Coumarin Metabolism by Rat Esophageal Microsomes and Cytochrome P450 2A3

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The rat esophagus is strikingly sensitive to tumor induction by nitrosamines, and it has been hypothesized that this tissue contains cytochrome P450 enzymes (P450s) which catalyze the metabolic activation of these carcinogens. The metabolic capacity of the esophagus is not well characterized. In the study described here, the products of ^{14}C-coumarin metabolism by rat esophageal microsomes were identified and quantified. Metabolite characterization was by LC/MS/MS and GC/MS and comparison to standards, quantification was by radioflow HPLC. The coumarin metabolites formed by rat esophageal microsomes were compared to those formed by P450 2A3. The major metabolites formed by esophageal microsomes were 8-hydroxycoumarin, *o*-hydroxyphenylacetaldehyde (*o*-HPA), and *o*-hydroxyphenylacetic acid (*o*-HPAA). A smaller amount of 5-hydroxycoumarin, about one-third the 8-hydroxycoumarin, was also formed. *o*-HPA and *o*-HPAA are products of coumarin 3,4-epoxidation. The relative rates of coumarin 8-hydroxylation and 3,4-epoxidation were similar. Coumarin 8-hydroxylation has not previously been reported as a major pathway in any tissue, and no P450s have yet been reported to catalyze this reaction. P450 2A3 catalyzed both the 7-hydroxylation and 3,4-epoxidation of coumarin. P450 2A3 was previously characterized as a coumarin 7-hydroxylase, however, in this study, we report that it catalyzes the formation of *o*-HPA more efficiently. The K_m and V_{max} were 1.3 ± 0.35 μM and 0.65 ± 0.06 nmol/min/nmol P450 for coumarin 7-hydroxylation and 1.4 ± 0.58 μM and 3.1 ± 0.46 nmol/min/nmol P450 for *o*-HPA formation.

Introduction

The rat esophagus is uniquely sensitive to tumor induction by nitrosamines (*1*, *2*). Most nitrosamines are metabolically activated by P450-catalyzed α-hydroxylation (*1*). It has been hypothesized by us, and others, that either a unique or relatively abundant P450 in the rat esophagus is responsible for this activation and therefore contributes to the sensitivity of the esophagus to nitrosamine carcinogenesis (*3*−*6*). It is an ongoing goal of our laboratory to characterize the metabolic capacity of the rat esophagus and specifically to identify P450s present in this tissue.

P450 enzymes present in the rat esophagus include 1A1, 2A3, and the recently cloned P450, 2B21 (*7*−*9*). P450 2B21 protein has not yet been either purified from the rat or expressed in a heterologous system (*9*). Therefore, its catalytic activities are unknown. P450 1A1 is relatively abundant in the esophagus but it does not catalyze metabolism of the potent esophageal carcinogen *N*-nitrosobenzylmethylamine (NBzMA)[1] (*10*). In contrast, P450 2A3, which is believed to be the ortholog of the human P450 2A6, is an efficient catalyst of both NBzMA and N′-nitrosonornicotine (NNN) metabolism (*11*, *12*). NNN is a tobacco specific nitrosamine considered a likely causative agent for esophageal cancer in smokers (*13*). In the past, we have suggested that P450 2A3 may be an important catalyst of nitrosamine metabolism in the rat esophagus (*8*). However, our more recent data do not support this conclusion (*11*, *12*).

P450 2A3 is an extrahepatic P450. cDNA for this enzyme was originally generated from rat lung mRNA (*14*). However, the enzyme is much more abundant in rat nasal mucosa (*8*, *15*). Very low levels of P450 2A3 mRNA, about 60-fold less than those in the lung have been detected in the rat esophagus (*8*). In addition, antibodies to the closely related mouse P450s, 2A4 and 2A5, detected an immunoreactive protein of the correct molecular weight in rat esophageal microsomes. The amount of this protein was similar to the amount detected in rat lung (*8*). This is in contrast to the very different levels of P450 2A3 mRNA in the esophagus and the lung (*8*). P450 2A3 is an efficient coumarin 7-hydroxylase (*11*). However, rat esophageal microsomes do not catalyze detectable levels of coumarin 7-hydroxylation (*11*). Therefore, the level of P450 2A3 in the rat esophagus must be quite low.

Despite the lack of coumarin 7-hydroxylase activity in the rat esophagus, coumarin inhibits NBzMA metabolism by rat esophageal microsomes (*11*). This inhibition is significantly increased when the enzyme is incubated with coumarin and NADPH prior to the addition of NBzMA. Therefore, a coumarin metabolite formed by rat esophageal microsomes is contributing to the observed inhibition. The P450 that catalyzes coumarin metabolism in the rat esophagus may also contribute to the catalysis of NBzMA metabolism. In addition, characterizing coumarin metabolism by rat esophageal microsomes will

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[1] Abbreviations: NBzMA, *N*-nitrosobenzylmethylamine; NNN, *N*-nitrosonornicotine; *o*-HPA, *o*-hydroxyphenylacetaldehyde; *o*-HPAA, *o*-hydroxyphenylacetic acid; *o*-HPPA, *o*-hydroxyphenylpropionic acid; APCI, atmospheric pressure chemical ionization; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; TMS, trimethylsilyl.

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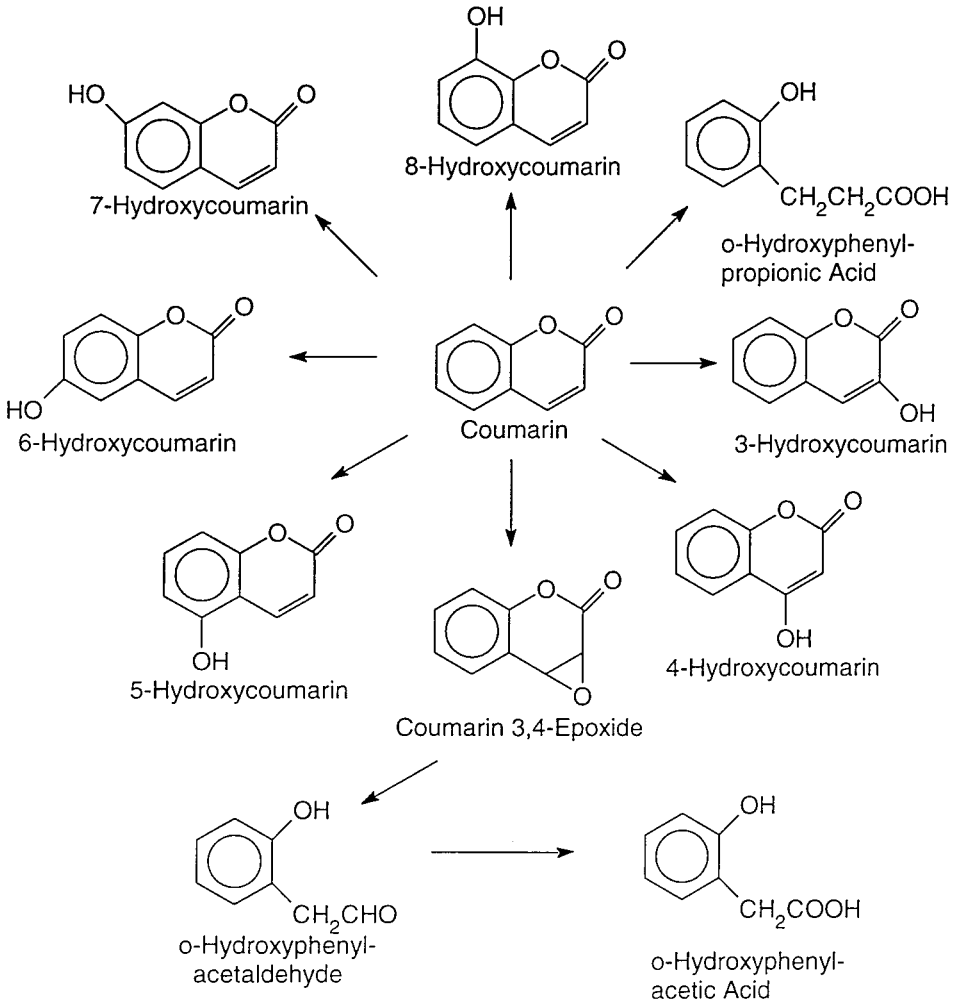
Scheme 1. Coumarin Metabolism



help to establish what P450s are present in this tissue.

Coumarin can be metabolized via many different pathways (ref *16* and Scheme 1). Six hydroxylated coumarins as well as *o*-HPA and *o*-HPAA, generated by lactone ring cleavage, have been identified as products of coumarin metabolism in various tissues and animal species (*16*, *17*). *o*-HPA is formed nonenzymatically from the unstable 3,4-epoxide of coumarin and *o*-HPA can be further metabolized to *o*-HPAA (*18*). In all the species and tissues studied to date, the predominate pathway of coumarin metabolism is either 7-hydroxylation or lactone ring cleavage, i.e., 3,4-epoxidation (*16*, *17*, *19*, *20*). Hydroxylated coumarins other than 7-hydroxycoumarin have been detected as minor metabolites in both in vivo and in vitro studies of coumarin metabolism in a number of species. Little is know about the enzymes involved in the formation of these products or their biological relevance.

In the rat, the predominant pathway of coumarin metabolism is 3,4-epoxidation. The major urinary coumarin metabolite is *o*-HPAA, and the major hepatic microsomal metabolite is *o*-HPA (*17*, *21*). Both 3,4-epoxycoumarin and *o*-HPA contribute to the hepatotoxicity that is observed in rats administered coumarin (*22*). We hypothesize that one of these metabolites may contribute to the NADPH-dependent coumarin inhibition of NBzMA metabolism by rat esophageal microsomes. In this study, we characterized the complete metabolism of coumarin by rat esophageal microsomes and compared the metabolites formed to the products of P450 2A3-catalyzed coumarin metabolism.

Materials and Methods

Chemicals. Esophagi from male Fisher 344 rats, age 7−13 weeks, were purchased from Harlan Bioproducts (Indianapolis, IN). [U-^{14}C−Benzyl]Coumarin (58 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). 4-Hydroxycoumarin, 2-hydroxyphenyl acetic acid, *o*-coumaric acid, and 6,7-dihydroxycoumarin were purchased from Fluka Chemical Co. (Ronkonoma, NY), 3-hydroxycoumarin was from Indofine Chemical Co. (Sommerville, NJ), *o*-HPA was a gift from Dr. Lois D. Lehman-McKeeman of Procter & Gamble (Cincinnati, OH), and 5-hydroxycoumarin was a gift from Dr. Takashi Harayama (Okayama University, Japan). BSTFA/TMCS (1%) was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI).

Synthesis of 6-Hydroxycoumarin and 8-Hydroxycoumarin. 6-Hydroxy- and 8-hydroxycoumarin were synthesized from 2,5-dihydroxybenzaldehyde or 2,3-dihydroxybenzaldehyde, respectively, by the method of Harayama et al. (*23*). The dihydroxybenzaldehyde (552 mg) was dissolved in 40 mL of diethylaniline. Carbethoxymethylenetriphenylphosphoran (1.67 g) was added, and the solution was refluxed for 40 min. Upon cooling, 40 mL of a 5% HCl solution was added and the mixture was extracted with diethyl ether (three times). The ether layer was extracted with a 5% NaOH solution. The aqueous layer was acidified with 5% HCl and again extracted with diethyl ether (three times). The ether was evaporated and the residue was

dissolved in ethyl acetate. The crude mixture was purified by preparative TLC on silica plates (Uniplate, Analtech, Newark, DE) with a mobile phase of 50% hexane:50% ethyl acetate. The products were further purified by HPLC system I. The identities of the products were confirmed by LC/MS and ^{1}H NMR (600 MHz). *8-hydroxycoumarin,* APCI/MS [*m/z*, (relative abundance)]: 163 ([M + H]$^+$, 100), 135 ([M + H-CO]$^+$, 17), 119 ([M + H-CO$_2$]$^+$, 10), 107 ([C$_7$H$_7$O]$^+$, 60), 91 ([C$_7$H$_7$]$^+$, 23). ^{1}H NMR (DMSO-d_6): δ 10.14 (s, 1H, OH); 7.96 (d, J = 9.3, 1H, 4-H); 7.12−7.04 (m, 3H, 5,6,7-H); 6.41 (d, J = 9.3, 1H, 3-H). *6-Hydroxycoumarin:* APCI/MS/MS [*m/z*, (relative abundance)]: 163 ([M + H]$^+$, 57), 135 ([M + H-CO]$^+$, 20), 119 ([M + H −CO$_2$]$^+$, 10), 107 ([C$_7$H$_7$O]$^+$, 100. ^{1}H NMR (DMSO-d_6): δ 9.69 (s, 1H, OH); 7.93 (d, J = 9, 1H, 4-H); 7.19 (d, J = 9.6, 1H, 5-H); 7.00−6.95 (s + d, 2H, 7,8-H); 6.39 (d, J = 9.6, 1H, 3-H). Proton assignment was confirmed by heteronuclear multiple quantum correlation (HMQC) experiments.

Coumarin Metabolism by P450 2A3 and Rat Esophageal Microsomes. Baculovirus (BV) expressed P450 2A3 was provided by Xinxin Ding (Wadsworth Center, Albany, NY) as a cell lysate, and coumarin metabolism was analyzed as previously described (*11*). Reactions were run under conditions where product formation was linear with time and protein. The products formed by the metabolism of [U-^{14}C benzyl]coumarin (0.5−10 μM) by P450 2A3 (2−10 pmol/mL) were analyzed by one of three HPLC systems. Detection was by radioflow analysis, and comparison to co-injected metabolite standards detected by UV absorbance at 254 nm. To determine the kinetic parameters of P450 2A3-catalyzed coumarin 7-hydroxylation and 3,4-epoxidation (*o*-HPA formation), duplicate reactions were run at five [U-^{14}C benzyl]coumarin concentrations (0.5, 1, 3, 5, and 10 μM). The concentration of P450 2A3 was 2 pmol/mL and the incubation time 10 min. The metabolites were analyzed on HPLC system I with radioflow detection. Two independent determinations of the kinetic parameters were carried out.

Rat esophageal microsomes were prepared as described previously (*3*). Four rat esophagus were used for each preparation of microsomes. Coumarin metabolism was analyzed by incubating microsomes (0.5−2 mg/mL) with [U-^{14}C benzyl] coumarin (3−20 μM) for 30−60 min at 37 °C in 200 μL of 100 mM Tris buffer (pH 7.4) in the presence of an NADPH generating system (*11*). The reaction was terminated by the addition of 20 μL 15% trichloroacetic acid. Samples, co-injected with standards, were analyzed by reverse-phase HPLC (system I, II, and III) with radioflow detection. Coumarin metabolism by rat esophageal microsomes was analyzed for seven separate microsomal preparations.

LC/MS and LC/MS/MS Analysis. Two esophageal microsomal preparations (4 esophagi each) were pooled for each experiment. The analysis was run in duplicate on two different pooled preparations. Coumarin metabolite samples were prepared as described for the radioflow analysis experiments except that nonradioactive coumarin was used at a concentration of 100 μM. Control reactions included reactions without NADPH, reactions without coumarin, and reactions without NADPH that were spiked with either 5-hydroxycoumarin or 8-hydroxycoumarin. At the termination of the reactions, the samples were centrifuged and 100 μL of the supernatant was analyzed directly by either LC/MS or LC/MS/MS. HPLC system I was used. LC/MS and LC/MS/MS was carried out on a Finnigan MAT model LCQ Deca instrument, operated in the positive ion APCI mode, interfaced with a Waters Alliance HPLC system. The APCI settings were capillary temperature, 200 °C; vaporizer temperature, 600 °C. The auxiliary gas (N_2) was applied at a pressure of 39.4 psi. For the analysis of samples, the instrument was tuned to 8-hydroxycoumarin.

HPLC Systems. Systems I and II used a Phenomenex (Torrance CA) Bondclone C$_{18}$ column (3.9 × 300 mm, 10 μm), and system III used a Phenomenex Luna C$_{18}$ column (4.6 × 250 mm, 5 μm). In system I, the metabolites were eluted isocratically with 69.3% H_2O:29.7% methanol:1% acetic acid, and the flow rate was 1 mL/min. System II was similar to that of Peters et al. (*24*). The mobile phase consisted of 21% tetrahydrofuran, 0.2% formic acid in water (A) and 0.2% formic acid in water (B). The metabolites were eluted with 32% A:68% B, for 5 min, followed by a linear gradient to 46% A:54% B in 15 min and then to 100% A over 10 min. The flow rate was 1 mL/min. In system III, the metabolites were eluted isocratically with 79.2% H_2O:19.8% methanol:1% acetic acid. Detection was by UV absorbance at 254 nm radioactivity, quantified on a β-ram radioflow detector (IN/US Systems, Inc., Tampa, FL).

GC/MS Analysis. Rat esophageal microsomes samples to be analyzed by GC/MS were prepared as described for the LC/MS analyses. To obtain sufficient material to purify coumarin metabolites by HPLC prior to GC/MS analysis, microsomes from four different preparations were pooled (16 esophagi) and incubated for 1 h with 100 μM coumarin in the presence of an NADPH generating system. P450 2A3 (20 pmol/mL) was incubated with 40 μM coumarin for 20 min as described above.

Both sample and control reactions were injected on HPLC system I. Two fractions of the eluant were collected from all reactions in duplicate. Fraction one contained *o*-HPA and *o*-HPAA (9−13 min), and fraction two contained 7-hydroxy and 8-hydroxycoumarin (13−17 min). Methanol was removed from each fraction by evaporation under a stream of nitrogen and the coumarin metabolites were extracted with ethyl acetate (three times). The organic phase was dried with Na_2SO_4, and the ethyl acetate was evaporated under nitrogen. The dry sample was dissolved in 20 μL of BSTFA:1% TMCS and heated at 65 °C for 30 min to generate TMS-derivatized metabolites. The recovery of standards (listed below), carried through the same procedure, was >95%. The derivatized samples were analyzed on a Hewlett-Packard (Wilmington, DE) GC/MS system consisting of a model G1530A gas chromatograph, with a model 6890 autosampler interfaced with a model 5973 mass selective detector operating in the electron impact ionization mode. A 30 m × 0.25 mm DB-5ms column (J & W Scientific, Folsom, CA) with 0.25 μm film thickness was used for separation of the metabolites. The inlet temperature was 280 °C. The GC oven temperature was held at 80 °C for 1 min, then increased by 5 °C/min to 300 °C. The mass spectrometer (MS quad temperature 150 °C, MS source temperature, 230 °C) was tuned against pentafluorobenzoic acid and operated in the full scan mode (*m/z* 100−400). The retention times of the derivatized standards under these conditions were *o*-HPA (12.11 min), *o*-HPAA (17.81 min), *o*-HPPA (20.19 min), 8-hydroxy- (20.69 min), 6-hydroxy- (23.16 min), 4-hydroxy- (23.39 min), 7-hydroxycoumarin (23.68 min), and 6,7-dihydroxycoumarin (28.52 min).

Kinetics and Statistical Analysis. K_m and V_{max} for coumarin metabolism by P450 2A3 were determined using the EZ-fit 5 kinetics program from Perrella Scientific [Amherst, NH (*25*)]. This program uses a nonlinear regression method of curve fitting and the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence. K_m and V_{max} values were compared using the *Z*-test.

Results

To characterize any coumarin metabolites formed by rat esophageal microsomes and P450 2A3, 6-hydroxy and 8-hydroxycoumarin, which were not available, were synthesized. Two HPLC systems (I and III) were developed to separate all coumarin metabolites. System I was essentially that which we have used previously to analyze 7-hydroxycoumarin (*11*). The acetic acid concentration was increased from 0.2 to 1.0%, and the methanol concentration was decreased to 29.7%. These relatively minor, but critical, changes allowed most coumarin metabolites to be separated by reverse-phase HPLC (Figure 1A). 8-Hydroxycoumarin eluted just prior to 7-hydroxycoumarin, and 6-hydroxycoumarin eluted before 8-hydroxycoumarin. However, 5-hydroxycoumarin

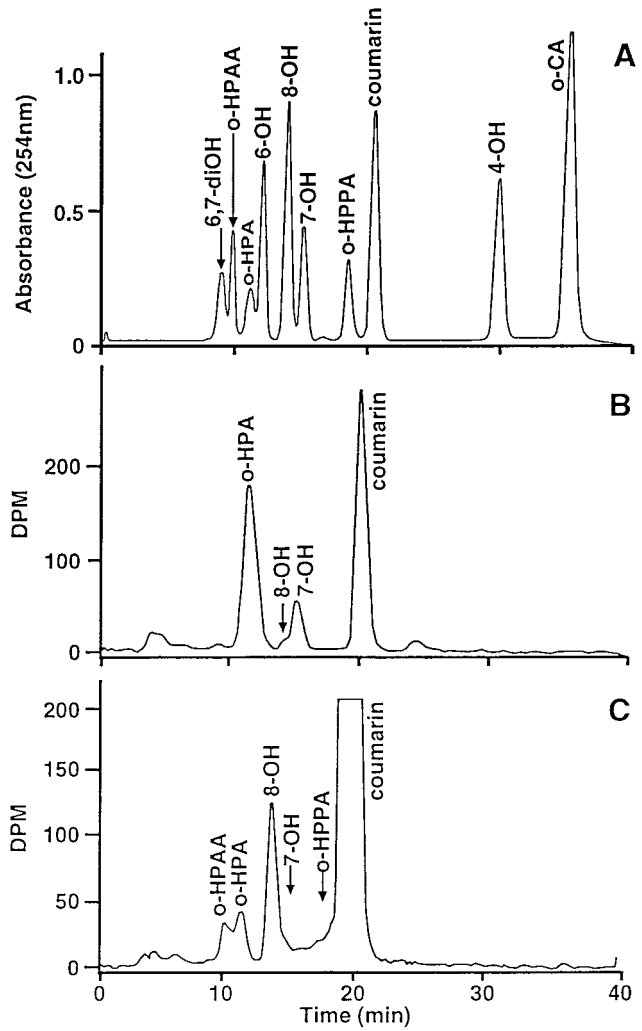


Figure 1. HPLC analysis of coumarin and coumarin metabolites. (A) Standards. (B) Products of P450 2A3 (2.1 pmol) catalyzed [U-^{14}C benzyl]coumarin (3 μM) metabolism (incubation time, 30 min). (C) Products of rat esophageal microsomes catalyzed [U-^{14}C benzyl]coumarin (20 μM) metabolism. (Incubation time, 60 min). Abbreviations: 4-, 6-, 7-, 8-OH, 4-, 6-, 7-, 8-hydroxycoumarin; 6,7-diol, 6,7-dihydroxycoumarin; *o*-CA, *o*-coumarin acid; *o*-HPPA, *o*-hydroxyphenylpropionic acid, *o*-HPA, *o*-hydroxyphenylacetaldehyde; *o*-HPAA, *o*-hydroxyphenylacetic acid. HPLC system I was used, details of the analysis and enzyme assays are as described in the Materials and Methods. All analyses were carried out in duplicate. Note: the retention times are slightly different in the three chromatograms due to the age of the HPLC column.

partially coeluted with coumarin in system I. To separate all the hydroxylated coumarin metabolites from one another and 5-hydroxycoumarin from coumarin, a second isocratic system (III) was used in which the methanol concentration was decreased to 19.8%. Good resolution of *o*-HPA, *o*-HPAA, and 6,7-dihydroxycoumarin was not obtained with system III.

We previously identified P450 2A3 as an efficient coumarin 7-hydroxylase. The analysis used was HPLC with fluorescence detection (*11*). In the present study, the total metabolism of coumarin by P450 2A3 was determined using [U-^{14}C benzyl]coumarin. The products of the reaction were analyzed by radioflow HPLC (Figure 1B). Two major metabolite peaks were detected. The larger one coeluted with *o*-HPA (11.5 min), a product of coumarin 3,4-epoxidation. No *o*-HPAA, the oxidation product of *o*-HPA, was detected. The metabolite eluting at 15.1 min coeluted with 7-hydroxycoumarin. The same two metabolites were detected with two other HPLC systems, system II used previously by Peters et al. (*24*) and system III which separates 5-hydroxycoumarin from coumarin. 5-Hydroxycoumarin was not detected as a product of P450 2A3-catalyzed coumarin metabolism. The kinetic parameters of P450 2A3-catalyzed coumarin metabolism were determined. P450 2A3 catalyzed the formation of *o*-HPA at five times the rate of coumarin 7-hydroxylation. The K_m and V_{max} were 1.3 $\pm$ 0.35 μM and 0.65 $\pm$ 0.06 nmol/min/nmol P450 for coumarin 7-hydroxylation and 1.4 $\pm$ 0.58 μM and 3.1 $\pm$ 0.46 nmol/min/nmol P450 for *o*-HPA formation. These two K_m values do not differ ($z = -0.124$, $p = 0.901$).

The products of [U-^{14}C benzyl]coumarin metabolism by rat esophageal microsomes were also analyzed with HPLC system I. The metabolites formed by esophageal microsomes were quite different then those formed by P450 2A3 (Figure 1, panels B and C). The major metabolite peak coeluted with 8-hydroxycoumarin (t_R, 14 min). Little, if any, 7-hydroxycoumarin was detected. Two additional metabolite peaks, coeluting with *o*-HPAA (10 min) and *o*-HPA (11 min), were also detected. The ratio of 8-hydroxycoumarin to the products of the 3,4-epoxide pathway, *o*-HPA and *o*-HPAA, was 1.3 for the sample illustrated in Figure 1C. Within the same microsomal preparation, this ratio did not differ between duplicates or change with coumarin concentration (1−20 μM). However, this ratio did vary between esophageal microsomal preparations, ranging from 1:1 to 2:1. In coumarin metabolite analyses using HPLC system III, a radioactive peak that coeluted with 5-hydroxycoumarin was also identified as a product of rat esophageal metabolism. In several independent analyses 5-hydroxycoumarin was formed at approximately one-third the rate of 8-hydroxycoumarin. The rate of coumarin metabolism increased 4-fold from 1−20 μM (data not shown). Kinetic parameters were not determined since, unlike P450 2A3, the enzyme(s) did not appear to saturate by 20 μM coumarin.

To further characterize the hydroxylated coumarin metabolites formed by rat esophageal microsomes, LC/MS and LC/MS/MS analysis conditions were developed. Using HPLC system I, LC/MS/MS analysis of all six hydroxylated coumarins was carried out. The relative abundance of the product ions obtained for *m*/*z* 163, the $(M + H)^+$ ion for each hydroxylated coumarin is presented in Table 1. The major fragment for both 6- and 8-hydroxycoumarin was *m*/*z* 135 loss of CO from the molecular ion, and the second most abundant fragment was *m*/*z* 119 loss of CO_2. In contrast, the major fragment obtained for 5-hydroxy- and 7-hydroxycoumarin was *m*/*z* 119. Essentially no *m*/*z* 135 fragment was detected. Neither *m*/*z* 135 nor *m*/*z* 119 was present in the product ion spectra of either 3 or 4-hydroxycoumarin, the major fragment for these two hydroxycoumarins was *m*/*z* 121.

Confirmation that the major coumarin metabolite formed by rat esophageal microsomes was 8-hydroxycoumarin was obtained by LC/MS and LC/MS/MS analysis. When the products of coumarin metabolism by esophageal microsomes were analyzed by LC/MS, a single metabolite peak with a molecular ion of *m*/*z* 163 was detected. The retention time and fragmentation pattern were identical to 8-hydroxycoumarin (data not shown). This metabolite was further characterized by LC/MS/MS. Rat esophageal microsomes were incubated with cou-

Table 1. Major Fragments Present in Product Ion Spectra of *m/z* 163 the M + H Ion of Hydroxylated Coumarins

	m/z (relative abundance) ion composition					
metabolite	135 $(C_8H_6O_2)H^+$	121 $(C_7H_5O_2)H^+$	119 $(C_8H_6O)H^+$	107 $C_7H_7O^+$	95 $(C_6H_6O)H^+$	91 $C_7H_7^+$
3-hydroxy		100			15	
4-hydroxy		100			20	
5-hydroxy			100			20
6-hydroxy	100		60	14		
7-hydroxy			100			7
8-hydroxy	100		95	28		55

marin, and the metabolites formed were analyzed by HPLC with MS/MS detection selecting for *m/z* 163 the molecular ion + 1 for monohydroxylated coumarins. The chromatogram illustrated in Figure 2A was obtained by monitoring only *m/z* 163 from the total daughter ion spectra obtained. Two peaks were detected with retention times of 13.2 and 17.9 min. The peak at 13.2 min coelutes with the 8-hydroxycoumarin standard. The product ion spectrum of *m/z* 163 for this metabolite (Figure 2B) is essentially identical to that of 8-hydroxycoumarin (Table 1).

The retention time and product ion spectrum of the metabolite peak at 17.9 min (Figure 2) is identical to 5-hydroxycoumarin (Figure 2C, Table 1). The major fragment present in the product ion spectra for this metabolite was *m/z* 119 generated by the loss of CO_2. 5-Hydroxycoumarin was initially not detected as a product of coumarin metabolism by rat esophageal microsomes using either radioflow HPLC or LC/MS analysis. This metabolite coelutes with coumarin in HPLC system I. Therefore, in the case of radioflow analysis, the much larger coumarin peak obscured this product. Similarly, its coelution with coumarin significantly increased the limit of detection for 5-hydroxycoumarin by LC/MS under the conditions used. As noted above, when the products of coumarin metabolism by rat esophageal microsomes were analyzed with a different HPLC system (III), a peak coeluting with 5-hydroxycoumarin was detected by radioflow HPLC. It was formed at about one-third the rate of 8-hydroxycoumarin (data not shown). *o*-HPA and *o*-HPAA were not detectable by LC/MS or LC/MS/MS with the conditions used to analyze the hydroxylated coumarins.

GC/MS analysis was used to characterize *o*-HPA and *o*-HPAA, as well as 7-hydroxy- and 8-hydroxycoumarin formed by P450 2A3 and esophageal microsomes. Mass spectral data and retention times of the silylated coumarin metabolite standards are listed in Table 2. The major fragment for all the hydroxylated coumarins was *m/z* 219, which corresponds to the loss of CH_3 from the TMS derivatized molecular ion, *m/z* 234. The molecular ion *m/z* 208 was the most abundant ion in the mass

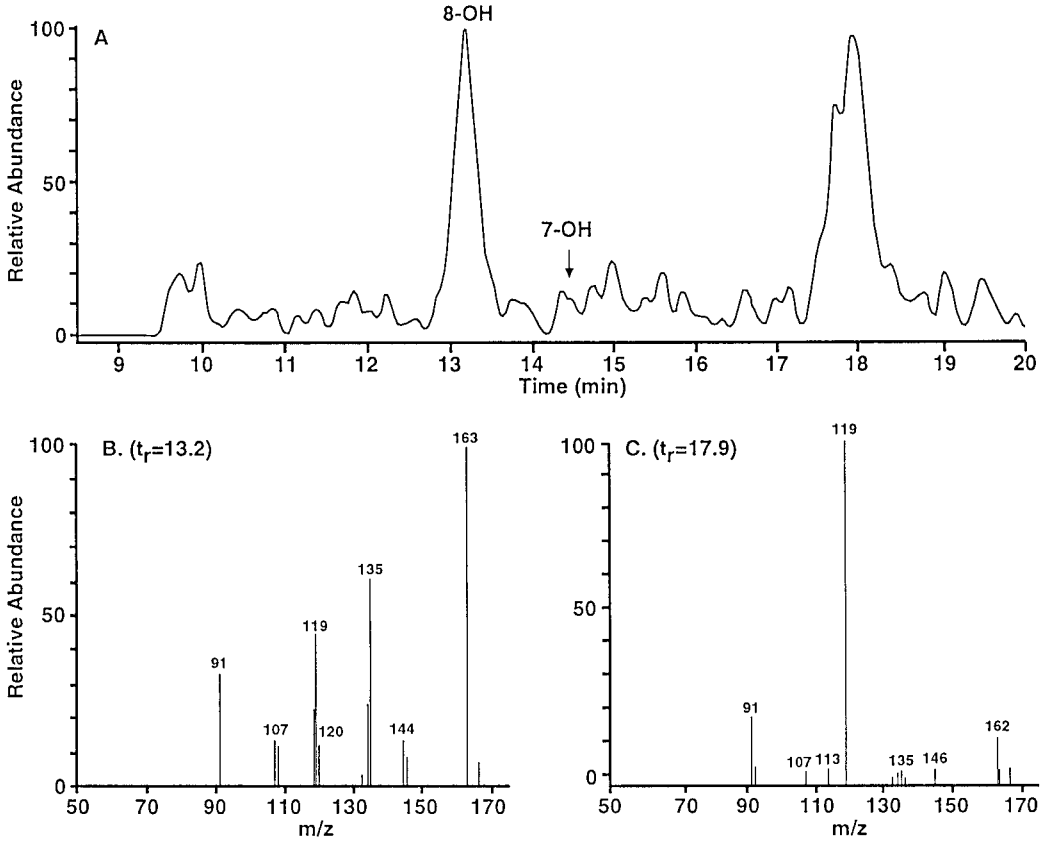


Figure 2. LC/MS/MS analysis of rat esophageal microsomes-catalyzed coumarin metabolism. Coumarin (100 μM) was incubated 60 min with rat esophageal microsomes and the products were analyzed by LC/MS/MS with selection for *m/z* 163, the protonated molecular ion of the hydroxylated coumarins. (A) Chromatogram monitoring *m/z* 163. (B) Product ion spectra of the metabolite peak eluting at 13.2 min. (C) Product ion spectra of the metabolite peak eluting at 17.9 min.

Table 2. Major Fragments Present in EI Spectra from GC/MS Analysis of TMS Derivatized Coumarin Metabolite Standards

metabolite	retention time (min)	*m/z* (percent relative abundance)
4-hydroxycoumarin	23.39	234(45), 219(100), 206(45)
6-hydroxycoumarin	23.16	234(55), 219(100), 191(10)
7-hydroxycoumarin	23.68	234(90), 219(100), 191(20)
8-hydroxycoumarin	20.69	234(20), 219(100), 191(45)
o-HPA	12.11	208(100), 193(40), 175(30)
o-HPAA	17.81	296(60), 253(80), 164(52), 147(100)

spectra of silylated *o*-HPA. The fragment ions detected were *m/z* 193 and *m/z* 165 corresponding to the sequential loss of CH_3 and CO. *o*-HPPA is derivatized twice and the major fragment detected was *m/z* 147 most likely a TMS dimer. A fragment ion of a TMS dimer is often detected in GC/MS analysis of compounds which are silylated at multiple sites (*26*). More unique ions in the *o*-HPAA spectrum included the molecular ion *m/z* 296 and a fragment ion generated by loss of CH_3 and CO, *m/z* 253.

To analyze the coumarin metabolites formed by P450 2A3 and rat esophageal microsomes with GC/MS, two fractions from each sample were collected following HPLC analysis. The first fraction, eluting between 9 and 13 min (Figure 2), should contain *o*-HPA and *o*-HPPA, and the second fraction, eluting from 9 to 13 min (Figure 2), 7-hydroxy and 8-hydroxy coumarin. Samples were extracted, derivatized with BSTFA/TMCS, then analyzed by GC/MS and compared to standards. The two coumarin metabolite fractions collected from P450 2A3 reactions contained *o*-HPA and 7-hydroxycoumarin, respectively. Whereas the first fraction collected from rat esophageal microsomal samples contained both *o*-HPA and *o*-HPAA and the second fraction 8-hydroxycoumarin. The spectra for each of these metabolites were identical to standards.

Discussion

The efficiency of coumarin 7-hydroxylation by the rat P450 2A3 is comparable to the closely related human and mouse enzymes P450 2A6 and 2A5 (*19*, *27*, *28*). However, clearly P450 2A3 does not have the specificity of these enzymes (*19*). We report here, that P450 2A3 catalyzes the 3,4-epoxidation of coumarin five times more efficiently than it does coumarin 7-hydroxylation. *o*-HPA, the decomposition product of coumarin 3,4-epoxide (*18*) is the major product of P450 2A3-catalyzed coumarin metabolism (Figure 1B). Previously, we characterized P450 2A3 as a coumarin 7-hydroxylase. In that study, HPLC with fluorescence detection was used to quantify 7-hydroxycoumarin (*11*). In the present study, the use of ^{14}C-coumarin allowed all the products of metabolism to be detected. The kinetic parameters for coumarin 7-hydroxylation from the two studies agree. The previously reported K_m was 1.7 ± 0.41 μM and V_{max}, 1.7 ± 0.008 nmol/min/nmol P450 (*11*) and the values reported here are 1.3 ± 0.35 μM and 0.65 ± 0.06 nmol/min/nmol P450.

This is the first report of a P450 2A enzyme catalyzing the 3,4-epoxidation of coumarin. However, several human P450s have been reported to catalyze this reaction. P450s, 1A1, 1A2, 2E1, and 3A4 all catalyze the formation of *o*-HPA with reasonable efficiencies (*19*). The K_m values range from 12 to 54 μM and V_{max} from 2 to 7 nmol/min/nmol P450. None of these P450s catalyzed the 7-hydroxylation of coumarin. The mouse P450 2G1, like P450 2A3, catalyzes both the formation of *o*-HPA and 7-hydroxycoumarin with a similar difference in efficiency for the two reactions (*19*).

Rat liver microsomes catalyze the 3,4-epoxidation of coumarin (*21*). However, what rat P450 catalyzes this reaction has not been reported. Rat nasal mucosa microsomes metabolize coumarin to both 7-hydroxycoumarin and *o*-HPA (*19*). The K_m of coumarin 7-hydroxylation by rat nasal microsomes is 1.8 μM, similar to the K_m of P450 2A3 (*11*, *29*). The ratio of *o*-HPA to 7-hydroxycoumarin formation in the nasal mucosa was reported to be about 2:1 (*19*), less than the 5:1 ratio for P450 2A3. P450 2A3 is abundant in the nasal mucosa (*8*, *15*) and most likely contributes to coumarin metabolism in this tissue.

We previously reported that in contrast to both rat nasal microsomes and P450 2A3, rat esophageal microsomes do not catalyze the 7-hydroxylation of coumarin (*11*). In the present study, we confirmed this and identified the major coumarin metabolite formed by esophageal microsomes as 8-hydroxycoumarin. This is the first report of 8-hydroxycoumarin as a major metabolite of coumarin in any tissue. However, 8-hydroxycoumarin may be one of several unidentified hydroxylated coumarins formed during coumarin metabolism by rat nasal microsomes (*19*). All the hydroxylated coumarins in the rat nasal mucosa were formed to a lesser extent than was *o*-HPA.

Rat esophageal microsomes catalyzed the formation of *o*-HPA and its oxidation product, *o*-HPAA, i.e., coumarin 3,4-epoxidation, to almost the same extent as the 8-hydroxylation of coumarin. However, the relative rates of 3,4-epoxidation and 8-hydroxylation varied between different preparations of esophageal microsomes. There are two possible explanations for the observed variation. One is that different enzymes catalyze these reactions and that one of these is denatured to varying extents during the isolation of microsomes. We and others have previously reported significant variation in the metabolic capacity of rat esophageal microsomal preparations (*3*, *30*). The second possibility is that that some of the 3,4 epoxide which is formed reacts with protein instead of decomposing to *o*-HPA and *o*-HPAA and that this is variable between preparations of microsomes. This would also result in the inaccurate quantitation of the 3,4-epoxidation pathway. In preliminary experiments, we were able to trap the epoxide with GSH and therefore to measure 3,4-epoxidation as the formation of a GSH adduct instead of the sum of *o*-HPA and *o*-HPAA (data not shown). In those experiments, the rate of 3,4-epoxidation rate did not increase to greater than the rate of 8-hydroxylation.

A single P450 may or may not catalyzes the 8-hydroxylation and 3,4 epoxidation of coumarin by rat esophageal microsomes. P450 1A1 is present in the rat esophagus and may contribute to coumarin 3,4-epoxidation in this tissue (*7*, *19*). The small amount of P450 2A3 present in the esophagus (*8*) could also contribute to this pathway. Neither P450 1A1 nor 2A3 catalyzes the 8-hydroxylation of coumarin (ref *19*, Figure 1). Therefore, a second enzyme would have to catalyze the 8-hydroxylation of coumarin. One candidate for this reaction is the recently cloned esophageal P450 2B21 (*9*). We are in the process of expressing this enzyme in a baculovirus/Sf9 system and will in the future determine if it is a catalyst of either coumarin 8-hydroxylation or 3,4-epoxidation. P450 2B21

is present in rat liver and a small amount of 8-hydroxycoumarin may be formed by rat liver slices (*20*).

The possibility exists that one enzyme may catalyze both the 8-hydroxylation and 3,4-epoxidation of coumarin, analogous to the catalysis of both coumarin 7-hydroxylation and 3,4-epoxidation by P450 2A3. However, the variation we observed in the ratio of 8-hydroxylation to 3,4-epoxidation of coumarin between individual esophageal preparations suggests that two distinct enzymes catalyzing this reaction in the esophagus. Future studies will determine the identity of the enzyme or enzymes that catalyze the 3,4-epoxidation and the 8-hydroxylation of coumarin in the esophagus and whether these same enzymes are catalysts of nitrosamine metabolism in this tissue.

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