

Arachidonate epoxygenase: identification of epoxyeicosatrienoic acids in rabbit kidney

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Abstract Epoxyeicosatrienoic acids were isolated and purified from female rabbit kidneys. They were identified as a group, prior to resolution, by packed column gas-liquid chromatography-mass spectroscopic techniques as their methyl esters as well as their trimethylsilyl bromohydrin methyl esters. Initial capillary gas-liquid chromatography-mass spectral analysis of the corresponding hydrogenated pentafluorobenzyl esters revealed the presence of the 8,9- and 14,15-epoxyeicosatrienoate regioisomers. These results, in conjunction with the documented *in vitro* biological activities of the arachidonate epoxygenase metabolites, suggest a role for them in renal function.—**Falck, J. R., V. J. Schueler, H. R. Jacobson, A. K. Siddhanta, B. Pramanik, and J. Capdevila.** Arachidonate epoxygenase: identification of epoxyeicosatrienoic acids in rabbit kidney. *J. Lipid Res.* 1987. 28: 840–846.

Supplementary key words renal • mass spectroscopy • regioisomeric composition • pentafluorobenzyl ester

An active arachidonic acid epoxygenase activity has been demonstrated in isolated rat renal cells (1) and subcellular fractions obtained from rat and rabbit kidney cortex and medulla (1–4). A role for non-cyclooxygenase arachidonic acid metabolites in renal function has been proposed (3, 5, 6). A recent report has indicated that at least two epoxygenase metabolites are formed by a preparation of cells isolated from the medullary portion of the rabbit thick ascending limb of the loop of Henle. Their formation was stimulated by arginine vasopressin and salmon calcitonin (7). Additionally, their production was enhanced in cells isolated from rabbits made hypertensive by aortic constriction (5). One of the metabolites is a potent *in vitro* inhibitor of Na,K-ATPase and the other relaxes blood vessels (7).

In our laboratory, we have demonstrated that luminal perfusion of the isolated rabbit cortical collecting tubule with 1 μ M 5,6-epoxyeicosatrienoic acid (5,6-EET) dramatically alters Na⁺/K⁺ flux and transepithelial voltage (3). Critical to the evaluation of the significance of the epoxygenase pathway in renal function is the demonstra-

tion that its metabolites are endogenous constituents of renal tissue. Herein, we report conclusive chemical evidence for the presence of EETs in rabbit kidney extracts and initial analysis of their regioisomeric composition.

MATERIALS AND METHODS

Female New Zealand White rabbits (2.0–3.0 kg) were kept at 22°C with alternating cycles of 12 hr of light and darkness and fed *ad libitum* Purina rabbit chow and water. Rabbits were killed by decapitation and their kidneys (5–10 g wet weight) were freed of connective tissue and directly immersed in liquid nitrogen (8). The frozen tissue was minced and 20% (w/v) homogenates were immediately prepared in ice-cold 40% CH₃OH–59.9% H₂O–0.1% HOAc containing 0.01% (w/v) of BHT utilizing a Tissumizer (Cole Parmer, Chicago, IL). After adding 0.2–0.4 μ Ci of [5,6,8,9,11,12,14,15-³H]14,15-EET (150–170 Ci/mmol) in ethanol as a recovery standard, an equal volume of cold CHCl₃ was added with vigorous mixing. Following phase separation, the aqueous layer was extracted again with two volumes of CHCl₃–CH₃OH 2:1. The combined organic phases were filtered through a plug of packed glass wool and immediately evaporated. To the resulting oily residue, 20 ml of an argon-saturated 0.2 N solution of NaOH in 80% CH₃OH was added and the suspension was maintained at 45°C overnight under argon. After acidification to pH 4 with dilute HCl, two volumes of ethyl acetate containing 0.01% BHT were added followed by H₂O to effect phase separa-

Abbreviations: EET, epoxyeicosatrienoic acid; EET-CH₃, methyl epoxyeicosatrienoate; EET-C²H₃, trideuteromethyl epoxyeicosatrienoate; DHET, *vic*-dihydroxyeicosatrienoic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; PFB, pentafluorobenzyl; HPLC, high pressure liquid chromatography; GLC, gas-liquid chromatography; pCI, positive ion chemical ionization; nCI, negative ion chemical ionization; MS, mass spectroscopy; TIC, total ion current; amu, atomic mass unit.

tion. The aqueous layer was additionally extracted twice with equal volumes of ethyl acetate. The combined organic phases were washed twice with 2 N NaCl and then evaporated by an argon stream. The recovery of radioactivity was always greater than 85%. The samples were bulk-purified by dissolving in ethanol and placing them onto a column of 2–4 g of silica gel (230–400 mesh, E. Merck, Darmstadt, Germany) which was subsequently eluted with 50 ml of ethanol. The eluate was evaporated and the residue was loaded onto a column containing 4 g of C₁₈ Sephalyte (40 μ m, Analytichem International, Harbor City, CA) equilibrated with 50% CH₃OH–49.9% H₂O–0.1% HOAc. The column was washed with 100 ml of the equilibration solvent using gentle suction followed by 50 ml of 70% CH₃OH–29.9% H₂O–0.1% HOAc. The radiolabeled recovery standard and EET fraction were then eluted with 50 ml of 90% CH₃OH–9.9% H₂O–0.1% HOAc. The recovery standard and EET fraction were pooled and evaporated, and the residue was transferred by repeated washes with 99.0% hexane–0.1% HOAc onto a silica gel column (9 g) equilibrated with the same solvent. The column was washed by gravity flow with approximately 100 ml of this solvent and then the radiolabeled recovery standard and EET fraction were eluted with 50 ml of 2% isopropanol–97.9% hexane–0.1% HOAc. Following solvent evaporation under argon, the samples were dissolved in a minimal volume of ethanol and purified by HPLC using a uBondapak C₁₈ column (3.9 \times 300 mm, Waters Associates, Milford, MA) and a linear solvent gradient of 49.95% CH₃CN–49.95% H₂O–0.1% HOAc to 99.9% CH₃CN–0.1% HOAc over 40 min at a flow rate of 1 ml/min. Fractions eluting with a retention time similar to synthetic EETs (20 to 24 min) were collected, pooled, and evaporated under argon. The residue as well as synthetic standards were esterified with diazomethane as previously described (9). GLC analysis on a 6-ft packed glass column of 3% SP-2100 DOH on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA) at 210°C with a helium flow of 20 ml/min revealed a component with a retention time similar to an equimolar mixture of the four regioisomeric methyl EET standards. Samples were submitted to pCI-GLC-MS analysis without further purification. The total overall recovery of the radiolabeled tracer varied between 20–40% as a range of values.

Standards of 5,6-, 8,9-, 11, 12-, and 14,15-EET were synthesized from [1-¹⁴C]arachidonic acid (0.01 μ Ci/ μ mol) or from [5,6,8,9,11,12,14,15-³H]arachidonic acid (150–170 Ci/mmol) according to published methods (10, 11). Radioisotopes were purchased from Amersham (Arlington Heights, IL). Arachidonic acid was from Nu-Chek-Prep (Elysian, MN). Each EET was purified by reverse phase HPLC prior to use. A sample of the 14,15-EET standard was converted to its trideuteromethyl ester (14,15-EET-C²H₃) as previously described (9). Catalytic

hydrogenations were performed as previously described (12). Aliquots of the biological samples and of an equimolar mixture of the four regioisomeric EET-CH₃ standards were transformed under identical conditions to their corresponding bromohydrins (11) and silylated (12). For packed column pCI-GLC-MS, analyses were done exactly as previously described (9) using CH₄ as reagent gas. The total EET-CH₃ concentration in the esterified biological samples was estimated by comparisons with a standard curve of the ion intensities at m/z 317/320 versus 14,15-EET-CH₃/14,15-EET-C²H₃ molar ratio constructed by mass fragmentographic analysis (13). The 317/320 ions are the most prominent EET-derived fragments that still contain an intact methyl ester moiety. Under these conditions of pCI-GLC-MS analysis, no significant loss or transfer of the deuterium label was observed (9, 13). Hydrogenated aliquots of the biological samples and of the synthetic standards were converted to the corresponding pentafluorobenzyl esters according to Turk, Colca, and McDaniel (14). For these samples, nCI-GLC-MS analyses were performed by splitless on-column injection onto a 30 m \times 0.20 μ m SP-2250 open tubular fused silica capillary column (Supelco Inc., Bellefonte, PA) using He as carrier gas at a linear flow rate of 20 cm/min and CH₄ as reagent gas. Samples were injected at an initial column temperature of 100°C. After 4 min, the oven temperature was raised to 250°C at a rate of 20°C/min. The injector and transfer line temperatures were 230 and 220°C, respectively.

RESULTS AND DISCUSSION

Based on our previous experience with rat liver (9) and to maximize detection sensitivity, the EETs were isolated and initially identified as a group without resolution of the individual isomers. Our strategy was first to obtain unequivocal chemical evidence for the presence of arachidonic acid epoxides in rabbit renal tissue and then to analyze their regioisomeric composition. Rabbit kidneys were extracted and the EET fraction was isolated, purified, and derivatized by the procedures outlined in Methods.

A summary of the chemical evidence for the presence of EETs in the purified rabbit kidney samples analyzed by packed column pCI-GLC-MS is as follows. *i*) Mass fragmentographic analysis of an aliquot of the esterified biological sample revealed coelution of EET-CH₃-derived ions at m/z 285, 303, 317, 335, and 363 (9) with a retention time of 7.1 min (data not shown). Under these conditions, the four regioisomeric EET-CH₃s coelute together with a retention time of 7.1 min (9). The relative ion intensities of the fragmentograms obtained with the biological samples were similar to those of authentic EET-CH₃s (9). *ii*) MS analysis of the material eluting at 7.1 min showed a fragmentation pattern typical of

EET-CH₃s (Fig. 1A, B). *iii*) Mass fragmentographic analysis after catalytic hydrogenation showed an increase of 6 amu for the characteristic EET-CH₃ ions previously observed in Fig. 1A and 1B. The relevant ions were now present at *m/z* 291, 309, 323, 341, and 369 (data not shown). For the biological and standard samples, these ions had an identical retention time of 9 min. *iv*) An aliquot of the biological sample and a comparable amount of an equimolar mixture of the four EET-CH₃ standards were individually converted into their corresponding regioisomeric bromohydrins and subsequently silylated. pCI-GLC-mass fragmentographic analysis of the resultant methyl bromotrimethylsilyloxyeicosatrienoates confirmed the presence of EETs in the biological sample. Under the reaction conditions utilized for bromohydrin formation, only an epoxide can lead to a molecule containing a *vic*-bromoalcohol. The fragmentograms in Fig. 2 show coelution of the isotopic bromine (⁷⁹Br/⁸¹Br) containing ions for the biological sample and the EET-CH₃ standards at *m/z* 397/399, 471/473, and 487/489. Both the

biological sample and the synthetic material coeluted with a retention time of approximately 11.2 min. Analogous methodology utilizing chlorohydrins has been previously exploited for the identification of EETs (15).

An estimation of the total EET content in rabbit kidney was obtained by coinjection of a known quantity of 14,15-EET-C²H₃ standard with a measured aliquot of the biological sample (Fig. 3). The ion intensities at *m/z* 317 and 320, as measured by the areas under the corresponding fragmentograms, were compared with a standard curve of ion intensities versus molar ratio. Based on the yield of the radiolabeled recovery standard, it was found that the concentration of total EET in 1 g of kidney (wet weight) obtained from different animals varied between 1–20 ppm. The total EET concentration reported for rat liver is also within this range (9) as are the levels of prostaglandins in rabbit kidney as determined by mass spectroscopy (16).

To improve chromatographic resolution of the regioisomeric EETs and to increase their detection sensitivity, we

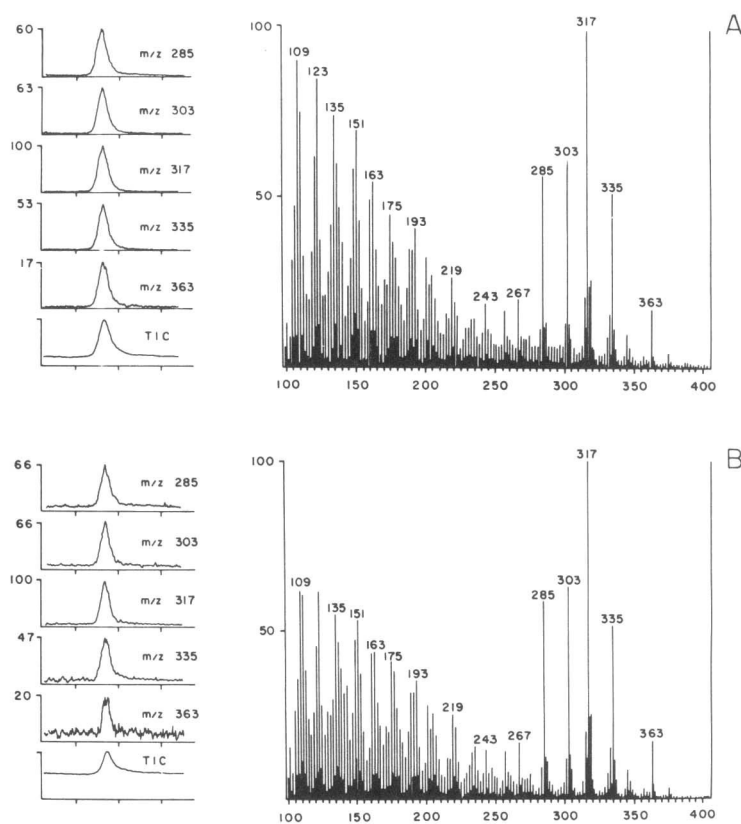


Fig. 1. Mass spectrum of the biological material isolated from rabbit kidneys. An aliquot of the sample extracted and purified from rabbit kidneys and an equimolar mixture of synthetic 5,6-, 8,9-, 11,12-, and 14,15-EET were esterified as described in Methods and then injected onto the packed GLC column. The eluents were analyzed by pCI-MS. Left: pCI-GLC-MS ion elution profiles. Right: mass spectra of the material eluting with a retention time of 7.1 min. Abscissa: mass scale, *m/z*; ordinate: abundance in percent of the base peak. A) Mass spectrum of the biological sample. B) Mass spectrum of the mixture of synthetic EET-CH₃s.

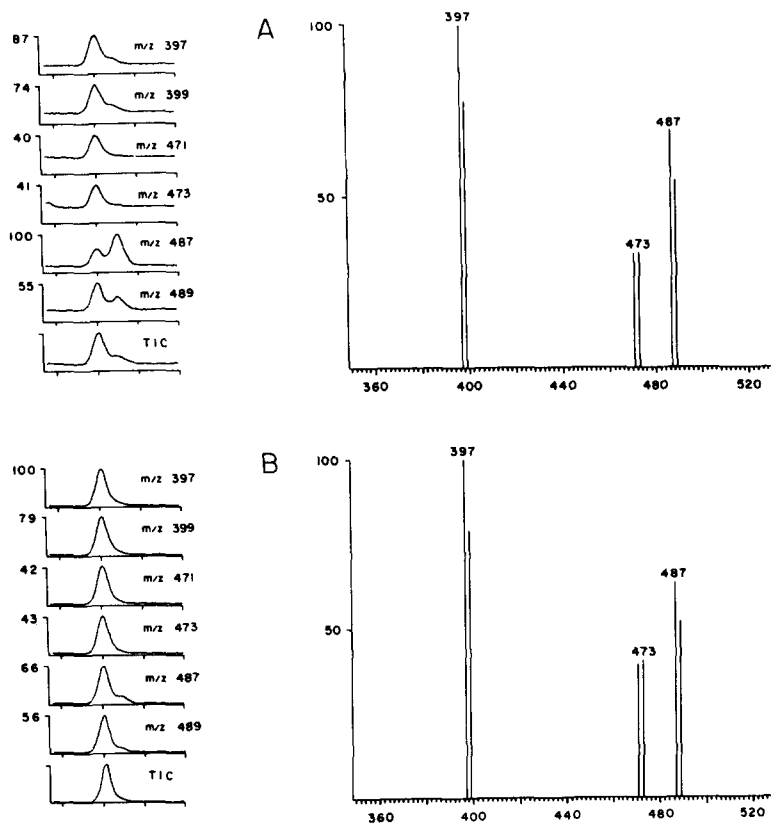


Fig. 2. Mass fragmentographic analysis of the methyl bromotrimethylsilyl derivative of the material purified from rabbit kidney. The methyl bromotrimethylsilyl derivatives of the biological sample and of an equimolar mixture of synthetic 5,6-, 8,9-, 11,12-, and 14,15-EET were prepared as described in Methods, dissolved in hexane, and injected onto the packed GLC column. The eluent was monitored by pCI-mass fragmentography at m/z 397, 399, 471, 473, 487, and 489. Left: elution profiles of the selected ions and of the TIC. Right: mass fragmentograms of the material eluting at 11.2 min. Abscissa: mass scale, m/z ; ordinate: abundance in percent of the base peak. A) Biological sample. B) Mixture of synthetic EETs.

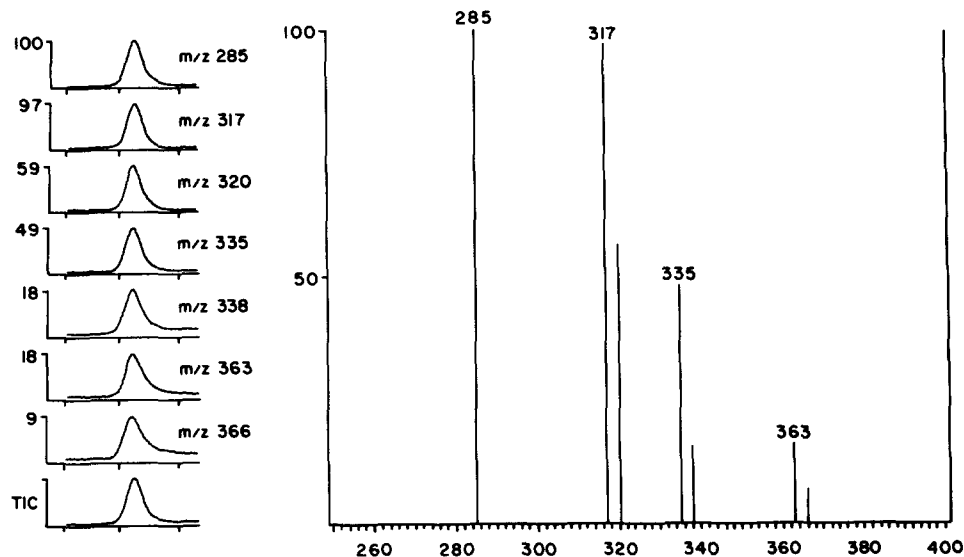


Fig. 3. Mass fragmentographic analysis obtained by cochromatography of the sample purified from rabbit kidney and synthetic 14,15-EET- C^2H_3 . A measured aliquot of the methyl ester of the biological sample was mixed with a known quantity of synthetic 14,15-EET- C^2H_3 and the mixture was then injected onto the packed GLC column. The eluent was monitored by pCI-mass fragmentography at m/z 285, 317, 320, 335, 338, 363, and 366 (left). The mass fragmentogram constructed with the data is shown on the right. Abscissa: mass scale, m/z ; ordinate: relative abundance in percent of the base peak.

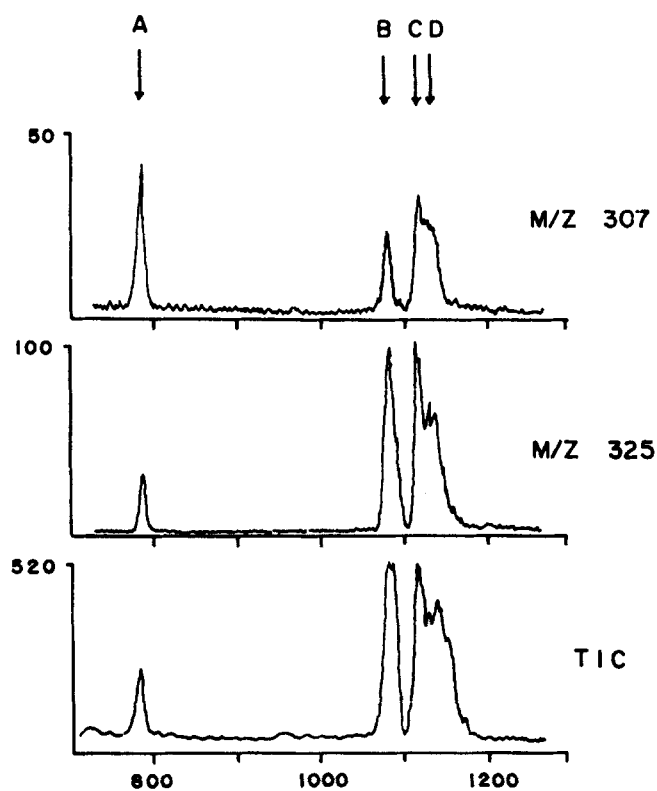


Fig. 4. Capillary GLC chromatogram of an equimolar mixture of synthetic PFB epoxyeicosanoates. An equimolar mixture of synthetic EETs was hydrogenated and derivatized as described in Methods and then injected onto the capillary GLC column. The eluent was monitored by nCI-MS using TIC and ion intensities at m/z 307 and 325. The 5,6-, 8,9-, 11,12-, and 14,15-isomers eluted in the order: A (26.2 min), B (36.0 min), C (37.1 min), and D (37.3 min), respectively. Abscissa: relative intensity; ordinate: scan number (30 scans per min).

prepared the PFB esters of hydrogenated and nonhydrogenated samples of synthetic 5,6-, 8,9-, 11,12-, and 14,15-EET and analyzed them by nCI-GLC-MS using various capillary columns. Best results were obtained utilizing the PFB esters of hydrogenated EETs and a 30-m SP-2250 capillary column. After hydrogenation, the 5,6-, and 8,9-epoxyeicosanoate regioisomers were resolved with base line separation, but only partial resolution of the 11,12- and 14,15-epoxyeicosanoate regioisomers was achieved (**Fig. 4**). Capillary nCI-GLC-MS analysis of the individual PFB epoxyeicosanoates showed two predominant ions in common at m/z 325 (base peak, loss of PFB) and 307 (loss of PFB and H_2O) (**Fig. 5**). In addition, unique diagnostic ions were present at m/z 178 for the 5,6- isomer, 141 and 157 for the 8,9- isomer, 183 for the 11,12- isomer and 225 for the 14,15- isomer (**Fig. 5**). The fragments at m/z 141, 157, 183, and 225 are consistent with loss of PFB and transannular cleavage of the respective oxido groups. Hydrogenated and PFB esterified aliquots of the biological samples were analyzed by capillary column

nCI-GLC-MS under identical conditions. The presence of 8,9- and 14,15-EET in the biological samples was inferred from: *a*) coelution of the characteristic ions at m/z 325, 307, 157, and 141 with a retention time identical to synthetic PFB 8,9-epoxyeicosanoate (36.0 min) (**Fig. 6**); *b*) nCI-GLC-MS analysis of the material eluting from the capillary column at 36.0 min showed a fragmentation pattern similar to that of authentic PFB 8,9-epoxyeicosanoate (**Fig. 6**); *c*) coelution of the characteristic ions at m/z 325, 307, and 225 with a retention time identical to synthetic PFB 14,15-epoxyeicosanoate (37.3 min) (**Fig. 6**); and *d*) nCI-GLC-MS analysis of the material eluting from the capillary column at 37.3 min showed a fragmentation pattern similar to that of authentic PFB 14,15-epoxyeicosanoate (not shown). No evidence was obtained for the presence of the 11,12- and 5,6- isomers. However, careful control experiments demonstrated that, due to its chemical lability, 5,6-EET undergoes extensive decomposition during the extraction and purification protocol described in Methods. Consequently, the failure to detect 5,6-EET may be due to technical limitations and therefore its presence in renal tissue can not be conclusively excluded at this time.

The mass spectroscopic, chromatographic, and chemical data summarized above are taken as conclusive evidence for the presence of EETs in rabbit kidney. Previously, Sevanian, Mead, and Stein (17) reported the presence of arachidonic acid epoxides in lung tissue extracts obtained from rats exposed to high concentrations of nitrogen oxides. Oliw and Moldeus (1) have reported the presence of small amounts of DHETs, hydrolysis products of the EETs, in the cytosol isolated from rabbit kidney cortex. Additionally, epoxides from fatty acids other than arachidonate have been isolated or detected in animals (17, 18) and plant tissues (19). Studies utilizing polyunsaturated fatty acid monolayers on silica gel have demonstrated that epoxides can be formed by autooxidation (17, 20). However, we previously established that, under the conditions of our isolation and purification protocol, autooxidation of endogenous arachidonic acid to EETs is negligible and does not account for our results (9). Specifically, addition of high specific activity [3H_8] arachidonic acid (10 μCi , 170 Ci/mmol) to the initial tissue homogenate does not result in a radioactive EET sample after final purification. Moreover, the observed isomeric composition of the EETs in the kidney samples suggests that they do not arise by autooxidation.

The above results establish arachidonate epoxidation as an additional pathway for the oxidative transformation of this physiologically important fatty acid. In conjunction with the aforementioned *in vitro* biological effects of the epoxygenase metabolites (3, 5-7), our findings give relevance to the cytochrome P-450 epoxygenase branch of the arachidonate cascade and lend credence to its postulated role in renal function.

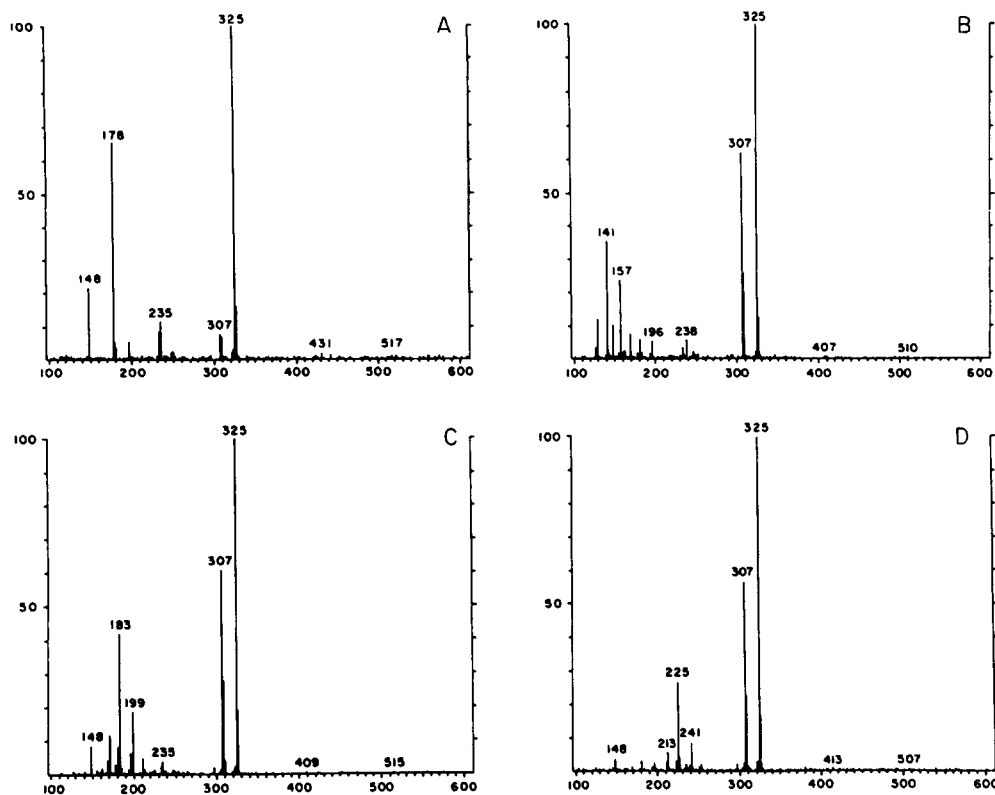


Fig. 5. Mass spectra of synthetic PFB epoxyeicosanoates. The individual PFB epoxyeicosanoates were prepared and analyzed by capillary nCI-GLC-MS as described in Methods. A: 5,6-isomer; B: 8,9-isomer; C: 11,12-isomer and D: 14,15-isomer. Abscissa: mass scale, m/z ; ordinate: abundance in percent of the base peak.

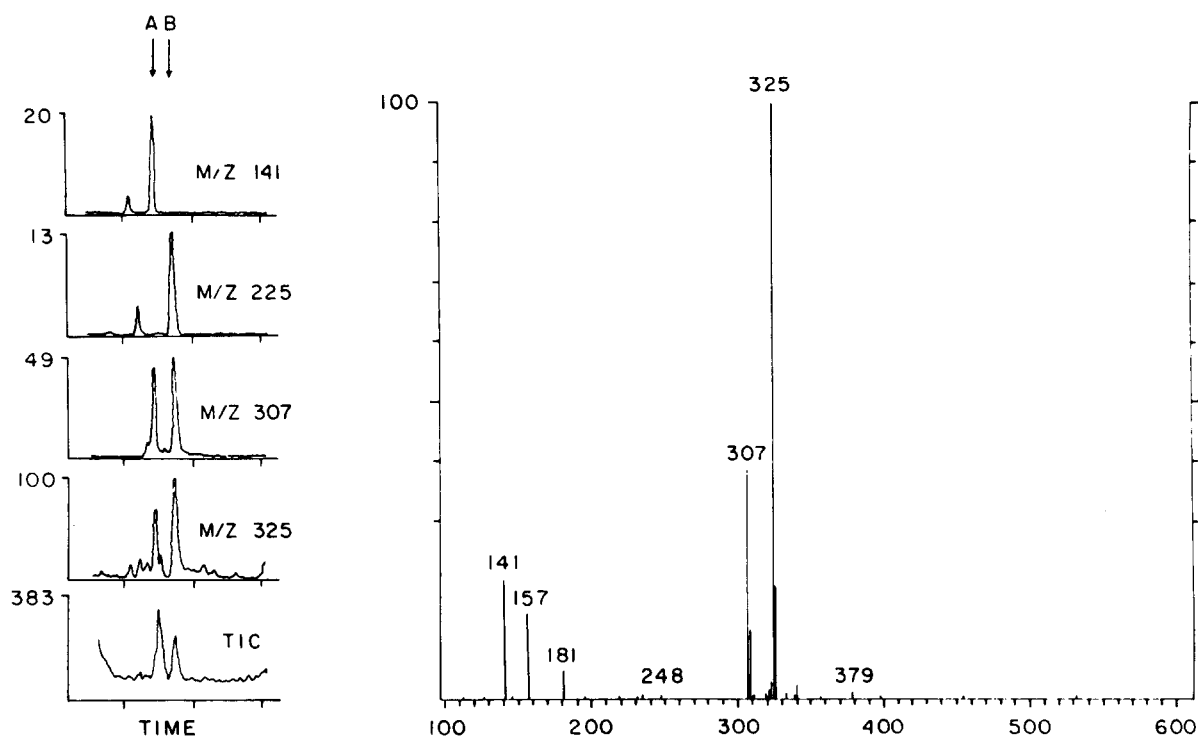


Fig. 6. Capillary nCI-GLC-MS analysis of the hydrogenated and PFB derivatized material purified from rabbit kidney. An aliquot of the biological sample was hydrogenated, esterified, and analyzed by nCI-GLC-MS as described in Methods. Left: elution profile for ions at m/z 141, 225, 307, and 325 and for the TIC. The arrows at A and B designate 36.0 and 37.3 min, respectively. Right: mass spectrum of the material eluting with a retention time (36.0 min) similar to authentic PFB 8,9-epoxyeicosanoate. Abscissa: mass scale, m/z ; ordinate: abundance in percent of the base peak.

Preliminary evidence indicates that the EETs in rabbit kidney are partially esterified to cellular glycerolipids. Current studies are focusing on establishing: *a*) the nature and distribution of the EET bound lipids, *b*) the presence or absence of 5,6-EET utilizing improved methodology compatible with its chemical lability, and *c*) the physiological relevance of arachidonate epoxygenase in renal function. ■■

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