Cytochrome P-450 can metabolize arachidonic acid (5,8,11,14-eicosatetraenoic acid) to four epoxides. One of them, cis-5(6)oxido-8,11,14-eicosatetraenoic acid, has been reported to possess biological activity. To ascertain whether this epoxide could be a substrate for the enzyme fatty acid cyclooxygenase, synthetic 3H-labeled cis-5(6)oxido-8,11,14-eicosatetraenoic acid was incubated with microsomes of ram seminal vesicles and the products were separated by reversed phase high performance liquid chromatography. The substrate was enzymatically transformed into products, which were more polar than 5,6-dihydroxy-8,11,14-eicosatrienoic acid. The biosynthesis was strongly inhibited by indomethacin or diclofenac sodium, two inhibitors of fatty acid cyclooxygenase. Two of the major metabolites could be identified by capillary gas chromatography-mass spectrometry as two stereoisomers of 5-hydroxyprostaglandin I\(_2\), viz. (5R,6R)-5-hydroxyprostaglandin I\(_2\) and (5S,6S)-5-hydroxyprostaglandin I\(_2\). The structures were established by comparison with the mass spectra of authentic material and by the retention time on capillary gas chromatography using deuterated internal standards. The two stereoisomers were presumably formed nonenzymatically from the intermediate 5(6)oxido prostaglandin endoperoxides or from 5(6)oxido prostaglandin F\(_{2\alpha}\) during the isolation procedure.

The enzyme fatty acid cyclooxygenase is abundant in the renal medulla, the lungs, and the seminal vesicles but it has been demonstrated in almost all tissues (1). Cytochrome P-450 has also been described as ubiquitous, but the highest levels of these monoxygenase enzymes are found in the liver, the renal cortex, the lungs, and the gut (2). Both enzymes can metabolize arachidonic acid. Fatty acid cyclooxygenase forms the PG\(_6\) endoperoxides PG\(_G\) and PGH\(_6\), which are of paramount biological interest (3), while certain monoxygenases (e.g., cytochrome P-450 PB-B) metabolize arachidonic acid to four epoxides by oxygenation of each of its four double bonds (4,5). One of these epoxides, cis-5(6)oxido-C\(_{20}\), was recently reported to be a poor substrate for the epoxide hydrolase enzymes, and to possess interesting biological activities (6-9).

It is presently unknown whether the biological effects of 5(6)oxido-C\(_{20}\) is mediated by the epoxide itself or, in analogy with leukotrienes, by formation of conjugates or other metabolites. The 5(6)epoxide differs from the other monoxygenase epoxides of arachidonic acid by having the three cis double bonds, which is one of the characteristics of substrates of fatty acid cyclooxygenase. The object of the present work was therefore to determine whether 5(6)oxido-C\(_{20}\) is metabolized by this enzyme and to determine the major products.

**EXPERIMENTAL PROCEDURES**

**Materials—**Arachidonic acid (99%) was from Sigma, St. Louis, MO. [5,6,8,9,11,12,14,15-\(^3\)H]Arachidonic acid (100 Ci/mmol) and [1-\(^14\)C]arachidonic acid (68 mCi/mmol) were from the Radiolabeled Chemical Centre, Amersham, England. PG\(_{2\alpha}\), PG\(_{2\alpha}\), methyl (5R,6R)-5-hydroxy-PG\(_{1\alpha}\), methyl (5S,6R)-5-hydroxy-PG\(_{1\alpha}\), and methyl (5S,6S)-5-hydroxy-PG\(_{1\alpha}\), were gifts from Dr. John E. Pike at the Upjohn Company, Kalamazoo, MI. [5,6,8,9,11,12,14,15-\(^3\)H]PG\(_{2\alpha}\) (160 Ci/mmol) was obtained from New England Nuclear. N-O-Bis(trimethylsilyl)trifluoroacetamide was obtained from Supelco. N(O-Bis(trimethylsilyl)acetamide (99% purity) was from Stohler Isotope Chemicals, Wakefield, MA. Glass plates, precoated with 0.25-mm silica gel (Kieselgel 60) for TLC was from Merck, Darmstadt, F. G. R. Equipment for HPLC was from Laboratory Data control (cf. Ref. 10) and the following columns were used (Waters and Associates): 10-μm silica gel (μPorasil; 3.9 × 300 or 7.8 × 300 mm). Octadeccasilane bonded to 10-μm silica gel (μBondapak/C\(_{18}\); 7.8 × 500 mm). Solvents for HPLC were from Rathburn Chemicals, Walkerburn, Scotland. Diclofenac sodium was from Ciba-Geigy and indomethacin from Sigma. Radioactivity was counted by liquid scintillation (PLD Prias, Packard) using Ready-solvHP (Beckman) as scintillator. Other chemicals were from Merck, RSV, stored at −60 °C, were obtained from the Department of Physiological Chemistry, Karolinska Institute. \(^1\)H-labeled cis-5(6)oxido-C\(_{20}\) (21 μCi/mmol) and \(^1\)C\(_{14}\)cis-5(6)oxido-C\(_{20}\) (16 μCi/mmol) were synthesized according to Corey and co-workers (11,12), purified by reverse phase HPLC, and characterized as described (4,10). The epoxides were stored in CH\(_2\)Cl\(_2\) with 5% pyridine at −20 °C and used within a few weeks.

**Experimental—**4–12 g of RSV were thawed, sliced into small pieces, and homogenized in 4 volumes of cold 0.1 M Na\(_2\)HPO\(_4\)/buffer (pH 7.4) in a glass homogenizer with several strokes of a Teflon pestle. The homogenate was centrifuged for 10 min at 15,000 × g (+4 °C). The supernatant was centrifuged for 70 min at 100,000 × g (+4 °C). The pellet was washed once with phosphate buffer and resuspended in sodium phosphate buffer so that 1 ml contained 10 mg of tissue. Soluble ferritin (0.1 mg/ml) was added to RSV, and homogenates were prepared as described above. Twenty microliters of homogenate, made up to 20 μl with buffer, was then added to 10 μl of PG\(_{2\alpha}\) (50 mCi/ml) in buffer, and the mixture was incubated at 37 °C for 45 minutes. The reaction was stopped by the addition of 100 μl of buffer, and the mixture was then applied to a column of Sephadex G-25 (Bio-Rad) equilibrated with buffer. Fractions of 1 ml were collected, and PG\(_{2\alpha}\) was determined by a fluorometric assay (cf. Ref. 10). To determine the effect of inhibitors, the microsomes were incubated with 5 μg of cyclooxygenase inhibitor per mg of tissue, and the reaction was stopped by the addition of 100 μl of buffer. The isolation procedure (4) was then used to determine whether the metabolites were formed with or without inhibitor.

**Incubation and Extraction—**(a) 0.5–1 ml microsome suspension was incubated with \(^1\)H-labeled cis-5(6)oxido-C\(_{20}\) (21 μCi/mmol) for...
were either added together with the radiolabeled substrate or added water for 2 min. The incubations were terminated by addition of 4 volumes of ethanol. The proteins were then precipitated by centrifugation. The supernatant was evaporated in vacuo and the residue was dissolved in water and extracted twice with ethyl acetate at pH 5 (0.5 M HCl). The organic layers were then dried over Na2SO4 and evaporated to dryness. The residue was dissolved in methanol with 27% water and centrifuged. The supernatant was purified by reversed phase HPLC (see below). 

(b) A 1.5 ml microsomal suspension of RSV was incubated with 150 μg of [3H]5(6)oxido-C20:3 (21 mCi/mmol) and a 10 ml suspension of 2 mg of 5(6)oxido-C20:3 (added in 50 μl of ethanol) as described above. After termination with 4 volumes of ethanol, the proteins were spun down and the supernatant was evaporated to dryness and methylated. After evaporation, the residue was dissolved in water and extracted twice at neutral pH with ethyl acetate. The combined organic layers were washed with water to neutrality, dried over Na2SO4, and evaporated. The residue was dissolved in methanol and used for synthesis of deuterated standards by the above procedure.

Indomethacin and diclofenac sodium inhibited the formation of metabolites in peak I. The effect of 0.1 mM diclofenac sodium, added at the same time as the substrate, is shown in Fig. 2A. Incubation with RSV microsomes, B, incubation with heat-denatured RSV microsomes, C, incubation with RSV microsomes, diluted 1:10 with buffer, D, the same conditions as in C except for the presence of 0.1 mM diclofenac sodium, an inhibitor of fatty acid cyclooxygenase (24). After extractive isolation, the metabolites were separated by reversed phase HPLC (methanol:HzO:acetate acid, 73:27:0.2; 2 ml/min/fraction) as shown. Peak I contained fatty acid cyclooxygenase products, peak II, 5,6-dihydroxy-C20:3, peak III, the 65-lactone of 5(6)oxido-C20:3, and peak IV, the epoxide.

FIG. 1. Reversed phase chromatograms of metabolites formed from [3H]5(6)oxido-C20:3 by microsomes of RSV. A, incubation with RSV microsomes. B, incubation with heat-denatured RSV microsomes. C, incubation with RSV microsomes, diluted 1:10 with buffer. D, the same conditions as in C except for the presence of 0.1 mM diclofenac sodium, an inhibitor of fatty acid cyclooxygenase (24). After extractive isolation, the metabolites were separated by reversed phase HPLC (methanol:HzO:acetate acid, 73:27:0.2; 2 ml/min/fraction) as shown. Peak I contained fatty acid cyclooxygenase products, peak II, 5,6-dihydroxy-C20:3, peak III, the 65-lactone of 5(6)oxido-C20:3, and peak IV, the epoxide.

FIG. 2. Straight phase chromatogram of the polar metabolites formed by microsomes of RSV and [3H]5(6)oxido-C20:3. The metabolites were methylated prior to extractive isolation and then purified by reversed phase HPLC as shown by the inset. Peak A in the inset contained the polar metabolites, which were further purified. Peak B contained excess substrate, which was eluted with methanol. The material in peak A was separated by straight phase HPLC on silica gel by a linear gradient of methanol in CHCl3 as shown by the main figure (flow, 1 ml/min/fraction). The two major peaks are I and II.
Fig. 1D and the formation of polar metabolites was inhibited by over 80% as compared with the control (Fig. 1C). Without preincubation, the ED_{50} was 0.04 and 0.05 mM for diclofenac sodium and indomethacin, respectively. When the microsomal suspension was preincubated with 1 or 3 μM indomethacin for 2 min at 37 °C, the formation of polar metabolites from 10 μM [3H]5(6)oxido-C_{20:3} was inhibited by 54 and 89%, respectively, and formation of prostaglandins from 10 μM [3H]arachidonic acid by 45 and 86%, respectively.

The metabolites used for structural analysis were obtained from experiments where the products were methylated with diazomethane prior to extractive isolation in order to avoid formation of 65-lactones. The metabolites were first purified by reversed phase HPLC as shown by the inset in Fig. 2. The inset demonstrates that little radioactivity eluted between 40 and 60 ml (where the 65-lactone of 5(6)oxido-C_{20:3} was expected) and that excess substrate was eluted with methanol (peak B). The polar metabolites in peak A were further separated by straight phase HPLC as shown by Fig. 2. Two major peaks of radioactivity, which eluted with 56-57 ml (peak I) and 61-63 ml (peak II) were characterized by GC-MS.

**Compound I (5-Hydroxy-PGI_{2a})—**A mass spectrum of the Me_{3}Si ether derivative of the material in peak I (cf. Fig. 2) is shown in Fig. 3 (top). This mass spectrum shows the same structurally important fragments as the mass spectra of the Me_{3}Si ether derivatives of each of the four methyl-5-hydroxy-PGI_{2} isomers (see below). Strong signals are noted at m/z 600 (M^+), 585 (M^+ - 15), 569 (M^+ - 31), 529 (M^+ - 71, loss of C-16 to C-20), 510 (M^+ - 90), 495, 439 (M^+ - 90 + 71)), 420 (M^+ - 90 + 90)), 397 (presumably cleavage between C-5 and C-6), 349 (397 - 90), 307 (397 - 90), 217 (397 - 90 + 90)), 203 (cleavage between C-5 and C-6), 173 (cleavage between C-14 and C-15), 129, and 73. The fragmentation is shown by the inset in Fig. 3. The C value was 25.1. The mass spectrum of the d_{5}-Me_{3}Si ether derivative of compound I showed signals inter alia at m/z 627 (M^+), 609 (M^+ - 18, loss of C^13H), 556 (M^+ - 71), 528 (M^+ - 99, loss of HOSi(C^13H)_3), 457 (M^+ - 99 + 71)), 429 (528 - 99), 415 (cleavage between C-5 and C-6), 358 (457 - 99), 331, 317, 316 (415 - 99), 217 (415 - 99 + 99)), 212 (cleavage between C-5 and C-6), 182 (cleavage between C-14 and C-15), 180, 138, and 82. This mass spectrum showed the same structurally important fragments as the mass spectra of the corresponding derivatives of synthetic methyl-5-hydroxy-PGI_{2a} and 5-hydroxy-PGI_{2p}. Compound I

![Fig. 3. Mass spectra of two stereoisomers of methyl-5-hydroxy-PGI_{2}. Top, Me_{3}Si ether derivative of compound I (methyl-5-hydroxy-PGI_{2a}), which was isolated from peak I of the chromatogram in Fig. 2. Bottom, Me_{3}Si ether derivative of compound II (methyl-5-hydroxy-PGI_{2p}) obtained from peak II in Fig. 2. The inset at the top shows formation of important fragments of both compounds.](http://www.jbc.org/)

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TABLE I
Carbon values of four stereoisomers of methyl-5-hydroxy-PGI\(_1\) and their mobility on TLC

<table>
<thead>
<tr>
<th>Stereoisomer of methyl-5-hydroxy-PGI(_1)</th>
<th>C value</th>
<th>R(_f) acetone:CH(_2)Cl(_2)</th>
<th>R(_f) acetone:hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5R,6R)</td>
<td>25.35</td>
<td>0.40</td>
<td>0.43</td>
</tr>
<tr>
<td>(5S,6S)</td>
<td>25.25</td>
<td>0.52</td>
<td>0.59</td>
</tr>
<tr>
<td>(5S,6R)</td>
<td>25.35</td>
<td>0.51</td>
<td>0.51</td>
</tr>
<tr>
<td>(5S,6S)</td>
<td>25.05</td>
<td>0.51</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The stereoisomers were analyzed as the Me\(_3\)Si ether methyl ester derivatives by mass fragmentography on a capillary GC column (20-m, SE-54 CB). C values were determined from the retention times of saturated fatty acid methyl esters. TLC was performed on glass plates, precoated with 0.25-mm silica gel, and the plates were developed twice with either acetone:CH\(_2\)Cl\(_2\) (1:1, v/v) or acetone:hexane (1:1, v/v). The (5S,6S) stereoisomer was synthesized as described in the text. The (5S,6S) and (5R,6R) stereoisomers were also named 5-hydroxy-PGI\(_{1a}\) and 5-hydroxy-PGI\(_{1b}\), respectively.

The stereoisomers had the same elution volume on straight phase HPLC as methyl 5-hydroxy-PGI\(_{1a}\). Furthermore, analysis by capillary GC using selective ion monitoring of two pairs of fragments (m/z 439/443 and 510/514) showed that compound I had the same retention time as methyl-5-hydroxy-[3,3,4,4-\(^2\)H\(_4\)]PGI\(_{1b}\). The other three stereoisomers of methyl-5-hydroxy-PGI\(_1\) had considerably longer retention times (see below) and compound I was therefore identified as the (5S,6S) stereoisomer, i.e. methyl-5-hydroxy-PGI\(_{1b}\).

Compound II (5-Hydroxy-PGI\(_{1b}\))—A mass spectrum of the Me\(_3\)Si ether derivative of the material in peak II is shown in Fig. 3 (bottom). The C value was 25.3. A comparison between the two mass spectra of Fig. 3 reveals a very similar fragmentation with differences mainly in relative intensities. This fragmentation was discussed above. The mass spectrum of compound II thus indicated that it could be another stereoisomer of 5-hydroxy-PGI\(_1\). In addition to the mass spectrum, the C value as well as the polarity on straight phase HPLC of compound II were similar to that of authentic methyl-5-hydroxy-PGI\(_{1b}\). The biological product also had the same retention time on capillary GC as methyl-5-hydroxy-[3,3,4,4-\(^2\)H\(_4\)]PGI\(_{1b}\) (Me\(_3\)Si ether derivative) as judged from selective ion monitoring (m/z 510/514 and 439/443). All these observations were consistent with compound II being the (5R,6R) stereoisomer methyl-5-hydroxy-PGI\(_{1b}\).

Comparison with Authentic Methyl-5-hydroxy-PGI\(_1\)—The structural analysis of compounds I and II was based on comparison with mass spectra of authentic material and on retention times on capillary GC using deuterated internal standards. The two stereoisomers of methyl-5-hydroxy-PGI\(_1\),
which were obtained by chemical synthesis as described above, and the three stereoisomers with known configuration, had mass spectra, which were almost identical with those of the biological products (Fig. 3; Me3Si derivatives). The C values are summarized in Table I. Furthermore, the &-Me3& ether derivatives of the two synthesized products showed almost identical mass spectra, consistent with the structure of two methyl-5-hydroxy-PGI1 stereoisomers (see compound I for discussion of the fragmentation).

One of the synthesized stereoisomers had the same mobility on TLC as methyl-(5R,6R)-5-hydroxy-PGI1, in two systems (cf. Table I) and proton NMR analysis (δ, CDCls) showed inter alia the characteristic quartet at 4.466 (14). This product was thus identified as the (5R,6R) stereoisomer, methyl-5-hydroxy-PGI1α. A mass spectrum of methyl-5-hydroxy-[3,3,4,4-2H4]PGI1α is shown in Fig. 4 (top). Several fragments were increased by four mass units in comparison with the mass spectrum of the Me3Si ether derivative of the protium form (e.g. m/z 604 (M+), 589 (M+ − 15), 514 (M+ − 90), 443 (M+ − 90 + 71), and 207), while other fragments remained unchanged (e.g. m/z 173, 217, 307, and 397). The deuterated compound had the same C value as the authentic material.

The other chemically synthesized stereoisomer was identified as methyl-5-hydroxy-PGI1α, i.e. (5S,6S), since it had a smaller C value than the three stereoisomers with known configuration (Table I). A mass spectrum of the Me3Si ether derivative of methyl-5-hydroxy-[3,3,4,4-2H4]PGI1α is shown in Fig. 4 (bottom).

The deuterated standards contained less than 0.5% of the protium form and the fragments m/z 510/514 and 439/443 were used for mass fragmentography. From the kinetic experiments, compound I was found by mass fragmentography to have the same retention time as deuterated methyl-5-hydroxy-PGI1α, and the configuration of compound I could thus be defined (cf. Table I). Compound II had the same retention time as deuterated methyl-5-hydroxy-PGI1α. However, the latter could not be separated from the (5S,6S) and (5R,6R) stereoisomers were separated on straight phase HPLC. The time curve for the microsomal formation of the two stereoisomers, analyzed after incubation for 15, 30, 120, and 300 s, showed that the highest levels were reached after 30 s and then declined. The protium:deuterium ratios of methyl-5-hydroxy-PGI1α were 0.52, 0.79, 0.34, and 0.24, respectively, and the other stereoisomer showed a parallel time curve.

**DISCUSSION**

The main finding of the present study is that 5(6)oxido-C203 is metabolized to many polar products by microsomes of RSV. This biosynthesis was inhibited by indomethacin in the low micromolar range and this drug concentration also inhibited the metabolism of arachidonic acid to the same extent. Higher concentrations of indomethacin were needed to inhibit the metabolism, when the drug and the epoxide were added to the incubation at the same time. Furthermore, the time curve for formation of the two stereoisomers of 5-hydroxy-PG1 showed that maximal levels were obtained already after incubation for 30 s. These observations strongly indicate that 5(6)oxido-C203 is metabolized by fatty acid cyclooxygenase, since the oxygenation of arachidonic acid by this enzyme has similar characteristics. The metabolism of arachidonic acid rapidly reaches its maximal velocity and the oxygenation ends completely within 1–2 min due to self-catalyzed inactivation.
of the enzyme (15). Preincubation with indomethacin suppresses the oxygenation at low concentrations, but the drug is considerably less potent when it is added to the enzyme at the same times as arachidonic acid (15).

Two of the major metabolites of 5(6)oxido-C_{20:3} were identified as 5-hydroxy-PG_{1a}, and 5-hydroxy-PG_{1b}. These prostaglandins have previously been synthesized (14), but they have not been identified as products of the cyclooxygenase pathway. A likely scheme for their formation is shown in Fig. 5.

5(6)oxido-C_{20:3} is postulated to be metabolized by fatty acid cyclooxygenase to an intermediate epoxy prostaglandin endoperoxide, 5(6)oxido-PGG_{1a}, 5-Hydroxy-PGI_{1a}, and 5-hydroxy-PGI_{1b}. These epoxides have previously been synthesized (14), but they have not been identified as products of the cyclooxygenase pathway. Fig. 5 shows the alternative mechanism. An intermediate for their formation is shown in Fig. 5. The renal cortex can form prostaglandins, which modulate renal blood flow and renal release of renin (20-24) as well as many unstable arachidonic acid metabolites that can be further metabolized to prostaglandins in the kidney and to investigate the biological effects of 5(6)oxido-PGG_{1} and its metabolites.

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E H Oliw


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