
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ANALYTICAL BIOCHEMISTRY 170, 83–93 (1988)

# Determination of Microsomal Lauric Acid Hydroxylase Activity by HPLC with Flow-Through Radiochemical Quantitation

MARIA C. ROMANO, KENNETH M. STRAUB, LEE ANN P. YODIS, REGINA D. ECKARDT, AND JOHN F. NEWTON

*Department of Drug Metabolism, Smith Kline & French Laboratories, 709 Swedeland Road, Swedeland, Pennsylvania 19479*

Received July 6, 1987

An assay for the microsomal hydroxylation of lauric acid (LA), based on HPLC with flow-through radiochemical detection, has been developed. Conditions were optimized for resolution and quantitation of three microsomal metabolites of LA, one of which has not been reported previously as a metabolite of LA in mammalian microsomal incubations. These products, 12-(ω)-hydroxy-LA, 11-(ω-1)-hydroxy-LA, and a novel metabolite, 10-(ω-2)-hydroxy-LA, were isolated by HPLC and identified by gas chromatography/mass spectrometry. In the presence of NADPH, the formation of all three metabolites was linear with time and microsomal protein concentration. Hydrogen peroxide also supported the microsomal metabolism of LA, although the ratio of metabolites was substantially different than that produced by NADPH-supported microsomes. Several biochemical probes (metyrapone, α-naphthoflavone, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride, and 10-undecynoic acid) were used to dissociate the three LA hydroxylase activities. These experiments suggest that the site-specific hydroxylation [ω-, (ω-1)-, (ω-2)-] of LA may be catalyzed by different isozymes of cytochrome *P*-450. 

KEY WORDS: lauric acid; cytochrome *P*-450; HPLC, lipids; ω-hydroxylation; microsomes; metabolism.

Lauric acid (LA),[1] like prostaglandins (PGs) and leukotrienes, is hydroxylated at the ω and (ω-1) positions by microsomal preparations isolated from both hepatic and extrahepatic tissue (1–6). The participation of cytochrome *P*-450 in LA metabolism has been demonstrated directly, in *in vitro* reconstituted cytochrome *P*-450 systems (7–9) and, indirectly, with the use of cytochrome *P*-450 inducers and inhibitors (10–12). These inhibitors and inducers have also been used to demonstrate that different cytochrome *P*-450 isozymes support the hydroxylation of LA at the ω and (ω-1) positions.

Some of the earliest techniques that were utilized to assay microsomal LA hydroxylase activity included radio-gas chromatography (1,2) and radio-thin-layer chromatography (8). However, many of these techniques did not provide sufficient resolution for quantitation of the individual ω- and (ω-1)-hydroxylated metabolites of LA (8,12). One of the most popular methods for quantitation of LA and its metabolites involves HPLC separation and radiochemical quantitation of the methyl esters of LA and its hydroxylated metabolites (13). This procedure has numerous steps and is therefore time consuming and subject to variability. Two groups have recently reported a simplified procedure for the quantitation of microsomal LA hydroxylation (11,14). This procedure involves HPLC separation of underivatized LA and the ω- and (ω-1)-hydroxylated metabolites.

[1] Abbreviations used: LA, lauric acid; PG, prostaglandin; UDYA, 10-undecynoic acid; SK&F 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; ACN, acetonitrile; PGE, prostaglandin E.



0003-2697/88 $3.00

Recent data suggest that each hydroxylation reaction is catalyzed by a different isozyme(s) of cytochrome *P*-450 (12). Therefore, quantitation of the individual metabolites of LA could be used for the characterization of a different isozyme(s) of cytochrome *P*-450. Studies by Kupfer and co-workers indicate that, in addition to hydroxylation at the $\omega$ and ($\omega$-1) positions, hepatic microsomes also catalyze the hydroxylation of $PGE_1$ and $PGE_2$ at the ($\omega$-2) position (15,16). Therefore, it is feasible that other lipids such as LA could also be hydroxylated at the ($\omega$-2) position by microsomal incubations. In this paper, we describe a technique which allows rapid separation and quantitation of three microsomal metabolites [$\omega$-, ($\omega$-1)-, and ($\omega$-2)-hydroxy] of LA. Furthermore, we describe an initial biochemical characterization of the three microsomal LA hydroxylases.

## MATERIALS AND METHODS

*Materials.* Chemicals and reagents were obtained from the following commercial sources: [$^{14}$C]lauric acid (sp act, 26 mCi/mmol; radiochemical purity is 98% as determined by HPLC; Amersham, Arlington Heights, IL); NADPH and metyrapone (Sigma Chemical Co., St. Louis, MO); glucose 6-phosphate and glucose-6-phosphate dehydrogenase (Boehringer-Mannheim Biochemicals, Indianapolis, IN; Fluka Chemicals Corp., Hauppauge, NY, and Sigma); lauric acid, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (for preparation of ethereal diazomethane), $\alpha$-naphthoflavone (Aldrich, Milwaukee, WI); SK&F 525-A (2-dithylaminoethyl-2,2-diphenylvalerate hydrochloride) (Smith, Kline & French, Philadelphia, PA); 10-undecynoic acid (Chemical Procurement Labs, College Point, NY); Flo-scint II (Radiomatic Instruments, Tampa, FL); Atomlight (New England Nuclear, Boston MA); and *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Suppelco, Bellefonte, PA). All other reagents were of the highest grade commercially available.

*Microsome preparation.* Male Fisher 344 rats (200–250 g) were obtained from Charles Rivers Labs (Wilmington, MA). Rats were delivered and maintained virus-antibody negative, as determined by screening of sentinel animals for rat corona virus/sialodacryocroadenitis, pneumonia virus of mice, and Sendai virus. Animals were housed in stainless steel wire cages in Bioclean rooms (Hazelton Systems, Aberdeen, MD) at a temperature of 72 ± 2°F and a relative humidity of 50 ± 10% with a 12-h light cycle (0700–1900). Food (Purina 5002, Ralston-Purina, St. Louis, MO) and water were available *ad libitum*. Rats were quarantined at least 2 weeks prior to use. Unfasted rats were killed by cervical dislocation and organs were excised immediately. Microsomes were prepared as reported (17) and stored at −80°C until use. Protein concentrations were determined according to Gornall *et al.* (18).

*Assay conditions.* Reaction vessels contained microsomal protein (0.3–0.6 mg/ml), $MgCl_2$ (5 mM), [$^{14}$C]lauric acid (0.1 mM, 10 mCi/mmol) and a regenerating system consisting of glucose 6-phosphate (12.5 mM) and glucose-6-phosphate dehydrogenase (2.2 units/ml) dissolved in 1 ml of Tris–HCl (0.05 M), pH 7.5. For inhibition experiments, SK&F 525-A and metyrapone were dissolved directly in a small volume of Tris–HCl prior to addition to incubations; $\alpha$-naphthoflavone was dissolved in acetone and coated on the walls of the incubation vessel, and the solvent was evaporated prior to addition of the microsomal suspension. After 1 min of preincubation, the reaction was started by addition of 1 $\mu$mol of NADPH. Samples were incubated for 10 min in a shaking water bath maintained at 37°C. Incubations were terminated with 0.4 ml of 10% $H_2SO_4$. The acidified incubation mixture was extracted twice with 3 ml of fresh ether and the ether was evaporated under nitrogen at 30°C. The dried residue was resuspended in 250 $\mu$l of 67.5% ACN in 1% acetic

acid. Extraction recovery was determined by counting 10 μl of the resuspended sample in 5 ml of Atomlight. Recovery of radioactivity from incubation mixtures was greater than 95%.

*Quantitation of LA and metabolites.* LA and metabolites were separated on an HPLC system consisting of two Model 510 solvent delivery systems, a Model 720 system controller, and a Model 710B WISP autoinjector (Waters Assoc., Inc., Milford, MA) equipped with a model IC Flo-One Beta radiochemical detector (Radiomatic Instruments) outfitted with a 0.4-ml liquid flow cell. Quantitation was carried out with a 5-μm Altex (Beckman Inst., Berkeley, CA) $C_{18}$ column (4.6 × 250 mm) by gradient elution with acetic acid (1%) and ACN. Following injection of sample (25 μl), the percentage of ACN was held at 36% for 14 min, then increased linearly to 90% over 4 min, and held at 90% for 3 min prior to reequilibration. The flow rate was maintained at 1.5 ml/min throughout the analysis. The scintillant (FLO-SCINT II) to HPLC effluent ratio was maintained at 3:1, which provided a counting efficiency of 94%. Gradient conditions did not affect counting efficiency by greater than 5%. The recovery of radioactivity from the column was 97 ± 1.2%.

*Isolation and identification of LA metabolites.* Hydroxylated metabolites of LA from the combined extracts of 20 incubations were separated on a 5-μm Altex (Beckman Inst.) $C_{18}$ column (10 × 250 mm) by gradient elution with acetic acid (1%) and ACN. Following injection, the percentage of ACN was held at 36% for 25 min, then increased linearly to 90% over 2 min, and held at 90% for 2 min prior to reequilibration. The flow rate was maintained at 3.5 ml/min throughout the analysis. Fractions were collected every 0.3 min. The radiochemical content of each fraction was determined and samples containing metabolites were evaporated under reduced pressure. The compounds were methylated with 250 μl of ethereal diazomethane, dried under nitrogen, and resuspended in hexane. Each metabolite was further purified by thin-layer chromatography; methylated metabolites were applied to silica gel TLC plates (Whatman, Hillsboro, OR) which were developed in hexane:ether:acetic acid, 70:30:1.5 (v/v). The plates were scanned with a Berthold Model LB 2832 TLC analyzer and the area corresponding to the radioactive peak was isolated and extracted twice in ether. The ether was dried under nitrogen and metabolites were resuspended in 5 μl of ACN and 5 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and were heated at 60°C for 35 min. Mass spectra of the trimethylsilyl ether methyl ester of each metabolite was obtained on a Finnigan 4500 gas chromatograph/mass spectrometer (Finnigan, San Jose, CA) equipped with a bonded phase fused silica capillary column (30 m × 0.32 mm, DB-5, 0.25-μm film, J&W Scientific, Folsom, CA) and an on-column injector which was maintained at room temperature. Following injection, the oven temperature was held at 50°C for 2 min, increased ballistically to 100°C, and then increased 4°C/min to 250°C.

## RESULTS

Incubation of rat hepatic microsomes with [$^{14}$C]LA, in the presence of NADPH, resulted in the formation of three metabolites (Fig. 1A). These metabolites were not formed when NADPH was omitted from the incubation mixture (Fig. 1B). The trimethylsilyl ether methyl ester of metabolite I had a C-value of 19.12; prominent ions were evident at *m/z* 287 ($M-CH_3$), 271 ($M-OCH_3$), 258 ($M-CHOCH_3$), 255, and 117 ($Me_3SiO^+ = CHCH_3$) (Fig. 2A). The trimethylsilyl ether methyl ester of metabolite II had a C-value of 17.93; prominent ions were evident at *m/z* 287 ($M-CH_3$), 273 ($M-CH_2CH_3$), 255, 244 ($M-CHOCH_2CH_3$), and 131 ($Me_3SiO^+ = CHCH_2CH_3$) (Fig. 2B). The trimethylsilyl ether methyl ester of metabolite III had a C-value of 18.35; prominent ions

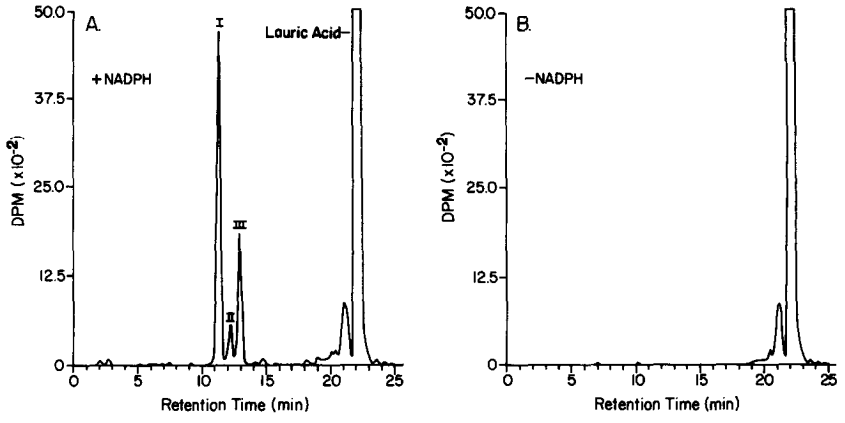


FIG. 1. HPLC radiochromatogram of ether extracts of hepatic microsomes incubated with [$^{14}$C]lauric acid. Incubations were conducted with (A) and without (B) NADPH. HPLC conditions are described under Materials and Methods.

were evident at *m/z* 287 (M–$CH_3$), 271 (M–$OCH_3$), 255 (M–$CH_3OH$, $CH_3$) and 103 ($Me_3SiO^+ = CH_2$) (Fig. 2C). On the basis of C-value and mass spectra, metabolite I was identified as 11-hydroxylauric acid [($\omega$-1)-hydroxylauric acid]; metabolite II was identified as 10-hydroxylauric acid [($\omega$-2)-hydroxylauric acid] and metabolite III was identified as 12-hydroxylauric acid ($\omega$-hydroxylauric acid).

Resolution of the three microsomal metabolites of LA by flow-through radiochemical detection was dependent upon flow cell size and the scintillant to HPLC effluent ratio. By maintaining the scintillant to HPLC effluent ratio at 2:1 and varying flow cell size it was possible to obtain baseline resolution of all three metabolites (Fig. 3). Peaks I and II could not be resolved with a 2.5 ml flow cell; some resolution of these peaks was observed when a 1.0 ml flow cell was used. With the larger flow cells, some improvement in resolution was obtained by increasing the scintillant to HPLC effluent ratio from 2:1 to 4:1 (Fig. 4). Quantitation of the three LA metabolites by flow-through radiochemical quantitation provided linear results over the ranges (0.375–26 nmol/ml) most often encountered in *in vitro* studies (Table 1). At a specific activity for [$^{14}$C]LA of 10 mCi/mmol, the effective limit of quantitation for each metabolite (assuming a 25-$\mu$l injection) was approximately 0.25 nmol/ml. Below these concentrations, the variability of results (CV >20%) obtained was too great to allow quantitation of each metabolite. In practical terms, rates as low as 12.5 pmol/mg protein/min can be accurately determined using standard assay conditions (10 min, 0.5 mg/ml, fourfold concentration following extraction). If metabolite formation was expected to be below this limit of quantitation, the specific activity of LA was increased to reduce the variability observed in quantitation of the metabolites or a larger volume was injected on column (volumes up to 75 $\mu$l could be injected without a noticeable decrease in resolution).

The formation of all three metabolites of LA by microsomal incubations was linear relative to time of incubation and protein concentration (Fig. 5). The formation of all three metabolites could be supported by NADPH added directly or by NADPH generated from glucose 6-phosphate (Table 2) or isocitric acid (data not shown) regenerating systems. However, certain glucose 6-phosphate regenerating systems (Sigma) resulted in substantially less microsomal metabolism of LA when compared to the metabolism supported by NADPH alone (Table 2). This inhibition of LA hydroxylases was due to an inhibitor present in the glucose 6-phosphate. Glucose 6-phosphate from other sources (Fluka,

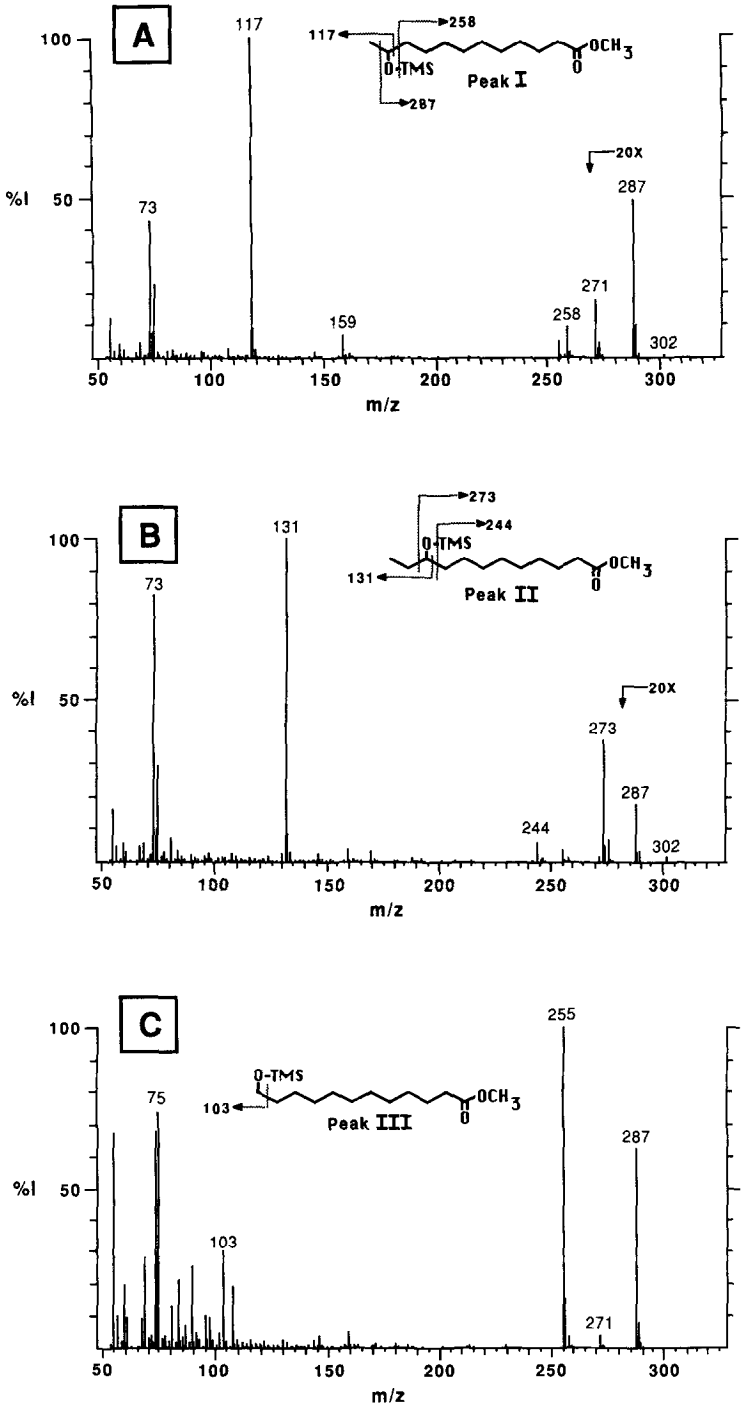


FIG. 2. Electron impact mass spectra of microsomal metabolites of lauric acid. The spectra of the trimethylsilyl (TMS) ether methyl ester of the (ω-1), (ω-2)-, and ω-hydroxylated metabolites are presented in (A), (B), and (C), respectively. Microsomal metabolites were isolated and derivatized as described under Materials and Methods.

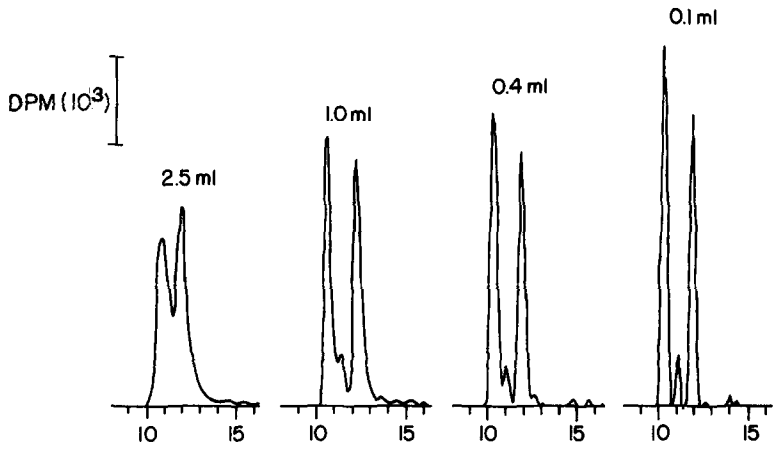


FIG. 3. Effect of flow cell size on resolution of lauric acid metabolites. Extracts of hepatic microsomes incubated with [$^{14}$C]lauric acid were analyzed by HPLC using flow cells of different volume. In all cases, the scintillant to HPLC effluent ratio was maintained at 2. Flow cell sizes and approximate flow cell residence times are as follows: 0.1 ml, 0.022 min; 0.4 ml, 0.089 min; 1.0 ml, 0.222 min; 2.5 ml, 0.556 min.

Boehringer-Mannheim) did not inhibit LA hydroxylases. The microsomal metabolism of LA could also be supported by hydrogen peroxide (Table 3). The formation of all three lauric acid metabolites increased as the concentration of $H_2O_2$ was increased in the incubation. However, the ratios of the metabolites formed were markedly different from those formed in the presence of NADPH. Very little ω-hydroxy-LA was formed while the formation of the (ω-1)- and (ω-2)-hydroxylated metabolites was similar to that supported by NADPH alone (Table 3).

In an effort to determine whether the dif-

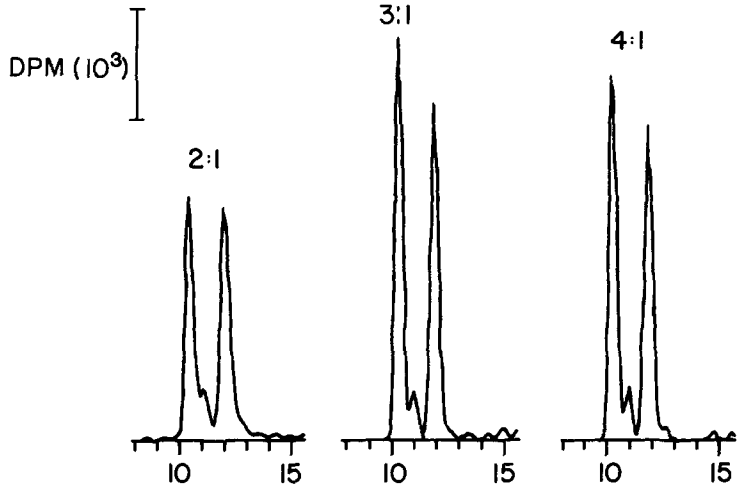


FIG. 4. Effect of scintillant flow rate on resolution of lauric acid metabolites. Extracts of hepatic microsomes incubated with [$^{14}$C]lauric acid were analyzed by HPLC using different ratios of HPLC effluent to scintillant. In all cases, the flow cell volume was maintained at 1 ml. HPLC effluent to scintillant ratios and approximate flow cell residence times are as follows: 4:1, 0.133 min; 3:1, 0.167 min; 2:1, 0.222 min.

TABLE 1

QUANTITATION OF MICROSOMAL METABOLITES OF LAURIC ACID BY FLOW-THROUGH RADIOCHEMICAL DETECTION: ASSAY STATISTICS[a]

| ω-Hydroxylaurate | | | (ω-1)-Hydroxylaurate | | | (ω-2)-Hydroxylaurate | | |
|---|---|---|---|---|---|---|---|---|
| Conc. (nmol/ml) | | | Conc. (nmol/ml) | | | Conc. (nmol/ml) | | |
| Actual | Observed | CV[b] | Actual | Observed | CV[b] | Actual | Observed | CV[b] |
| 18.000 | 18.061 | 3.6 | 26.000 | 26.093 | 2.8 | 3.000 | 2.984 | 8.1 |
| 9.000 | 9.531 | 3.4 | 13.000 | 13.231 | 2.8 | 1.500 | 1.542 | 12.8 |
| 4.500 | 4.575 | 8.2 | 6.500 | 6.369 | 4.2 | 0.750 | 0.760 | 15.0 |
| 2.250 | 2.157 | 3.8 | 3.250 | 3.384 | 8.6 | 0.375 | 0.355 | 16.7 |

[a] Values obtained (observed concentration, CV) are derived from five replicate injections of a mixture of lauric acid metabolites. The concentration of each individual metabolite in the mixture was adjusted to the actual concentration listed above. Metabolites were quantitated by flow-through radiochemical detection as described under Materials and Methods.

[b] Coefficient of variation.

ferent hydroxylations of LA were supported by a different isozyme(s) of cytochrome *P*-450, the effect of different inhibitors of cytochrome *P*-450 (SK&F 525-A, metyrapone, and α-naphthoflavone) on LA hydroxylases were evaluated (Fig. 6). SK&F 525-A inhibited all three LA hydroxylases in a concentration dependent manner; at $10^{-3}$ M, inhibition of all three enzyme activities was approximately 80%. Incubation with α-naphthoflavone decreased LA ω- and (ω-1)-hydroxylase activities by approximately 30%; similar concentrations had no effect on LA (ω-2)-hydroxylases. Incubation

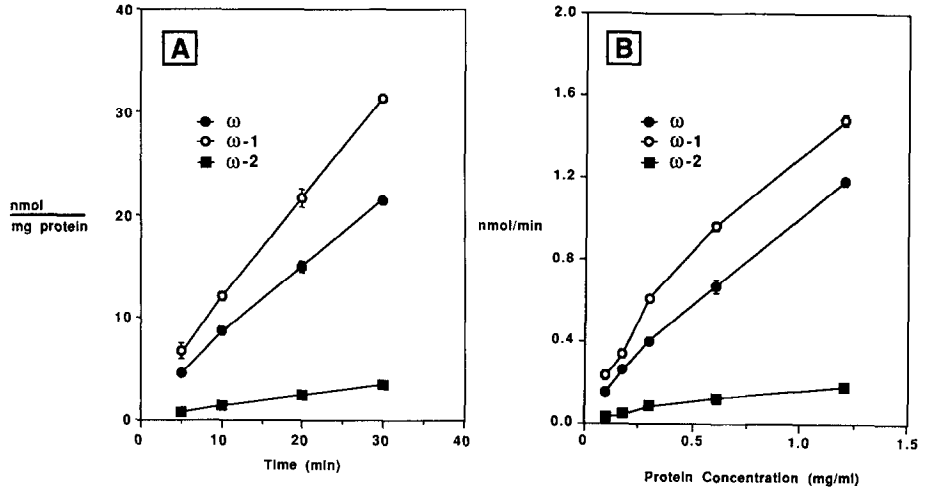


FIG. 5. Effect of incubation time (A) and protein concentration (B) on the microsomal metabolism of lauric acid. Protein concentration in time-dependence incubations was 0.4 mg/ml; protein dependence incubations were conducted for 10 min. Values reported are the mean ± SE of three determinations. (●) ω-, (○) (ω-1)-, and (■) (ω-2)-hydroxylases were measured as described under Materials and Methods.

TABLE 2

HYDROXYLATION OF LAURIC ACID BY HEPATIC MICROSOMES SUPPORTED WITH DIFFERENT GLUCOSE 6-PHOSPHATE REGENERATING SYSTEMS

| | Laurate hydroxylation[a] | | |
|---|---|---|---|
| | ω | ω-1 | ω-2 |
| NADPH | 100 ± 3 | 100 ± 5 | 100 ± 4 |
| G-6-P No. 1[b] | 42 ± 4 | 61 ± 3 | 63 ± 4 |
| G-6-P No. 2[c] | 115 ± 3 | 116 ± 4 | 104 ± 8 |
| G-6-P No. 3[d] | 124 ± 1 | 94 ± 2 | 88 ± 7 |

[a] Each value represents the mean ± SE of at least three different determinations. Data are expressed as a percentage of the activity measured in the presence of NADPH alone. Control values for ω-, (ω-1)-, and (ω-2)-hydroxylases were 1.579 ± 0.075, 1.084 ± 0.036, and 0.214 ± 0.008 nmol/min/mg protein, respectively.

[b] Glucose 6-phosphate (12.5 mM) and glucose-6-phosphate dehydrogenase (2.2 units/ml) were obtained from Sigma.

[c] Glucose 6-phosphate (12.5 mM) and glucose-6-phosphate dehydrogenase (2.2 units/ml) were obtained from Boehringer-Mannheim.

[d] Glucose 6-phosphate (12.5 mM) and glucose-6-phosphate dehydrogenase (2.2 units/ml) were obtained from Fluka.

with metyrapone resulted in a concentration-dependent reduction of LA (ω-1)- and (ω-2)-hydroxylases; at $10^{-3}$ M, inhibition of both enzyme activities was approximately 60%. In contrast, metyrapone resulted in very little inhibition (<10%) of LA ω-hydroxylases.

The acetylenic fatty acid, 10-undecynoic acid (UDYA), was also employed as a biochemical tool for dissociating LA hydroxylase activities. Preincubation of microsomes for periods up to 1 h resulted in a small (10–20%) increase in all LA hydroxylase activities when compared to nonpreincubated controls. This increase in hydroxylase activities is unexplained but may reflect metabolism, during the preincubation period, of lipids which are released from the microsomal membrane and inhibit LA hydroxylases. Rat liver microsomes contain 2–2.5 nmol of free fatty acids, both saturated and unsaturated, per milligram of microsomal protein (19,20). As postulated by Kupfer and others, the amounts and ratios of these fatty acids may affect cytochrome *P*-450-dependent activities such as LA hydroxylases (21). Preincubation of microsomes with UDYA resulted in a time-dependent loss of LA ω-, (ω-1)-, and (ω-2)-hydroxylase activities (Fig. 7A). Nearly 50% of LA ω-hydroxylase activity was inactivated by 50 μM UDYA. However, a much smaller percentage (20–30%) of LA (ω-1)- and (ω-2)-hydroxylases were destroyed under similar preincubation conditions. This preferential inactivation of LA ω-hydroxylases was observed over a large concentration range of UDYA (Fig. 7B). This differential inactivation of LA hydroxylases was not observed when a nonspecific suicide substrate for cytochrome *P*-450, 1-aminobenzotriazole (12), was preincubated with hepatic microsomes; approximately 70% of all three LA hydroxylase activities was lost following a 30-min preincubation with 100 μM 1-aminobenzotriazole (data not shown).

## DISCUSSION

The microsomal metabolism of LA has been investigated in a variety of *in vitro* sys-

TABLE 3

HYDROXYLATION OF LAURIC ACID BY HEPATIC MICROSOMES SUPPORTED WITH NADPH OR $H_2O_2$

| Cofactor | Laurate Hydroxylation (nmol/mg prot)[a] | | |
|---|---|---|---|
| | ω | ω-1 | ω-2 |
| NADPH | 8.42 ± 0.19 | 19.50 ± 0.34 | 2.22 ± 0.07 |
| $H_2O_2$ (50 mM) | 0.57 ± 0.03 | 5.84 ± 0.14 | 0.90 ± 0.11 |
| $H_2O_2$ (100 mM) | 0.71 ± 0.04 | 8.22 ± 0.15 | 1.08 ± 0.15 |
| $H_2O_2$ (200 mM) | 1.17 ± 0.10 | 10.59 ± 0.47 | 1.42 ± 0.06 |

[a] Each value represents the mean ± SE of quadruplicate incubations. The reactions were started with NADPH or $H_2O_2$ and conducted for 10 min, as described under Materials and Methods.

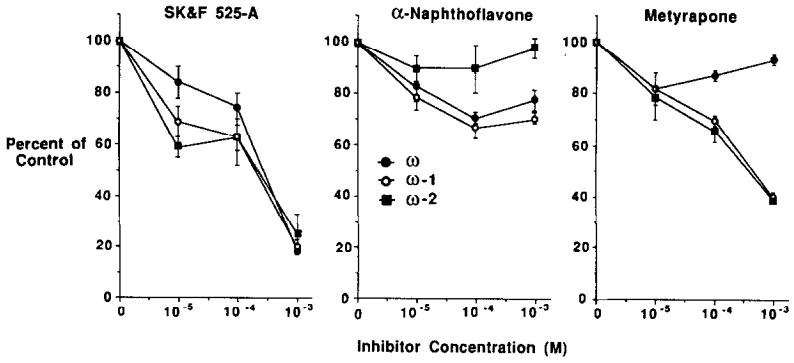


FIG. 6. *In vitro* effect of metyrapone, SK&F 525-A, and α-naphthoflavone on microsomal lauric acid hydroxylases. (●) ω-, (○) (ω-1)-, and (■) (ω-2)-hydroxylases were measured as described under Materials and Methods. Data are expressed as a percentage of the respective control (incubations in the absence of inhibitor). Values reported are the mean ± SE of four determinations. Control values for ω-, (ω-1)-, and (ω-2)-hydroxylases were 1.068 ± 0.047, 0.756 ± 0.027, and 0.136 ± 0.012 nmol/min/mg protein, respectively.

tems (1,2,7–12). The metabolism of LA has been used extensively to characterize a subpopulation of cytochrome *P*-450s, collectively referred to as cytochromes *P*-452 (8). These mixed function oxidases are preferentially induced by hypolipidemic agents, of which clofibrate is the prototypical inducer (22). The biochemical sequelae of this induction is a dramatic increase in LA ω-hydroxylase activity (22). Studies conducted with acetylenic analogs of LA by Ortiz de Montellano and Reich have demonstrated

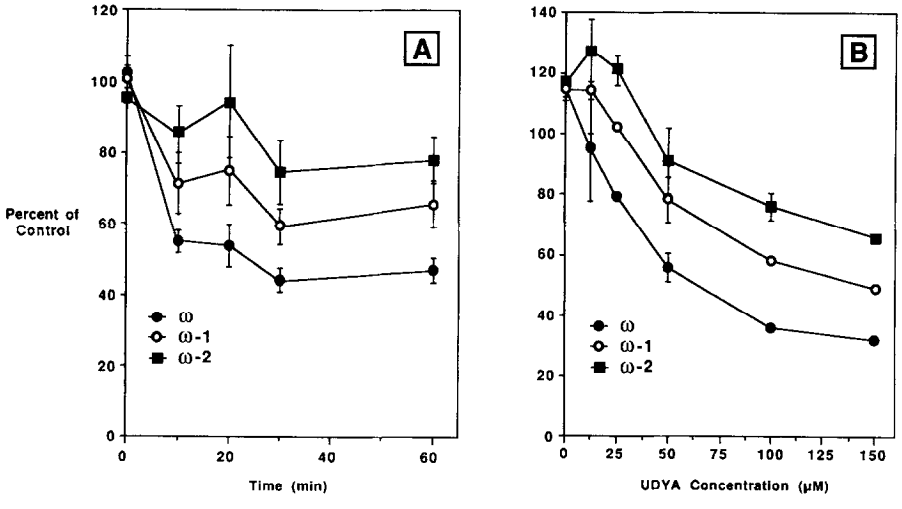


FIG. 7. Time- (A) and concentration-dependent (B) inactivation of microsomal lauric acid hydroxylases by 10-undecynoic acid. (●) ω-, (○) (ω-1)-, and (■) (ω-2)-hydroxylases were measured as described under Materials and Methods. In time dependence experiments, the time of preincubation was varied in the presence of 50 μM UDYA. In concentration dependence experiments, microsomes were preincubated with different concentrations of UDYA (10–150 μM) for 30 min. Data are expressed as a percentage of activities observed in nonpreincubated microsomes in the absence of UDYA. Values reported are the mean ± SE of four determinations. Control values for ω-, (ω-1)-, and (ω-2)-hydroxylases were 1.381 ± 0.124, 1.137 ± 0.244, and 0.160 ± 0.018 nmol/min/mg protein, respectively.

that at least three isozymes of cytochrome *P*-450 support the microsomal metabolism of LA (12). Furthermore, the position of hydroxylation is dictated by the specific forms of cytochrome *P*-450 which are present in rat hepatic microsomes. Therefore, the site-specific hydroxylation of LA could be used to document the presence or absence of specific isozymes of cytochrome *P*-450 in microsomal suspensions.

Many of the traditional methods used for assay of LA hydroxylase activity do not differentiate between the hydroxylated metabolites (8,12). However, the use of gas and, more recently, liquid chromatographic techniques has allowed the separation of the ω- and (ω-1)-hydroxylated metabolites of LA (1,2,10,13). Both techniques generally require the use of a radiolabeled substrate and fairly elaborate derivatization techniques for quantitation and separation of permethylated metabolites. Orton and Parker have utilized reverse phase HPLC with flow-through radiochemical detection to quantitate the ω- and (ω-1)-hydroxylated metabolites of LA without derivatization (11). Recently, Welch and coworkers have described a normal phase HPLC system for the separation of the ω- and (ω-1)-hydroxylated metabolites of LA (14). However, both of these analytical techniques have not been well characterized for precision, accuracy, and practical limits of quantitation. Furthermore, none of the above-mentioned techniques has allowed for the detection and quantitation of the (ω-2)-hydroxylated metabolite of LA in mammalian microsomal incubations. The techniques described for quantitation of the three microsomal metabolites of LA in the present study provide reproducible and linear results over a range of concentrations that are anticipated for most *in vitro* conditions. The practical limit of quantitation of each LA metabolite was approximately 0.25 nmol/ml. Using standard assay conditions, this would allow accurate quantitation of LA (ω-2)-hydroxylase activities one-20th of those observed in most microsomal incubations from rats.

The hydroxylation of LA at the (ω-2) position has been reported in certain plant and bacterial systems (23,24). Furthermore, the hydroxylation of $PGE_1$ and $PGE_2$ at the (ω-2) position has been reported previously by Kupfer and co-workers (15). However, the hydroxylation of LA at the (ω-2) position has not been reported previously as a significant pathway of LA metabolism in mammalian microsomes. This is probably due to the inability to adequately resolve (ω-2)-hydroxy-LA from (ω-1)-hydroxy-LA. Using reverse phase HPLC with flow-through radiochemical detection it is possible to adequately resolve (ω-2)- and (ω-1)-hydroxy-LA by decreasing the residence time within the radiochemical flow cell. As demonstrated in the present study, a decreased residence time can be achieved by increasing the flow rate through the cell as well as by decreasing the actual flow cell size. The (ω-2)-hydroxylation of $PGE_1$ and $PGE_2$ is also supported by hydrogen peroxide (15). A similar phenomena was observed when LA was incubated in the presence of hydrogen peroxide.

As has been previously reported for $PGE_1$ and $PGE_2$ (15), hydrogen peroxide-dependent metabolism of LA resulted in a ratio ((ω-1) or (ω-2)/ω) of hydroxylated metabolites that was markedly different than that produced when NADPH was used to support LA metabolism. This suggests that the ω-hydroxylation of LA was supported by different isozymes of cytochrome *P*-450 than those which support the (ω-1)- and (ω-2)-hydroxylations of LA. Previous investigators, utilizing suicide substrates and competitive inhibitors of LA metabolism, have demonstrated that the ω- and (ω-1)-hydroxylations of LA are supported by different isozymes of cytochrome *P*-450 (10,12). In the present study, similar approaches for dissociating the three (ω-, ω-1, ω-2) lauric acid hydroxylase activities were utilized in an attempt to demonstrate isozyme specific positional hydroxylation of LA. The competitive inhibitors, α-naphthoflavone, metyrapone, and SK&F 525-A, had differential effects on the various LA hydroxylases. While SK&F 525-A inhib-

ited all three hydroxylases uniformly, metyrapone inhibited only the (ω-1)- and (ω-2)-hydroxylases. α-Naphthoflavone inhibited only ω- and (ω-1)-hydroxylases although the separation was much less pronounced than with metyrapone. Preincubation of microsomes with 10-undecynoic acid inactivated LA ω-hydroxylase activity in a time- and concentration-dependent manner. Furthermore, the inactivation of ω-hydroxylase activity by 10-undecynoic acid was much more pronounced than the destruction of (ω-1)- and (ω-2)-hydroxylases. These data clearly indicate that a large portion of the ω-hydroxylation of LA in hepatic microsomes is supported by isozymes of cytochrome *P*-450 that are different from those which catalyze the (ω-1)- and (ω-2)-hydroxylations of LA. However, substantial biochemical separation of (ω-1)-hydroxylases from (ω-2)-hydroxylases could not be achieved with the probes used in the present study suggesting that a similar isozyme(s) of cytochrome *P*-450 supports these two hydroxylations. Further confirmation of these results will require purification of all three enzyme activities from hepatic microsomes.

In summary, an assay for the microsomal hydroxylation of LA, based on HPLC with flow-through radiochemical detection, has been developed. The products formed, (ω)-hydroxy-LA, (ω-1)-hydroxy-LA, and the novel mammalian metabolite (ω-2)-hydroxy-LA, could be quantitated with high precision and accuracy with minimal sample manipulation. The biochemical dissociation of ω-hydroxylase activity from (ω-1)- and (ω-2)-hydroxylases suggests that different isozymes of cytochrome *P*-450 support these reactions.

## REFERENCES


1. Bjorkhem, I., and Danielson, H. (1970) *Eur. J. Biochem.* **17,** 450–459.
2. Ellin, A., Orrenius, S., Pilotti, A., and Swahn, C.-G. (1973) *Arch. Biochem. Biophys.* **158,** 597–604.
3. Kupfer, D., Navarro, J., and Piccolo, D. E. (1978) *J. Biol. Chem.* **253,** 2804–2811.
4. Powell, W. (1978) *J. Biol. Chem.* **253,** 6711–6716.
5. Newton, J. F., Eckardt, R. D., Bender, P. E., Leonard, T. B., and Straub, K. M. (1985) *Biochem. Biophys. Res. Commun.* **128,** 733–738.
6. Romano, M. C., Eckardt, R. D., Bender, P. E., Leonard, T. B., Straub, K. M., and Newton, J. F. (1987) *J. Biol. Chem.* **262,** 1590–1595.
7. Lu, A. Y. H., Junk, K. W., and Coon, M. J. (1969) *J. Biol. Chem.* **244,** 3714–3721.
8. Gibson, G. G., Orton, T. C., and Tamburini, P. P. (1982) *Biochem. J.* **203,** 161–168.
9. Okita, R. T., Parkhill, L. K., Yasukochi, Y., Masters, B. S., Theoharides, A. D., and Kupfer, D. (1981) *J. Biol. Chem.* **256,** 5961–5964.
10. Okita, R. T., and Masters, B. S. (1980) *Drug Metab. Dispos.* **8,** 147–151.
11. Orton, T. C., and Parker, G. L. (1982) *Drug Metab. Dispos.* **10,** 110–115.
12. Ortiz de Montellano, P. R., and Reich, N. O. (1984) *J. Biol. Chem.* **259,** 4136–4141.
13. Fan, L. L., Masters, B. S., and Prough, R. A. (1976) *Anal. Biochem.* **71,** 265–272.
14. Clarke, M. J., Hawke, R. L., and Welch, R. M. (1986) *J. Liq. Chromatogr.* **9,** 1711–1725.
15. Holm, K. A., Engell, R. J., and Kupfer, D. (1985) *Arch. Biochem. Biophys.* **237,** 477–489.
16. Holm, K. A., Koop, D. R., Coon, M. J., Theoharides, A. D., and Kupfer, D. (1985) *Arch. Biochem. Biophys.* **243,** 135–143.
17. Dent, J. G., and Graichen, M. E. (1982) *Carcinogenesis* **3,** 733–738.
18. Gornall, R. J., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766.
19. Gibson, G. G., Cinti, D. L., Sligar, S. G., and Schenkman, J. B. (1980) *J. Biol. Chem.* **255,** 1867–1873.
20. Schenkman, J. B., Jansson, I., Gibson, G. G., Silgar, S. G., and Cinti, D. L. (1980) *in* Microsomes, Drug Oxidations and Chemical Carcinogenesis (Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., and O'Brien, P. J., Eds.), Vol. 1, pp. 479–487, Academic Press, New York.
21. Kupfer, D. (1980) *Pharmacol. Ther.* **11,** 469–496.
22. Parker, G. L., and Orton, T. C. (1980) *in* Biochemistry, Biophysics and Regulation of Cytochrome *P*-450 (Gustafsson, J. A., Duke, J. C., Mode, A., and Rafter, J., Eds.), pp. 373–377, Elsevier/North-Holland, Amsterdam.
23. Miura, Y., and Fulco, A. J. (1975) *Biochim. Biophys. Acta* **388,** 305–317.
24. Salaun, J. P., Benveniste, I., Reichhart, D., and Durst, F. (1981) *Eur J. Biochem.* **119,** 651–655.