Terminal acetylenic fatty acid mechanism-based inhibitors (Ortiz de Montellano, P. R., and Reich, N. O. (1984) J. Biol. Chem. 259, 4136-4141) were used as probes in determining the substrate specificity of rabbit lung cytochrome P-450 isozymes of pregnant animals in both microsomes and reconstituted systems. Lung microsomal and reconstituted P-450 form 5-catalyzed lauric acid ω- and (ω-1)-hydroxylation activities were inhibited by a 12-carbon terminal acetylenic fatty acid, 11-dodecynoic acid (11-DDYA), and an 18-carbon terminal acetylenic fatty acid, 17-octadecynoic acid (17-ODYA). Rabbit lung microsomal lauric acid ω-hydroxylase activity was more sensitive to inhibition by 11-DDYA than was (ω-1)-hydroxylase activity. In reconstituted systems containing purified P-450 form 5, both ω- and (ω-1)-hydroxylations of lauric acid were inhibited in parallel when either 11-DDYA or 17-ODYA was used. These data suggest the presence of at least two P-450 isozymes in rabbit lung microsomes capable of lauric acid ω-hydroxylation. This is the first report indicating the multiplicity of lauric acid hydroxylases in lung microsomes.

Lung microsomal prostanoid ω-hydroxylation, mediated by the pregnancy-inducible P-450m-m (Williams, D. E., Hale, S. E., Okita, R. T., and Masters, B. S. S. (1984) J. Biol. Chem. 259, 14600-14608) was subject to inhibition by 17-ODYA only, whereas 11-DDYA acid was not an effective inhibitor of this hydroxylase. We have recently developed a new terminal acetylenic fatty acid, 12-hydroxy-16-heptadecynoic acid (12-HHDYA), that contains a hydroxyl group at the ω-6 position. We show that 12-HHDYA possesses a high degree of selectivity for the inactivation of rabbit lung microsomal prostaglandin ω-hydroxylase activity which cannot be obtained with the long chain acetylenic inhibitor, 17-ODYA. In addition, 12-HHDYA has no effect on lauric acid ω- or ω-1-hydroxylation or on benzphetamine N-demethylation. The development of this new terminal acetylenic fatty acid inhibitor provides us with a useful tool with which to study the physiological role of prostanoid ω-hydroxylation in the rabbit lung during pregnancy.

Prostaglandins (PGs) and fatty acids are physiologically important compounds that are hydroxylated by cytochromes P-450 (P-450) at the ω, ω-1, and ω-2 positions (1, 2). Isozymes of P-450 that catalyze these reactions are widely distributed phylogenetically, having been studied in mammals, amphibians, fish, plants, yeast, and bacteria (1-9). Although the ω- and (ω-1)-hydroxylations of fatty acids and PGs have been the subject of a number of studies (reviewed in Refs. 1, 2), the physiological function of these P-450-dependent reactions remains obscure.

In order to study the physiological role of such reactions, it is necessary to ascertain which cytochrome P-450 isozymes are involved and how they are regulated. Various tools have been utilized in similar studies of P-450 specificity, including differential response to hormonal or xenobiotic inducers or inhibitors and inhibition by polyclonal or monoclonal antibodies (10-12). A relatively new approach for examining P-450 specificity involves the use of mechanism-based inactivators, also known as "suicide substrates" (13). A number of compounds such as 1-aminobenzotriazole and allylisopropylacetamide (2-isopropyl-4-pentenamide) are very effective at inhibiting microsomal cytochrome P-450-dependent activities. The inactivation is NADPH-dependent and time-dependent and follows pseudo-first order kinetics which is characteristic of mechanism-based inhibitors (reviewed in Ref. 14). Acetylenic and olefinic compounds make up a subset of P-450 suicide substrate inhibitors (15-17). Acetylenic derivatives of preferred substrates, such as 1-ethinylpyrene (for benzo(a)pyrene hydroxylase) or 20-(1,5-hexadienyl)-5-pregn-3,20a-diol (for cholesterol side chain cleavage), have been demonstrated to be highly selective toward the inactivation of a particular P-450 isozyme (18, 19). Ortiz de Montellano and Reich (20) have synthesized terminal acetylenic fatty acids that are highly selective inhibitors of rat liver P-450 isozymes which are active toward fatty acid substrates.

The abbreviations used are: PG, prostaglandin; P-450, cytochrome P-450; 11-DDYA, 11-dodecynoic acid; 17-ODYA, 17-octadecynoic acid; 12-HHDYA, 12-hydroxy-16-heptadecynoic acid; [1-14C] lauric acid, [1-14C]-dodecenoic acid; [5,6-3H]PGE, [5,6-3H]prostaglandin E1; DLPC, dilauroylphosphatidylcholine; HETE, hydroxyeicosatetraenoic acid; LTB4, leukotriene B4.
without affecting total P-450 levels or other P-450-dependent activities.

The rabbit lung is an excellent system for the study of the regulation and physiological function of P-450-dependent PG and fatty acid ω- and/or (ω-1)-hydroxylation. One advantage is that, although the P-450-specific content of lung microsomes is almost 50% that of the liver, the isozyme composition in rabbit lung is much less complex, with the liver containing at least 12 distinct isozymes (21). The majority of the cytochrome P-450 in lung microsomes from male or nonpregnant female rabbits consists of two isozymes, P-450 form 2 and P-450 form 5 (22). P-450 form 2 exhibits little or no activity toward either lauric acid or PGs (23). Rabbit lung P-450 form 5 has high activity toward the ω- and (ω-1)-hydroxylation of lauric acid but no detectable turnover with any PG (23). P-450 form 6 makes up 1–7% of the total cytochrome P-450 in untreated rabbits and is inducible by 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin (24). In lung microsomes from pregnant rabbits, a third major P-450 can be isolated which is nearly undetectable in males or nonpregnant females. This P-450 (P-450ωCωC) is induced 50- to 100-fold during pregnancy and comprises 15–30% of the total lung microsomal P-450 in pregnant rabbits (2, 23, 25). P-450ωCωC regiospecifically hydroxylates the ω-position of PGs and appears to be the only lung isozyme in pregnant rabbits active toward prostaglandins.

In this study, we have tested a series of terminal acetylenic fatty acids (11-dodecanolic acid, 17-octadecylic acid, and 12-hydroxy-16-heptadecylic acid) as inhibitors of lauric acid ω- and (ω-1)-hydroxylation and PGEω-ω-hydroxylation in pulmonary microsomes prepared from pregnant rabbits and with reconstituted P-450 form 5 and P-450ωCωC. The data presented demonstrate the selectivity obtained by designing inhibitors whose structure more closely resembles that of the natural substrates. The synthesis of 12-hydroxy-16-heptadecylic acid, which possesses an ω-6-hydroxyl group, a structural feature common to all prostaglandins, provides us with an excellent tool with which to examine the physiological significance of PG ω-hydroxylation in the rabbit lung during pregnancy. The further development of this type of suicide substrate will provide us with compounds that have great potential as research and therapeutic agents to selectively alter the production or metabolism of endogenous or exogenous compounds by the cytochrome P-450 system.

**EXPERIMENTAL PROCEDURES**

**Materials**—[11-C]Lauric acid, [5,6-H]PGAωC, and [5,6-H]PGEω were obtained from Amersham Corp. Sigma was the source of the unlabeled substrates, NADPH, DLPC, and isocitrate dehydrogenase. The acetylenic derivatives, 11-DDYA and 17-DDYA, were synthesized as described previously (26). 11-DDHYA was obtained by base-catalyzed rearrangement of the triple bond in 12-hydroxy-13-heptadecylic acid. The latter internal acetylenic alcohol was synthesized by condensation of pentyne with 12-oxododecanoic acid. 12-Oxododecanoic acid was obtained by pyridinium chlorochromate oxidation of 12-hydroxylauric acid. A description of the experimental procedures follows.

**Synthesis of 12-Oxododecanoic Acid**—A suspension of 12-hydroxylauric acid (2.15 g, 10 mmol) in 25 ml of cold methylene chloride was combined under nitrogen at room temperature with a suspension of pyridinium chlorochromate (3.25 g, 15 mmol) in 20 ml of methylene chloride. The resulting mixture was stirred for 3 h according to the general procedure of Corey and Suggs (27). Diethyl ether (200 ml) was then added and the black reaction mixture was filtered. The filtered solution was washed through Florisil, the ether removed on a rotary evaporator, and the residue purified by chromatography on a 1.5 × 14-cm column packed with 230–400 mesh silica gel (Alrich) and eluted with diethyl ether (0.83 g of 12-oxododecanonic acid, 3.9 mmol, 40% yield): 1H NMR 7.96 (t, 1H, J = 2 Hz, CHO), 2.35 (m, 4H, -CH2CO2H and -CH2CHO), 1.60 (m, 2H, -CH2CH2CO2H), and 1.29 ppm (s, 14H, methylenes); IR (nujol) 1708 (CO2H), 1736 cm−1 (CHO).

**Synthesis of 12-Hydroxy-13-Heptadecylic Acid**—A solution of 1-pentyn (0.25 g, 3.5 mmol) in 4 ml of dry tetrahydrofuran was added by syringe under nitrogen to 3 mmol of ethyl magnesium bromide (from Aldrich, 2.0 mol solution) in 2.5 ml of dry tetrahydrofuran in a flask equipped with a reflux condenser. The mixture was allowed to stand for 3 h at 25 °C before 0.21 g (1.0 mmol) of 12-oxododecanoic acid in 2 ml of dry tetrahydrofuran was added. The resulting solution was refluxed for 1.5 h and then cooled to 0 °C. Saturated ammonium chloride solution (3 ml) was added, and the mixture was extracted with diethyl ether. The combined extracts were dried over anhydrous MgSO4, and the solvent was removed on a rotary evaporator, yielding 0.25 g (0.81 mmol, 81%) of 12-hydroxy-13-heptadecylic acid: 1H NMR 4.36 (broad t, 1H, J = 10 Hz, CH2CHO), 2.27 (m, -CH2CO2H and -CH2CHO), 1.66–1.30 (m, 2H, methylenes), and 0.98 ppm (t, 3H, J = 6.8 Hz, Me); IR (CCl4) 1712 cm−1 (−CO2H); gas chromatography-electron impact mass spectrometry m/z 264 (M−H2O).

**Synthesis of 12-Hydroxy-16-Heptadecylic Acid**—The acetylenic function was shifted to the chain terminal as described for the synthesis of other terminal acetylenic acids (26). The reaction was carried out with 0.25 g (0.81 mmol) of 12-hydroxy-13-heptadecylic acid, 311 mg (6.5 mmol) of a 50% suspension of NaH in mineral oil, and a total of 11 ml of 1.3-mmol solution. The separated product was purified by chromatography on a 1.5 × 14-cm column of silica gel washed with 9:1 benzene/diethyl ether and eluted with diethyl ether. The reaction yielded 0.11 g (0.39 mmol, 48%) of 12-hydroxy-16-heptadecylic acid: 1H NMR 3.63 (broad t, 1H, CHO), 2.35 (m, 4H, -CH2CO2H and -C=CH2), 1.95 (t, 1H, J = 2.6 Hz, -C=CH2), and 1.29 ppm (m, 22H, methylenes); IR (CHCl4) 3304 (C=CH), 2955 (OH), 2114 (C=C stretch), and 1712 cm−1 (−CO2H); gas chromatography-electron impact mass spectrometry of methyl ester (70 eV) m/z 265 (M−MeO).

The structures of acetylenic fatty acids are shown in Scheme I.

**Purification of Rabbit Lung P-450ωωC and P-450 Form 5**—Cytochromes P-450ωωC and P-450 form 5 were purified from lung microsomes of pregnant (25th to 30th day of gestation) New Zealand White rabbits (3–5 kg) as described previously (23) utilizing a slightly modified procedure of the method of Guengerich (26). The isozymes appeared homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and exhibited specific contents of 12.1 (P-450ωωC) and 17.6 (P-450 form 5) nmol mg−1 (23).

**Inhibition of Rabbit Lung Microsomal and Reconstituted PG, and Lauric Acid Hydroxylation by Acetylenic Fatty Acids**—The acetylenic fatty acids were added to a 10-μl stock solution, a 10-min preincubation at 20 °C was included prior to the addition of the NADPH-regenerating system containing 20 μM isocitrate and 0.1 mg of isocitrate dehydrogenase was added, followed by buffer (0.1 M potassium phosphate, pH 7.5, containing 5 mM MgCl2 and 1 mM EDTA) to a final volume of 1 ml. For the reconstitution experiments, a 10-min preincubation at 20 °C was included prior to the addition of the NADPH-regenerating system and buffer. The samples were then allowed to equilibrate at 37 °C for 2 min, and NADPH was then added to a final concentration of 1 mM to initiate the reaction. At given time intervals following initiation, the amount of active P-450 remaining was determined as follows. One hundred microliter aliquots were removed (in 1.5 × 14-cm column packed with 230–400 mesh silica gel (Alrich)) and eluted with diethyl ether (0.83 g of 12-oxododecanonic acid, 3.9 mmol, 40% yield): 1H NMR 7.96 (t, 1H, J = 2 Hz, CHO), 2.35 (m, 4H, -CH2CO2H and -CH2CHO), 1.60 (m, 2H, -CH2CH2CO2H), and 1.29 ppm (s, 14H, methylenes); IR (nujol) 1708 (CO2H), 1736 cm−1 (CHO).
duplicate or triplicate at each time point) and added to 0.9 ml of 100 
μM [14C]lauric acid (2-3 μCi/μmol) or 50-100 μM [3H]PGE, (1-2 μCi/
μmol), 1 mM NADPH, and the regenerating system and buffer de-
scribed above. Reactions containing microsomes used 1.0 mg/ml of
microsomal protein and contained the same NADPH-regenerating
system and buffer as described for the reconstitution reactions. Con-
trol preincubations were performed either in the absence of each
acyclic inhibitor or NADPH. In some experiments, aliquots were
removed at various time points and assayed spectrophotometrically
for total P-450 or for benzphetamine-N-demethylase activity as de-
scribed previously (23).

High Pressure Liquid Chromatography Assay for [1,4C]Lauric Acid
ω- and (ω-1)-Hydroxylation and [5,6-3H]PGE, ω-Hydroxylation—The
microsomal or reconstituted P-450 incubation reactions were termi-
nated by the addition of 0.2 ml of 2.5% sulfuric acid or 0.065 ml of 1
N HCl and the samples were extracted with ethyl acetate and analyzed
by reverse-phase high pressure liquid chromatography as described
previously (23). The metabolites were quantitated by a FLO-ONE
Model HP radioactive flow detector (Radiomatic).

Purification of Other Enzymes—NADPH-cytochrome P-450 reduc-
tase was purified from liver microsomes of phenobarbital-treated
rabbits by the method of Strittmatter et al. (29). Cytochrome
bswas purified from rabbit liver microsomes by the method of

RESULTS

Time- and Concentration-dependent Inhibition by 11-
DDYA of Rabbit Lung Microsomal Laurate ω- and (ω-1)-
Hydroxylation—Preincubation of lung microsomes from preg-
rnant rabbits in the presence of 11-DDYA and NADPH pro-
duced a time- and concentration-dependent loss of lauric acid
(ω-1)- and ω-hydroxylation. Approximately 85% inactivation
of lauric acid ω-hydroxylation by 250 μM 11-DDYA was
observed within 60 min at 37 °C (Fig. 1A), whereas (ω-1)-
hydroxylation was inhibited by only 85% under these condi-
tions (Fig. 1B). The greater sensitivity of microsomal lauric
acid ω-hydroxylation to inhibition is suggestive of the pres-
ence of more than one cytochrome P-450 catalyzing the (ω-
1)-hydroxylation of lauric acid in rabbit lung microsomes.
This pattern of inhibition is similar to that observed with the
P-450-dependent lauric acid hydroxylation system of rat liver
microsomes (20). Note that PGE1 ω-hydroxylation was not
subject to inhibition by 250 μM 11-DDYA (Fig. 1A, closed
squares). In addition, the omission of NADPH from the
preincubation mixtures, in the presence of the inhibitor, did
not result in inhibition of either PG ω-hydroxylase or lauric
acid hydroxylase activities (data not shown) indicating that
catalysis is necessary to achieve inhibition.

It is apparent from the data presented in Fig. 1, A and B,
that the percent of control activity remaining at time 0 of
preincubation at the various inhibitor concentrations did not
always equal 100% (for example, at 250 μM 11-DDYA). This
was most likely due to incomplete quenching of the inhibition
reaction upon dilution of the test aliquot into the assay
mixture containing the radiolabeled substrate (see “Experi-
mental Procedures”). In other words, the concentration of
substrate in the assay mixture (100 μM) was not sufficient to
adequately compete with the inhibitor (final concentration,
25 μM) for binding to the enzyme. This situation obtained in
several experiments presented herein; however, in subsequent
experiments in which the KI for 12-HHDYA was determined
the ratio of substrate to inhibitor was maintained high enough
(at least 20:1) such that a lack of quenching was no longer a
factor.

Inhibition of Reconstituted P-450 Form 5 by 11-DDYA and
17-DDYA—Lauric acid ω- and (ω-1)-hydroxylation, catalyzed
by reconstituted P-450 form 5, were inhibited by both the
short chain (11-DDYA) and the long chain (17-DDYA) ace-
tylenic fatty acids (Fig. 2, A and B, respectively). The parallel
inhibition of ω- and (ω-1)-hydroxylation in the reconstituted

![Fig. 1. Time- and concentration-dependent inhibition of rabbit lung microsomal laurate hydroxylation by 11-DDYA. Rabbit lung microsomes were preincubated with 20 μM (closed circles), 100 μM (open circles), or 250 μM (closed triangles) 11-DDYA in the presence of NADPH at 37 °C. At 0, 15, 30, and 60 min, 0.1-ml aliquots were removed and assayed for the amount of [14C]-lauric acid ω-hydroxylation (A) or (ω-1)-hydroxylation activity remaining (B) as described under “Experimental Procedures.” The lack of effect of 250 μM 11-DDYA on the ω-hydroxylation of [3H]PGE1 (100 μM) is shown in A (closed squares).](image-url)
system differs from that observed in lung microsomes in which lauric acid ω-hydroxylase activity was more sensitive to inhibition than was (ω-1)-hydroxylase activity. This loss of differential inhibition upon purification of P-450 form 5 suggests that at least two cytochrome P-450 isozymes participate in the hydroxylation of lauric acid. One of these, P-450 form 5, catalyzes both ω- and (ω-1)-hydroxylation whereas the second enzyme catalyzes (ω-1)-hydroxylation.

Inhibition of Rabbit Lung Microsomal and Reconstituted P-450<sub>ω</sub>-dependent PGE<sub>ω</sub>-Hydroxylase Activity by 17-ODYA—Preincubation of lung microsomes from pregnant rabbits with 17-ODYA for 60 min at 37 °C produced a concentration- and time-dependent loss of PGE<sub>ω</sub>-hydroxylase activity (Fig. 3). Under these conditions, an inhibitor concentration of 100 μM was sufficient to inactivate approximately 75% of this activity. At higher concentrations of 17-ODYA there was increased inhibition (>95% at 500 μM); however, a portion of the inhibition observed at this high concentration of 17-ODYA was likely due to competition between the inhibitor and the substrate in the activity assay ([3H]PGE<sub>ω</sub> concentration was 50 μM) as alluded to earlier. Shown in Fig. 4 is the inhibition of reconstituted P-450<sub>ω</sub>-dependent PGE<sub>ω</sub>-hydroxylase activity by 17-ODYA. These data reflect that which was observed in the microsomal system (Fig. 3), i.e. a concentration- and time-dependent loss of PGE<sub>ω</sub>-hydroxylase. Also shown in Fig. 4 is the complete lack of inhibition by 250 μM 11-DDYA of purified P-450<sub>ω</sub>-catalyzed PGE<sub>ω</sub>-hydroxylation (closed squares), which demonstrates that 11-DDYA is not an inhibitor of PG ω-hydroxylation but is a specific inhibitor of lauric acid hydroxylation (Figs. 1 and 2).

In contrast, 17-ODYA inhibits both lauric acid ω- and (ω-1)-hydroxylation and PGE<sub>ω</sub>-hydroxylation (Figs. 2B and 4) which makes this acetylenic fatty acid a nonselective inhibitor with respect to these two rabbit lung microsomal P-450-catalyzed activities.

Inhibition of Rabbit Lung Microsomal PGE<sub>ω</sub>-Hydroxylase by 12-HHDYA—The lack of specificity of 17-ODYA for the inhibition of pregnant rabbit lung microsomal PG ω-hydroxylation led us to design an acetylenic suicide substrate (12-hydroxy-16-heptadecynoic acid) that possessed a structural feature common among substrates of the rabbit lung cytochrome P-450 PG ω-hydroxylase, i.e. the ω-6-hydroxy group (38). Preincubation of pulmonary microsomes (prepared from 25 to 28-day pregnant rabbits) in the presence of 12-HHDYA and NADPH produced a concentration- and time-dependent loss of PGE<sub>ω</sub>-hydroxylase activity (Fig. 5). The presence of 10 μM 12-HHDYA was sufficient to inactivate approximately 80% of this activity after 60 min of incubation at 37 °C. This acetylenic inhibitor had no significant effect on lauric acid ω- or (ω-1)-hydroxylase or benzphetamine N-demethylase activity at concentrations up to 200 μM (data not shown).

The data presented in Fig. 5 indicate that the kinetics of inhibition of PG ω-hydroxylation by 12-HHDYA do not strictly follow pseudo-first order kinetics. This is apparent from the nonlinearity of the semilog plots which actually suggest a biphasic behavior in the kinetic inhibition pattern. In fact, this general pattern has been observed in all experiments presented thus far, regardless of the inhibitor or the system being examined. In order to more accurately define the time dependence of inactivation, microsomes prepared...
from the lungs of pregnant rabbits were preincubated in the presence of 10 μM 12-HHDYA or 100 μM 17-ODYA, and the degree of inhibition of PG ω-hydroxylase activity was determined after 2, 4, and 8 min (Fig. 6). It is apparent that the time course of inactivation of PGE1 ω-hydroxylase in these microsomes is biphasic with a fast phase (0–8 min) followed by a slow phase (15–120 min) of inhibition. The time course of inactivation with 17-ODYA is identical to that observed with 12-HHDYA; however, in order to obtain a similar degree of inhibition one must use a 10-fold greater concentration of inhibitor or preincubation, was 2.6 nmol min⁻¹ mg⁻¹ microsomal protein.

**FIG. 3.** Suicide substrate inhibition of rabbit lung microsomal [3H]PGE1 ω-hydroxylase by 17-ODYA. Rabbit lung microsomes (1 mg/ml) were incubated at 37°C in the absence of inhibitor (closed circles) or in the presence of 25 mM (open circles), 100 μM (closed triangles), 250 μM (open triangles), and 500 μM (closed squares) 17-ODYA. At 0, 15, 30, and 60 min 0.1-ml aliquots were removed and assayed for the amount of ω-hydroxy-PGE1 formed. The mixture was incubated for 40 min at 37°C and the amount of ω-hydroxy-PGE1 formed was determined as described under "Experimental Procedures." PGE1 ω-hydroxylase activity, in the absence of inhibitor or preincubation, was 2.6 nmol min⁻¹ mg⁻¹ microsomal protein.

**FIG. 4.** Inhibition of reconstituted P-450<sub>PDL</sub> activity by 11-DDYA or 17-ODYA. Purified rabbit lung P-450<sub>PDL</sub> was reconstituted with DLPC, NADPH cytochrome P-450 reductase, and cytochrome b<sub>5</sub> in the presence of 250 μM 11-DDYA (closed squares) or 50 μM (open circles) or 250 μM (closed triangles) 17-ODYA. Enzymatic activity in the absence of any inhibitor is indicated by the closed circles. At 0, 15, 30, and 60 min 0.1-ml aliquots were removed and assayed for the amount of PG ω-hydroxylase activity remaining in the presence of 100 μM [3H]PGE1, as described under "Experimental Procedures." The P-450<sub>PDL</sub>-dependent PG ω-hydroxylase activity, in the absence of inhibitor or preincubation, was 2.0 nmol min⁻¹ nmol⁻¹ P-450.

**DISCUSSION**

The large number of P-450 isozymes within a given animal tissue and the relatively broad and overlapping substrate specificities of these monoxygenases have presented a challenge for the determination of the relative contribution of a particular P-450 isozyme to the oxidation of a given substrate. As a means of determining a P-450's involvement in a given reaction, chemical inhibitors, such as SKF-525A, metyrapone, and α-naphthoflavone, have often been employed. These compounds have varying degrees of specificity toward several forms of cytochrome P-450 and therefore are not particularly useful for such purposes. Within recent years, mechanism-based inactivators of cytochromes P-450, compounds which are irreversible inhibitors requiring catalytic turnover to produce inhibition, have been utilized to determine the substrate specificity of a number of P-450s. Constitutive P-450s that biosynthesize or catabolize endogenous compounds, such as steroids and fatty acids, are particularly well-suited for suicide substrate inhibition as they act upon a very limited array of substrates. Olefinic and acetylenic steroid or fatty acid suicide substrates have been demonstrated to be especially effective inhibitors of cytochromes P-450 which metabolize endogenous compounds (14). Acetylenic inhibitors have been utilized for the differentiation of isozyme-catalyzed metabolism of various endogenous substrates and of regiospecific metabolism of a single substrate. Ortiz de Montellano and Reich (20, 32) have synthesized a series of ω-acetylenic monocarboxylic fatty acids varying in length from 11 (10-undecynoic acid) to 18 (17-octadecynoic acid) carbons. The shorter chain deriva-
Acetylenic Fatty Acid Inhibitors of Lung Cytochromes P-450

Fig. 5. Inhibition of rabbit lung microsomal PGEl ω-hydroxylation activity by 12-HHDYA. Rabbit lung microsomes (1 mg/ml) were incubated at 37 °C in the absence of inhibitor (closed circles) or in the presence of 10 μM (open circles), 25 μM (closed triangles), 100 μM (open triangles), and 300 μM (closed squares) 12-HHDYA. At 0, 15, 30, and 60 min 0.1-ml aliquots were removed and assayed for the amount of PG ω-hydroxylase activity remaining in the presence of 50 μM [3H]PGEl as described in the legend to Fig. 3. PGEl ω-hydroxylase activity, in the absence of inhibitor or preincubation, was 2.1 nmol min⁻¹ mg⁻¹ microsomal protein.

Fig. 6. Kinetics of inhibition of rabbit lung microsomal PGEl ω-hydroxylation by 12-HHDYA and 17-ODYA. Rabbit lung microsomes (1 mg/ml) were incubated at 37 °C in the absence of inhibitor (closed squares), in the presence of 10 μM 12-HHDYA (open circles), or in the presence of 100 μM 17-ODYA (closed circles). At the indicated times 0.1-ml aliquots were removed (in triplicate) and transferred to a solution containing 100 mM potassium phosphate buffer, 1 mM EDTA, 5 mM MgCl₂, and 50 μM [3H]PGEl. This mixture was incubated for 60 min at 37 °C and the amount of ω-hydroxy PGEl was determined as described under "Experimental Procedures." The error bars represent the standard deviation.

In addition to the fatty acid acetylenic suicide substrates, a modified version of these straight chain inhibitors was utilized in this study: namely 12-hydroxy-16-heptadecynoic acid. The development of the 12-HHDYA was based primarily upon data accumulated in collaboration with Dr. R. T. Okita (Medical College of Wisconsin) regarding the substrate specificity of another rabbit lung enzyme, the NAD⁺-specific prostaglandin dehydrogenase. This cytosolic enzyme participates in the initial step in the metabolic inactivation of prostaglandins of the E and F series approximately 50-fold during gestation (36). It has been shown that prostaglandin dehydrogenase displays activity not only toward PGs of the E, F, and A series, but also to 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid (15-HETE) and also 12-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (37). Okita et al. (38) have subsequently shown that long microsomes of pregnant rabbits also catalyze the ω-hydroxylation of 15-HETE to yield the 15,20-diHETE. These investigators also demonstrated that 5-HETE, 12-HETE, and LTB₄ were not converted to ω-hydroxy metabolites. The substrates metabolized by the PG dehydrogenase and by pulmonary microsomes (prepared from the lungs of pregnant rabbits) possess a single common structural feature, that is they all contain a hydroxyl group at the ω-6 position. Those eicosanoids that were not ω-hydroxylated contain hydroxyl groups at the ω-16 position (5-HETE) or the ω-12 position (12-HETE and LTB₄). In addition to the compounds mentioned above, Powell (39) demonstrated that lung microsomes isolated from pregnant rabbits were capable of catalyzing the ω-
Acetylenic Fatty Acid Inhibitors of Lung Cytochromes P-450

hydroxylation of thromboxane B₂ which also contains an ω-6-hydroxyl group. Taking advantage of this common structural feature among substrates that are ω-oxidized by the rabbit lung microsomal PG ω-hydroxylase, we designed a suicide substrate that proved to be more potent and also more specific toward the inactivation of microsomal PG ω-hydroxylase activity than either 11-DDYA or 17-ODYA. The 12-HHDYA inhibited rabbit lung microsomal PG ω-hydroxylase without affecting lauric acid ω- or (ω-1)-hydroxylation or benzphetamine N-demethylase activity. Furthermore, as shown in Fig. 7, 10 μM 12-HHDYA was capable of inactivating 80% of the PG ω-hydroxylase activity, whereas a 10-fold higher concentration of 17-ODYA was required to achieve the same degree of inhibition under identical experimental conditions.

Lung microsomes from pregnant rabbits hydroxylate lauric acid at the ω- and (ω-1)-positions (23) and prostaglandins at the ω-position (23, 40, 41). Reconstitution experiments performed with P-450 forms purified from these microsomes suggest that lauric acid ω- and (ω-1)-hydroxylation and prostaglandin ω-hydroxylation are catalyzed by two distinct isozymes, P-450 form 5 and P-450eca, respectively (23). Acetylenic fatty acid derivatives were employed as probes to explore further the substrate specificities of rabbit pulmonary microsomes prepared from the lungs of pregnant animals and of these two cytochrome P-450 isozymes. Preliminary experiments had indicated that 11-DDYA could differentially inhibit fatty acid hydroxylation without affecting prostaglandin hydroxylation (34). We have shown in this study that 11-DDYA effectively inhibits rabbit lung microsomal lauric acid ω- and (ω-1)-hydroxylation without affecting PGE₂ ω-hydroxylation (Figs. 1, A and B). The incomplete inactivation of lauric acid (ω-1)-hydroxylation by 11-DDYA (Fig. 1B) suggests the presence of at least one other P-450 form in lung which regioselectively hydroxylates lauric acid at this position. Reconstituted cytochrome P-450 form 5-dependent lauric acid ω- and (ω-1)-hydroxylation are inhibited in parallel. This indicates that a single P-450 isozyme, that being P-450 form 5, is capable of catalyzing both types of hydroxylations (Fig. 2A).

The long chain suicide substrate, 17-ODYA, is also an effective inhibitor of reconstituted P-450 form 5-mediated lauric acid hydroxylations at both the ω- and (ω-1)-positions (Fig. 2B). Currently, little is known regarding the fatty acid substrate specificity of P-450 form 5, but the ability of the 18-carbon acetylenic fatty acid, 17-ODYA, to inhibit cytochrome P-450 form 5-catalyzed lauric acid hydroxylation suggests that this P-450 is active toward long chain, as well as medium chain, fatty acids (Figs. 1 and 2). 17-ODYA also inhibited rabbit lung microsomal and reconstituted P-450eca-mediated PG ω-hydroxylation (Figs. 3 and 4). A similar situation can be found in the human polymorphonuclear leukocyte LTB₄ ω-hydroxylation system which is insensitive to 10-undecenoic acid, only slightly inhibited by 11-DDYA, but readily inactivated by the long chain acetylenic inhibitor 17-ODYA (26).

The inactivation of both lauric acid hydroxylase activities and PG ω-hydroxylation activity, in microsomal and reconstituted systems, by the short and/or the long chain acetylenic fatty acid inhibitors exhibited biphasic kinetics. This biphasicity is most apparent in Fig. 6 in which the inactivation of rabbit lung microsomal PG ω-hydroxylase activity was examined at shorter preincubation times. The reason for the biphasicity of the inhibition kinetics is unknown at present. It is not due to the presence of more than one P-450 PG ω-hydroxylase in lung microsomes of pregnant rabbits as our purified preparations of this enzyme were homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and PG ω-hydroxylase activity was not detected with the other P-450s isolated from these microsomes (23). It is important to note that the general pattern of inhibition of PG ω-hydroxylation in rabbit lung microsomes in the presence of 17-ODYA and 12-HHDYA was essentially the same (Fig. 7). This rules out the possibility that the biphasic nature of the inhibition by 12-HHDYA was due to the presence of both 12(R) and 12(S)-hydroxy derivatives, that is that the enzyme had a greater affinity for one enantiomer over the other with the weaker-binding isomer acting as a competitive inhibitor. This possibility cannot exist with 17-ODYA. Such biphasic inhibition patterns have been observed by Miller and Halpert (42), who have examined the suicidal inactivation of phenobarbital and 3-methylcholanthrene-inducible rat liver P-450α by chloramphenicol analogues, and by Macdonald et al. (43), who have investigated the suicidal inactivation of the major phenobarbital-inducible rat liver P-450 by cyclopropylamines. These investigators were unable to adequately explain the nature of the biphasicity of inactivation; however, Macdonald et al. (43) suggested that a metabolite of the inhibitor was generated that subsequently competes for metabolism.

The inactivation of cytochrome P-450 by terminal acetylenes is often the result of N-alkylation of the prosthetic heme group by the activated inhibitor. This N-alkylation of heme produces green pigment and loss of the P-450 chromophore (15, 44). Evidence presented by Ortiz de Montellano and Reich (20, 32) suggests that terminal acetylenic inhibitors of the fatty acid hydroxylating P-450s do not result in loss of the P-450 chromophore even though they inhibit catalytic activity very efficiently (20). This result can be explained if the acetylene is oxidized to a species that can react with the protein. Ortiz de Montellano et al. (45) have demonstrated that terminal acetylenes can be oxidized by cytochromes P-450 to ketenes. The ketene can then react with water to produce the carboxylate derivative or with nucleophilic moieties resulting in alkylation. Caday et al. (46) have recently demonstrated that the terminal acetylenic fatty acid, 10-undecenoic acid, inhibits the rat liver clofibrate-inducible P-450 (P-450α₄) via alkylation of the protein and not of the heme. This P-450 shows a unique preference for the hydroxylation of lauric acid at the ω-position. The ratio of hydroxylation of lauric acid by P-450α₄ at the ω- and (ω-1)-positions is 1:1 (46). Ortiz de Montellano and Reich (47) have shown that oxidation of terminal acetylenes at the penultimate carbon results in formation of the enzyme adduct but that oxidation at the terminal carbon results in metabolite formation, i.e. the ketene as described above. It is not surprising, therefore, that the mechanism of inactivation of the P-450α₄ involves protein alkylation since this P-450 shows a high preference for oxidation of the terminal methyl group. Since the rabbit lung P-450 prostaglandin ω-hydroxylase shares this type of regio-specificity with P-450α₄, it is not unreasonable to assume that the mechanism of inactivation of this enzyme by the terminal acetylenic inhibitors used in this study proceeds by a similar mechanism, i.e. inhibition through protein modification.

The demonstration that the ketene, produced from the terminal acetylene through the action of the P-450, can react with water suggests that it is capable of being released from the active site without reacting with the enzyme further. The ketene may then rebind to the enzyme and react with a nucleophilic moiety at or near the active site resulting in inactivation. This nonspecific second order process could
result in the biphasic kinetics observed. The synthesis of 14C-labeled 12-HHDYA,2 will allow us to determine the stoichiometry of the inactivation process, leading to the measurement of the partition ratio of this compound for the inactivation of PG ω-hydroxylation, as well as to the possible elucidation of the mechanism of inhibition which results in the biphasic kinetics described.

The development of a highly selective inhibitor of rabbit lung microsomal PG ω-hydroxylase activity presents us with an excellent probe with which to examine the physiological role of this activity. In order to obtain an inhibitor that would be specific for either rabbit P-450 ω4 or human PMN LTB4 ω-hydroxylase, one needs to design terminal acetylenic compounds with structures more closely resembling the natural substrates, rather than the straight chain fatty acid derivatives. Isozyme-specific cytochrome P-450 suicide substrates have great potential, as research and therapeutic agents, to alter selectively the production or metabolism of endogenous or exogenous compounds by this important monooxygenase system.

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REFERENCES

31. Deleted in proof

1 P. R. Ortiz de Montellano, unpublished data.