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Biochemistry

Metabolism of arachidonate through NADPH-dependent oxygenase of renal cortex

(cytochrome *P*-450/monooxygenase/hydroxy fatty acid/metyrapone)

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ABSTRACT **In normal kidneys the renal medulla very efficiently converts arachidonic acid to prostaglandins. Although the renal cortex has only trace amounts of cyclooxygenase activity, we report here the existence of an active cortical NADPH-dependent monooxygenase that converts arachidonate primarily into 19-hydroxy- and 20-hydroxyarachidonate as well as 19-ketoarachidonate and a dicarboxylic acid. The enzyme is presumably a cytochrome *P*-450 monooxygenase and demonstrated marked resistance to inhibition by 2-diethyaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525A), metyrapone, and carbon monoxide. In the rabbit kidney these products are produced only by the cortex in the presence of NADPH and represent the major metabolic products of arachidonate metabolism.**

The kidney metabolizes arachidonic acid to oxygenated products through the cyclooxygenase into the unstable intermediate prostaglandin (PG)H_2 (1), which can be further metabolized to PGE_2, $PGF_{2\alpha}$ (2), thromboxane A_2 (3), and PGI_2 (4). The rank order of renal cyclooxygenase activity is papilla > medulla > cortex (in the rabbit). In incubations of renal cortical homogenates with [^{14}C]arachidonic acid we have frequently observed nonpolar peaks on thin-layer plates that were not cyclooxygenase products, because their synthesis was not inhibited by indomethacin. The recent demonstration of arachidonic acid metabolism through lipoxygenase to several novel monohydroxy and dihydroxy compounds (5, 6) has rekindled our interest in defining the nature of the nonpolar arachidonate metabolites synthesized by the renal cortex. We have therefore determined the regional distribution, metabolic potential, and identity of the renal noncyclooxygenase products.

METHODS AND MATERIALS

New Zealand White male rabbits weighing 2.5–3.0 kg were anesthetized with pentobarbital (30 mg/kg intravenously) and the kidneys were removed and placed in ice-cold saline. The cortex and medulla were separated and each was homogenized (Tekmar Homogenizer, Cincinnati, OH) in 4 parts (wt/vol) 50 mM Tris·HCl buffer, pH 7.5, containing 2 mM EDTA. In some experiments indomethacin was included in the homogenizing buffer at 2 μg/ml. The homogenate was then sedimented at 10,000 × *g* in a refrigerated Sorval centrifuge for 15 min at 4°C. The supernatant was used as a source of enzyme. Incubations were carried out with [^{14}C]arachidonic acid (specific activity 55 mCi/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) as the substrate. At the end of incubation the lipids were extracted after acidification to pH 3.5 with 1 M HCl and subjected to chromatography. For thin-layer chromatography (TLC) separations, the extraction was with two equal volumes of ethyl acetate followed by centrifugation at 2500 × *g*, then the supernatant was evaporated to dryness under a stream of N_2. For high-pressure liquid chromatography (HPLC), the extraction was carried out with 2 vol of methanol. After centrifugation, the lower phase was extracted with 1 vol of methanol, the supernatants were pooled and acidified to pH 3.5 with 0.1 M HCl, and 6 vol of diethyl ether and 4 vol of water were added. The ether phase was removed and washed with 2 vol of water, ethanol was added to the ether phase (1 ml of ethanol to 10 ml of ether) so that the water could be removed azeotropically, and the solution was evaporated to dryness in a rotary evaporator. The total lipids were then reconstituted in a small volume (500 μl) of appropriate organic solvent prior to chromatography. For experiments with microsomes, the 10,000 × *g* supernatant was centrifuged at 100,000 × *g* for 60 min and resuspended in one-fourth the original wet weight of 50 mM potassium phosphate buffer.

Chromatography. (*i*) TLC was carried out on silica gel G (Brinkman Sil G) plates developed in the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100, vol/vol). The developed plates were exposed to Kodak X-Omat film for 48 hr. Alternatively, we used a system of benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, vol/vol), which more effectively separated the nonpolar products of arachidonic acid. (*ii*) For HPLC we used two Waters 6000A solvent delivery systems, a U6K injector with a 2-ml loading loop, a model 600 programmer, Waters μPorasil and μBondpak analytical columns and a Waters semipreparative μPorasil column. Aliquots (50 μl) of the fractions were removed for determination of radioactivity in a liquid scintillation counter. The solvents used will be detailed in *Results*. Protein was measured by the method of Lowry *et al.* (7).

Gas Chromatography/Mass Spectrometry. The radioactive compounds eluting during the HPLC were converted to the methyl esters with ethereal diazomethane and compounds containing hydroxyl groups were converted to the trimethylsilyl ethers with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane or were converted to *t*-butyl derivatives with *t*-butyldimethylsilylchlorosilane/imidazole reagent (Applied Science). The reaction was carried out at 40°C for 2 hr or overnight at room temperature in a 50:50 mixture of BSTFA and pyridine for the trimethylsilyl ether. For *t*-butyl derivatives the reaction was carried out overnight at room temperature. For isothermal gas chromatography we used a 3% OV-101 (Applied Science) column 2 m long and 2 mm inside diameter, with injector at 250°C and column at 220°C and carrier N_2. The gas chromatograph was interfaced with a Finnigan 3300 mass spectrometer. The ionization voltage was 70 eV.

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Abbreviations: TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; HETE, hydroxy-5,8,11,14-icosatetraenoic acid; PG, prostaglandin; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride.

Materials. Standards of the positional isomers of hydroxy-5,8,11,14-icosatetraenoic acid (HETE) 5-HETE, 11-HETE, 8-HETE, and 9-HETE were a generous gift from William Stenson of the Division of Gastroenterology, Washington University, and were added in dimethyl sulfoxide (Sigma). Arachidonic acid was purchased from Nu Check Prep (Elysian, MN) and $PGF_{2\alpha}$, PGE_2, and PGD_2 were purchased from Sigma. SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) (lot number SJB-7468-42) was a gift of Smith, Kline & French. Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propanone] was purchased from Sigma. BSTFA/pyridine was obtained from Pierce. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was obtained from Aldrich.

RESULTS

Fig. 1 shows an autoradiograph obtained from incubation of [^{14}C]arachidonate (3 μM) with the 10,000 × *g* supernatant of rabbit cortical tissue prepared in the absence of indomethacin. The incubations were carried out in 50 mM Tris buffer, pH 7.5, in total volume of 1 ml containing 4 mg of protein for 20 min at 37°C. In addition to the classic prostaglandins produced ($PGF_{2\alpha}$, PGE_2) there are two nonpolar zones of radioactivity with R_f values of 0.75 (compound I; probably two compounds) and 0.79 (compound II). These zones of radioactivity are not significantly influenced by Ca^{2+} (0–20 mM) or 1 mM glutathione. No arachidonate metabolites were formed by the boiled enzyme (10,000 × *g* supernatant). These products were not inhibited in the presence of indomethacin at concentrations of 2 μg/ml, but 5,8,11,14-icosatetraynoic acid at concentrations of 100 μg/ml inhibited conversion by 45%. The enzymatic conversion of arachidonate to the nonpolar metabolites was linear with protein concentration between 0.25 and 4 mg/ml. In addition, at concentrations of protein greater than 4 mg/ml a third zone of radioactivity was noted: compound III, which had a chromatographic mobility in the TLC system indicating it was less polar than compound I and had an R_f value of 0.82.

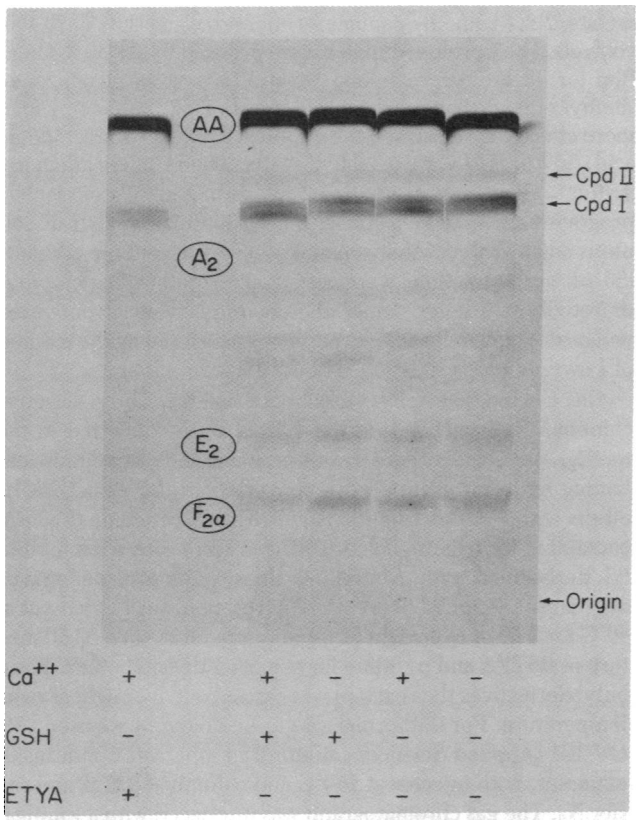

FIG. 1. TLC of products of [^{14}C]arachidonate incubated with 10,000 × *g* supernatant of renal cortex run in ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10). The compounds of interest migrate between arachidonic acid (AA) and PGA_2 (A_2). Compound I, R_f 0.75; compound II, R_f 0.79. GSH, glutathione; ETYA, 5,8,11,14-icosatetraynoic acid.

A double reciprocal plot of velocity of reaction against substrate concentration indicated a K_m for arachidonate of 166 μM and V_{max} of 8.7 nmol per mg of protein per 60 min for compound I. Experiments with a 10,000 × *g* supernatant obtained from the renal medulla homogenate did not demonstrate any ability to metabolize arachidonic acid to compounds with similar R_f values on TLC.

Cortical whole homogenates, 10,000 × *g* pellets, 10,000 × *g* supernatants, 100,000 × *g* supernatants, and 100,000 × *g* pellets were incubated with [^{14}C]arachidonic acid. The 10,000 × *g* supernatant converted [^{14}C]arachidonate to at least three nonpolar compounds. The 100,000 × *g* supernatant and the 100,000 × *g* pellet did not alter arachidonate, but when mixed together they essentially reconstituted the 10,000 × *g* supernatant and converted the arachidonate to the nonpolar products previously described. One explanation for the mixing experiment was that the 100,000 × *g* supernatant contained a cofactor or cofactors necessary for full expression of a membrane-bound enzyme present in the 100,000 × *g* pellet. To test this hypothesis we added NADPH and NADH (1 mM) to the incubations of 100,000 × *g* pellet with arachidonic acid (10 μM). The addition of NADPH to the 100,000 × *g* pellet stimulated conversion of arachidonate to nonpolar compounds with R_f values similar to those obtained when the 10,000 × *g* supernatant was used. The enzyme had an absolute requirement for NADPH; NADH did not stimulate conversion of arachidonate. SKF-525A at concentrations up to 100 μg/ml (0.28 mM) and metyrapone at 0.1 mM and 1 mM did not inhibit product formation, but at 10-fold higher concentrations there was 50% inhibition of activity. SKF-525A and metyrapone were used because they are inhibitors of the cytochrome *P*-450 mixed-function oxidase system.

The R_f values of the nonpolar compounds formed from arachidonate were very similar to the R_f values reported for the 12-OH, 8-OH, and 9-OH icosanoids of the lipoxygenase pathways (8). However, they were not detected by UV absorbtion at 233 or 280 nm, which suggested that these compounds lacked the conjugated diene or triene structure characteristic of the lipoxygenase products. Furthermore, the 45% inhibition by 5,8,11,14-icosatetraynoic acid at a concentration of 100 μg/ml indicated a greater resistance to inhibition than the lipoxygenase enzymes and suggested that the compounds produced did not involve the lipoxygenase pathways. In an attempt to identify the structure of these compounds we incubated 2.5 mg of arachidonic acid containing 20 μCi of [^{3}H]arachidonate in 10 small incubation mixtures with 100,000 × *g* pellet from renal cortex in the presence of 1 mM NADPH. Each tube contained 250 μg of arachidonate and 2 μCi of [^{3}H]arachidonate in 500 μl with 5 mg of microsomal protein. The tubes were then extracted with 2 vol of methanol and the contents were centrifuged. The pellet was reextracted with 1 vol of methanol and the extracts were pooled, acidified to pH 3.5 with 1 M HCl, and extracted with diethyl ether. After evaporation of the ether the residue was subjected to HPLC in a linear gradient of 100% hexane/ethanol/glacial acetic acid (993:6:1, vol/vol) to 100% hexane/ethanol/acetic acid (983:16:1) over 90 min at 2 ml/min. Fig. 2 shows the elution patterns of the arachidonate products obtained from HPLC on a 300 × 7.8 mm μPorasil column.

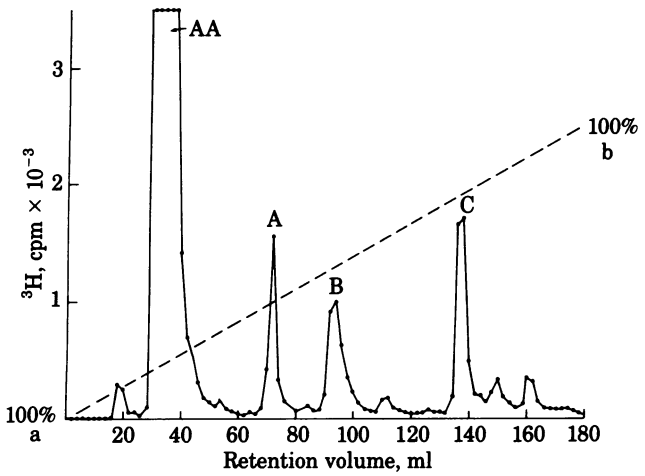

FIG. 2. HPLC run of incubation of arachidonic acid (AA) with renal cortical microsomes in the presence of 1 mM NADPH. Linear gradient of hexane/ethanol/acetic acid (993:6:1) (solvent a) to hexane/ethanol/acetic acid (983:16:1) (solvent b). Radioactivities were measured with 50-μl samples.

Three major peaks (compounds A, B, and C) were obtained with retention volumes of 72, 92, and 136 ml.

The mass spectra of compounds A, B, and C are shown in Fig. 3 *A*, *B*, and *C*. Fig. 3*A* represents the mass spectrum of the methyl ester of compound A from HPLC. The mass spectrum showed ions of high intensity at mass-to-charge ratios (m/z) 332 (M^+, molecular ion), 300 ($M^+ - 32$), 289 ($M^+ - 43$) α cleavage and loss of -C(O)CH_3, 274 ($M^+ - 58$) McLafferty rearrangement and loss of -$CH_2C(O)CH_3$ + H, 247 ($M^+ - 85$) loss of -$[CH_2]_3C(O)CH_3$, 220 due to rearrangement and loss of 112, 207 ($M^+ - 125$) loss of -$CH_2CH{=}CH[CH_2]_3C(O)CH_3$, 180 due to rearrangement and loss of 152, 175 [$M^+ - (125 + 32)$], and 173 [$M^+ - (85 + 74)$]. The spectrum suggests this compound is 19-ketoicosatetraenoic acid.

Fig. 3*B* represents the mass spectrum of the methyl ester of compound B. The mass spectrum showed ions of high intensity at m/z 362 (M^+), 331 ($M^+ - 31$), loss of -OCH_3, 302 ($M^+ - 60$) loss of -C(O)OCH_3 + H, 299 ($M^+ - 63$) loss of 2 OCH_3 + H, 288 ($M^+ - 74$) McLafferty rearrangement and loss of -$CH_2C(O)OCH_3$ + H, 275 ($M^+ - 87$) loss of -$[CH_2]_2C(O)OCH_3$, 261 ($M^+ - 101$) loss of -$[CH_2]_3C(O)OCH_3$, 257 [($M^+ - (74 + 31)$] loss of -$CH_2C(O)OCH_3$ + OCH_3 + H, 247 ($M^+ - 115$) loss of -$[CH_2]_4C(O)OCH_3$, 221 ($M^+ - 141$) loss of $CH{=}CH[CH_2]_4C(O)OCH_3$, 215 [$M^+ - (115 + 32)$], 207 ($M^+ - 155$) loss of -$CH_2CH{=}CH[CH_2]_4C(O)OCH_3$, 201 [$M^+ - (87 + 74)$], and 175 [$M^+ - (155 + 32)$]. This spectrum suggests the compound is 5,8,11,14-icosatetraene-1,20-dioic acid methyl ester.

Fig. 3*C* represents the mass spectrum of compound C methyl ester trimethylsilyl ether. The mass spectrum showed ions of high intensity at m/z 406 (M^+) 391 ($M^+ - 15$) loss of CH_3, 375

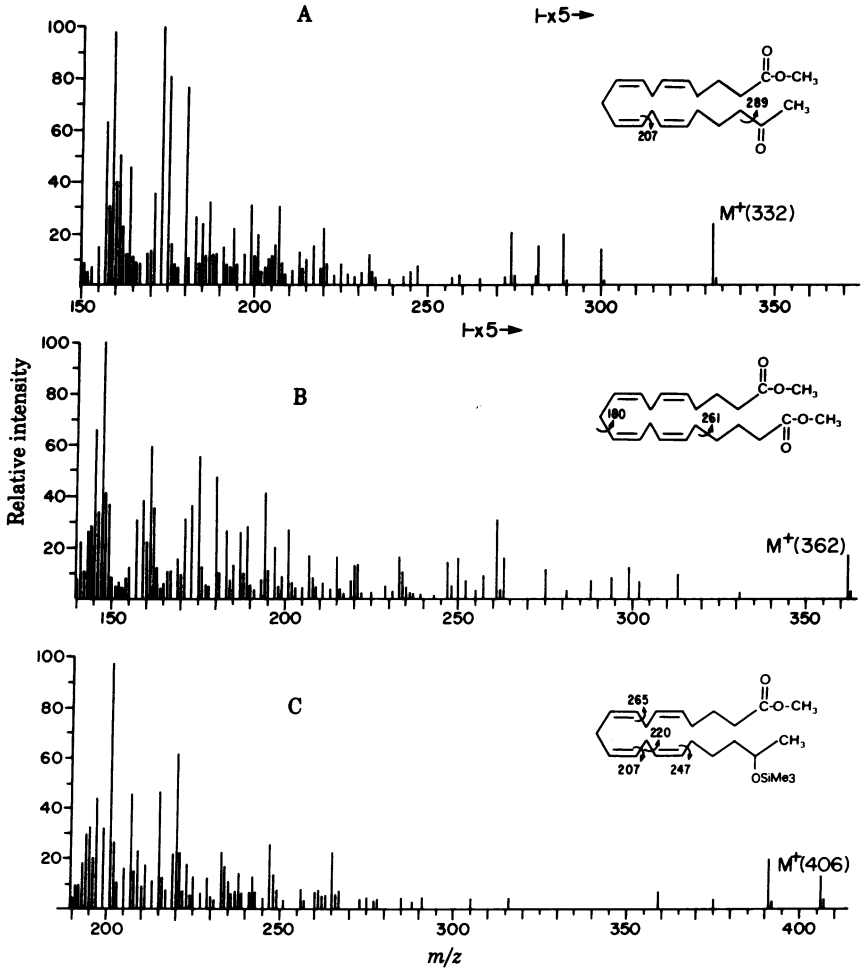

FIG. 3. Mass spectra of methyl ester of compound A (*A*), methyl ester of compound B (*B*), and methyl ester trimethylsilyl ether of compound C (*C*).

($M^+ - 31$) loss of OCH_3, 359 ($M^+ - 15 - 32$), 316 ($M^+ - 90$) loss of $HOSi(CH_3)_3$, 305 ($M^+ - 101$) loss of $-[CH_2]_3C(O)OCH_3$, 285 [$M^+ - (31 + 90)$], 275 ($M^+ - 131$) loss of $-CH_2CH(OSi(CH_3)_3)CH_3$, 265 ($M^+ - 141$) loss of $-CH_2CH=CH[CH_2]_3C(O)OCH_3$, 247 ($M^+ - 159$) loss of $-[CH_2]_3CH(OSi(CH_3)_3)CH_3$, 220 ($M^+ - 186$) due to a rearrangement and loss of $-CH=CH[CH_2]_3CH(OSi(CH_3)_3)CH_3$, 215 [$M^+ - (101 + 90)$], 207 ($M^+ - 199$), and 201 [$M^+ - (131 + 74)$]. This spectrum suggests the compound is 19-hydroxyicosatetraenoic acid. The gas chromatographic column used separated the products of the most polar peak (peak C) into two compounds. The major product was the 19-hydroxy compound and the lesser one, with a longer retention time, had a molecular weight of 448 as the *t*-butyl derivative and may represent the 20-hydroxylated compound. The molecular weight of compound C was further confirmed by making a *t*-butyldimethylsilyl ether, which gave a prominent $M^+ - 57$ ion at 391 on mass spectrometry.

DISCUSSION

The observations presented suggest that arachidonic acid is a natural substrate for the rabbit renal cortical monooxygenase systems and the fatty acid undergoes hydroxylations at positions 19 and 20. These hydroxylations are similar to those described in liver for PGE_1 and PGE_2 (9) and PGA_1 and PGE_1 (10) and suggest that they were mediated by NAD(P)H-dependent cytochrome *P*-450 enzymes. It is of some interest that these hydroxylating enzymes demonstrated an absolute requirement for NADPH, and that NADH at 1 mM could not support their oxygenase activity. Thus these enzymes are similar in cofactor requirements to the guinea pig renal cortical monooxygenase with PGE_1 as substrate (11). The major hydroxylations appeared to be at position 19. Orrenius has studied the various fatty acid oxygenations and found a decrease in $\omega/(\omega - 1)$ hydroxylation ratio with increasing carbon atom chain length (12). However, he did not report on the oxygenation of arachidonate. This polyunsaturated fatty acid ($C_{20:4}$), which has been demonstrated to be a substrate of another oxygenase (the cyclooxygenase) in renal cortex (13), can thus be metabolized to classic prostaglandins and thromboxanes. We have demonstrated metabolism through a NADPH-dependent cytochrome *P*-450 system to 19-hydroxy and 20-hydroxy fatty acids, and in addition arachidonate is converted to 19-keto arachidonate and a dicarboxylic acid. These products appear to be the major metabolites of arachidonate by the renal cortex *in vitro* when the reactions proceed in the presence of NADPH.

The kinetic parameters obtained refer to synthesis of 19-hydroxylation, because in our hands this appears to be the major product formed (compound **I**) and here a K_m of 166 μM and a V_{max} of 8.7 nmol per mg of protein per 60 min were obtained. Our studies using inhibitors of the cytochrome *P*-450 system are also very interesting and different from the results obtained from guinea pig liver monoxygenase using PGA_1 and PGE_1 as substrates. Kupfer *et al.* (10) showed 60% inhibition of hepatic monooxygenase activity with SKF-525A (1 mM) and metyrapone (5 mM) and about 80% inhibition with $CO/O_2/N_2$ (5:1:4, vol/vol). In our hands SKF-525A at 100 μg/ml (0.28 mM) and metyrapone at 1 mM had no effect on monooxygenase activity. However, at 10-fold higher concentrations SKF-525A and metyrapone oxygenase activity was inhibited by 50%. Thus the rabbit renal cortical enzyme appeared to be fairly resistant to several cytochrome *P*-450 enzyme inhibitors. This was also demonstrated in experiments with carbon monoxide when we used a $CO/O_2/CO_2$ mixture (1:1:0.5, vol/vol) and found no inhibition of oxygenase activity. This observation was similar to that observed for hydroxylation of PGE_1 by guinea pig renal cortical microsomes (total lack of inhibition by carbon monoxide) but is in contrast to the marked inhibition by CO of laurate hydroxylation with rat kidney cortex microsomes (11).

The biology of these compounds awaits further definition; however, the kidney appears to have a clear separation in cortical and medullary arachidonate metabolic activity.

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