cytochrome *b*₅ oxidoreductase and cytochrome *b*₅ in order to serve as electron transfer components to facilitate this reduction reaction.

Inhibitors to Identify Reductive Enzymes—As described above, Levine and coworkers utilized various P450-specific inhibitors to determine which P450s catalyzed azo reduction reactions (Levine and Lu, 1982; Raza and Levine, 1986b). Initially, non-specific inhibitors of P450s, cyanide and azide were tested and only cyanide at high concentration inhibited azo dye reduction. Subsequently, specific P450-specific inhibitors were tested to establish which P450s among many were capable of catalyzing azo dye reduction. While many compounds at high concentration inhibit the P450s, some inhibitors display isoform-specificity at low concentration (Table 1). Such a strategy has been successfully applied to defining the form of P450 involved in specific reactions. Specific mono-clonal antibodies have also been applied to defining the roles of specific P450s (Shou et al., 2000). As new enzymes are described that catalyze these oxidation reactions, they can also be tested. In Table 1, we have also included flavoprotein-specific inhibitors used by others.

Critical Parameters—It is useful to perform the standard curve development for 9-ACA and 9-A-MeOH as a first step in establishing this assay, since the procedures are nearly identical to the actual assays. This will insure that the manipulations required for the assay have been perfected. To optimize the assays, one should perform the assays with rodent liver microsomal fractions from mice or rats treated with 0.05% phenobarbital in the drinking water for 5 days. An initial experiment should be to perform assays with several time points and protein concentrations to confirm whether the reactions are linear with time and protein concentration. The reactions can be run for longer times, but a time course of reaction should be considered to ascertain the linear results at the longer times. If one utilizes NADP⁺ for the assay of 9-ACA/9-A-MeOH production, one should compare the reactions initiated with NADPH vs. NADP⁺, prior to performing critical experiments. Authentic standards for HNA and DHN can be obtained by incubating HNE in the presence of yeast aldehyde dehydrogenase (82884, Sigma Aldrich, St. Louis, MO) or expressed aldose reductase.

Troubleshooting—To ensure the samples for 9-AA metabolism can be easily measured, development of the standard curve for 9-A-MeOH and 9-ACA will insure that the extraction assay and all procedures are optimized. The reactions with rodent liver microsomal fractions from mice and rats treated with 0.05% phenobarbital in the drinking water for 5 days should be used to verify that adequate data can be obtained before initiating other experiments with unknown sources of protein from tissue fractions or recombinant P450s.

Anticipated Results—Using the conditions discussed above, we anticipate you should be able to show whether the control P450s or various tissue fractions display linear enzyme reactions with time and protein concentration. These assays should allow one to document that a new source of enzyme is capable of aldehyde reduction or oxidation and that the results are similar, larger than, or less than that seen with liver microsomal fractions.

Time Considerations—The preparation of all reagents except NADPH-regenerating solutions and glucose oxidase-catalase reaction mixtures can be performed in less than a half day. These later two solutions should be prepared the morning of the experiment. The incubation tubes should be placed on ice until they are complete and then placed in a water bath. Assuming less than 20 samples will be run, this procedure can easily be completed early in the morning and the reaction performed prior to lunch or immediately after lunch. The procedure can be stopped after addition of base or quick freezing, respectively. The extractions and analytical analyses should be completed in a single step. Routinely, the 9-ACA assay can be completed in a single day. For 4-HNE metabolism the samples can be processed ready for HPLC analysis in 1 day or less. The HPLC assays are the slow step (1.5 h per sample) due to the long time required for resolution of the product. MS analysis would take another day. The 9-ACA assay has to date correlated with the results obtained for 4-HNE assay and is technically a less time-consuming assay [amunom and prough references].

Acknowledgments

The Authors wish to acknowledge the assistance of Laura Stevens and Dan Riggs for their technical expertise in these studies. Supported in part from NIH Grants 1 PO1 ES11860 (SS/RAP), HL95593 (SS), and 5P30ES014443.

Internet Resources: Several commercial concerns have kits for measurement of various cytochromes P450 and various Phase II enzyme reactions including specific substrates and inhibitors. There are also websites that provide more information about drugs that serve as inhibitors of cytochromes P450. Two internet resources to read is using specific inhibitors include psychresidentonline.com and BD GenTest [http://www.bdbiosciences.com/research/admetox/services/discovery_service.jsp].

Reference List

- Amunom I, Stephens LJ, Tamasi V, Cai J, Pierce WM Jr, Conklin DJ, Bhatnagar A, Srivastava S, Martin MV, Guengerich FP, Prough RA. Cytochromes P450 Catalyze Oxidation of Alpha, Beta-Unsaturated Aldehydes. *Arch Biochem Biophys*. 2007; 464:187–196. [PubMed: 17599801]
- Chandra A, Srivastava SK. A Synthesis of 4-Hydroxy-2-Trans-Nonenal and 4-(3H) 4-Hydroxy-2-Trans-Nonenal. *Lipids*. 1997; 32:779–782. [PubMed: 9252968]
- Fuller AT. Is p-aminoazobenzenesulfonamide the active agent in prontosil therapy? *Lancet*. 1937; i: 194–198.
- Guengerich FP. Oxidation-Reduction Properties of Rat Liver Cytochromes P-450 and NADPH-Cytochrome P-450 Reductase Related to Catalysis in Reconstituted Systems. *Biochemistry*. 1983; 22:2811–2820. [PubMed: 6307349]
- Guengerich FP. Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. *Chem Res Toxicol*. 2001; 14:611–650. [PubMed: 11409933]
- Guengerich FP. Mechanisms of Cytochrome P450 Substrate Oxidation: MiniReview. *J Biochem Mol Toxicol*. 2007; 21:163–168. [PubMed: 17936929]
- Guengerich FP. Cytochrome P450 and Chemical Toxicology. *Chem Res Toxicol*. 2008; 21:70–83. [PubMed: 18052394]
- Havemeyer A, Grunewald S, Wahl B, Bittner F, Mendel R, Erdelyi P, Fischer J, Clement B. Reduction of N-Hydroxy-Sulfonamides, Including N-Hydroxy-Valdecocix, by the Molybdenum-Containing Enzyme MARC. *Drug Metab Dispos*. 2010; 38:1917–1921. [PubMed: 20699408]
- Levine WG. Metabolism of Azo Dyes: Implication for Detoxication and Activation. *Drug Metab Rev*. 1991; 23:253–309. [PubMed: 1935573]
- Levine WG, Lu AY. Role of Isozymes of Cytochrome P-450 in the Metabolism of N,N-Dimethyl-4-Aminoazobenzene in the Rat. *Drug Metab Dispos*. 1982; 10:102–109. [PubMed: 6124393]
- Levine WG, Zbaida S. Two Classes of Azo Dye Reductase Activity Associated With Rat Liver Microsomal Cytochrome P-450. *Adv Exp Med Biol*. 1991; 283:315–321. [PubMed: 1906222]
- Lind C, Hochstein P, Ernster L. DT-Diaphorase As a Quinone Reductase: a Cellular Control Device Against Semiquinone and Superoxide Radical Formation. *Arch Biochem Biophys*. 1982; 216:178–185. [PubMed: 6285828]
- Mallett AK, King LJ, Walker R. A Continuous Spectrophotometric Determination of Hepatic Microsomal Azo Reductase Activity and Its Dependence on Cytochrome P-450. *Biochem J*. 1982; 201:589–595. [PubMed: 7092814]
- Mallett AK, King LJ, Walker R. Solubilisation, Purification and Reconstitution of Hepatic Microsomal Azoreductase Activity. *Biochem Pharmacol*. 1985; 34:337–342. [PubMed: 3918538]
- Marini S, Grasso E, Longo V, Puccini P, Riccardi B, Gervasi PG. 4-Biphenylaldehyde and 9-Anthraldehyde: Two Fluorescent Substrates for Determining P450 Enzyme Activities in Rat and Human. *Xenobiotica*. 2003; 33:1–11. [PubMed: 12519690]
- Masters BS, Bilimoria MH, Kamin H, Gibson QH. The Mechanism of 1- and 2-Electron Transfers Catalyzed by Reduced Triphosphopyridine Nucleotide-Cytochrome c Reductase. *J Biol Chem*. 1965; 240:4081–4088. [PubMed: 4378860]
- Milton MN, Elcombe CR, Gibson GG. On the Mechanism of Induction of Microsomal Cytochrome P450IVA1 and Peroxisome Proliferation in Rat Liver by Clofibrate. *Biochem Pharmacol*. 1990; 40:2727–2732. [PubMed: 2260995]
- Prough RA, Amunom I, Tamasi V, Conklin DJ, Bhatnagar A, Srivastava S. Cytochrome P450s catalyze reduction of lipid aldehydes metabolites. *Drug Metab Rev*. 2009; 41
- Prough RA, Coomes MW, Cummings SW, Wiebkin P. Metabolism of Procarbazine [N-Isopropyl-Alpha-(2-Methylhydrazino)-p-Toluamide HCl]. *Adv Exp Med Biol*. 1981; 136(Pt B):983–996. [PubMed: 7046391]
- Raza H, Levine WG. Azoreduction of N,N-Dimethyl-4-Aminoazobenzene (DAB) by Rat Hepatic Microsomes. Selective Induction by Clofibrate. *Drug Metab Dispos*. 1986a; 14:19–24. [PubMed: 2868860]

- Raza H, Levine WG. Effect of Phenobarbital and Beta-Naphthoflavone on Oxidative Metabolism of N,N-Dimethyl-4-Aminoazobenzene by Regenerating Rat-Liver Microsomes and Its Response to Sulphydryl Compounds. *Xenobiotica*. 1986b; 16:827–837. [PubMed: 3094256]
- Shou M, Lu T, Krausz KW, Sai Y, Yang T, Korzekwa KR, Gonzalez FJ, Gelboin HV. Use of Inhibitory Monoclonal Antibodies to Assess the Contribution of Cytochromes P450 to Human Drug Metabolism. *Eur J Pharmacol*. 2000; 394:199–209. [PubMed: 10771285]
- Srivastava S, Ramana KV, Bhatnagar A, Srivastava SK. Synthesis, Quantification, Characterization, and Signaling Properties of Glutathionyl Conjugates of Enals. *Methods Enzymol*. 2010; 474:297–313. [PubMed: 20609918]
- Trefouele J, Trefouel J, Nitti F, Bovet D. Activite du p-aminophenylsulfamide sur les infections streptococciques. Experimentales de la souris et due lapin. *Comptes Rendus Seances Societe Biology*. 1935; 120:756–762.
- Tweedie DJ, Erikson JM, Prough RA. Metabolism of Hydrazine Anti-Cancer Agents. *Pharmacol Ther*. 1987; 34:111–127. [PubMed: 3310042]

Figure 1. Fluorescence excitation and emission spectra of 9-anthracene-carboxylic acid and 9-hydroxymethyl-anthracene

Emission spectra of 9-anthracenecarboxylic acid (green line) and 9-hydroxymethyl-anthracene (pink line) were scanned in a Perkin Elmer Model 50B spectrofluorometer using 255 nm as an excitation spectra. The excitation spectra were obtained using 411 nm as the emission wavelength to measure fluorescence for 9-hydroxymethyl-anthracene (blue line) and 455 nm as the emission wavelength to measure fluorescence for 9-anthracenecarboxylic acid (red line).

Figure 2. Schematic drawing of reaction vessel to perform experiments under anaerobic (argon) or carbon monoxide (CO) atmosphere

Gas regulators for air, argon, or carbon monoxide are attached to Tygon tubing. The other end is attached to a modified outer syringe tube that allows one to insert the needle on the syringe part into a septum stopper capping a 50 mL Erlenmeyer flask. Note in upper right figure how to trim the syringe after removing the plunger. Insert an 18 gauge needle into the serum stopper allowing release of gas into a hood. The trimmed end of the syringe tube can then be inserted into an appropriate sized Tygon tubing and after attaching an 18 gauge needle, it should be inserted into the serum stopper on the Erlenmeyer flask. Intramedic tubing (non-reactive polyethylene tubing) can be attached to the needle to allow the gases to bubble through the solutions, until the various enzymes are added. The reagents can be added using syringes with 18 gauge needles.

Table 1**Inhibitors of various Reductive Enzymes**

Enzyme	Substrate	Inhibitor
CYP1A	ethoxyresorufin	7,8-naphthoflavone
CYP1A	ethoxyresorufin	furaflavone
CYP2A	coumarin	8-methoxypsoralen; trans-cyclopromine
CYP2B	ethylmorphine	ketoconazole, SKF525A
CYP2C8/9	tolbutamide	sulfaphenazole; phenytoin
CYP2D6	dextromethorphan	quinidine
CYP2E	chlorzoxazone	pyridine, chlormethiazole, diethyldithio-carbamic acid
CYP3A	midazolam; testosterone	ketoconazole; Troleandomycin
CYP4A	lauric acid	clofibrate; 10-undecyenoic acid
NADPH:cytochrome P450 oxidoreductase	dichlorophenol indophenol; cytochrome c	diphenyliodonium chloride
NAD(P)H:quinone oxidoreductase	menadione plus cytochrome c or dichlorophenol indophenol	dicoumarol; diphenyliodonium chloride