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Lipoxygenase activity in the gills of the freshwater mussel, *Ligumia subrostrata*

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Isolated gill tissue from freshwater mussels acclimated to pondwater, metabolize exogenous arachidonic acid via the lipoxygenase pathways. 5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) was identified as the principal metabolite using high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), and gas chromatography mass spectrometry. Using HPLC and RIA, we also found 12-HETE to be produced at about 50% of the level of 5-HETE. Using HPLC, other lipoxygenase metabolites appeared to be synthesized by isolated gills but cyclooxygenase products were notably absent. However, both cyclooxygenase- and lipoxygenase-derived products of arachidonic acid metabolism are demonstrable in crude homogenates of mussel gills.

Introduction

Arachidonic acid is metabolized to oxygenated metabolites with potent biological activity. These compounds are collectively termed eicosanoids and include products of the cyclooxygenase pathway (prostaglandins, thromboxanes), the lipoxygenase pathways (hydroxyeicosatetraenoic acids, leukotrienes), and cytochrome P_{450}-dependent oxidations (epoxyeicosatetraenoic acids, dihydroxyeicosatetraenoic acids). The metabolism of arachidonic acid has been extensively studied in mammalian systems, and almost all mammalian cells and tissues have the ability to metabolize arachidonate via one or more of the above-mentioned pathways.

Although one of the earliest sources of copious amounts of a prostaglandin (15-epi-prostaglandin A_2) was the gorgonian coral, *Plexaura homomalla* [1], the metabolism of arachidonic acid by lower vertebrates and invertebrates has received less attention [2]. The widespread occurrence of prostaglandins throughout the animal kingdom suggests that prostaglandin regulation of fundamental biological processes is an important and conserved evolutionary event [3,4]. Thus, the study of arachidonic acid metabolism by tissues of lower vertebrates and invertebrates will be useful in understanding the evolution and function of the eicosanoids.

Prostaglandins may be involved in the regulation of ion and water transport in lower vertebrates and invertebrates. Tissues involved in these functions tend to have a relatively high capacity for the production of prostaglandins [3]. More direct evidence for such a role comes from work on two species of molluscs. In the marine mussel, *Modiolus demissus*, the production of prostaglandin E_2 (PGE_2) was higher in animals exposed to a hypoosmotic medium or to magnesium-free seawater [5]. In the freshwater mussel, *Ligumia subrostrata*, blood levels of PGE_2, as determined by RIA, were shown to be inversely correlated with sodium influx [6]. Additionally, injection of PGE_2 resulted in a decline in sodium uptake by the animal, while the injection of the cyclooxygenase inhibitor, indomethacin, had the opposite effect [7]. The primary site of sodium uptake by freshwater mussels is the gills and this tissue has been shown to contain high levels of arachidonic acid [6,8]. Gill homogenates from *L. subrostrata* metabolize [^{3}H]arachidonic acid into compounds that co-chromatograph in several different thin-layer chro-

Abbreviations: PG, prostaglandins; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; 5(*S*),12(*S*)-diHETE, 5(*S*),12(*S*)-dihydroxy-6-*trans*-8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; AA-861, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl chloride.

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matography systems with authentic PGE_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) standards [6]. The addition of meclofenamate to the homogenate inhibited the production of these compounds, indicating that they are produced by the cyclooxygenase pathway.

While products tentatively identified as PGE_2 and $PGF_{2\alpha}$ were produced by gill homogenates from *L. subrostrata*, the major metabolite appeared to be a lipoxygenase-derived product. This compound had an R_F value in several TLC systems that was intermediate between those of PGE_2 and arachidonic acid, and its production was increased in the presence of meclofenamate. The goal of the present study was to identify this metabolite.

Materials and Methods

[^{3}H]Arachidonic acid (5,6,8,9,11,12,14,15-^{3}H) (80–135 Ci/mmol), 5-[^{3}H]HETE (5,6,8,9,11,12,14,15-^{3}H) (150–240 Ci/mmol) and 12-[^{3}H]HETE (5,6,8,9,11,12,14,15-^{3}H) (150–240 Ci/mmol) were obtained from New England Nuclear. Arachidonic acid was obtained from Nu-Check Prep or Cayman Chemical. Prostaglandins E_2, $F_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, indomethacin, NDGA, propyl gallate, diphenyl disulfide, glutathione and methyl-*N*-nitro-*N*-nitrosoguanidine were supplied by Sigma Chemical Co. 5-HETE, 12-HETE and LTB_4 were from Biomol or Cayman Chemical. Takeda Pharmaceutical Co., Japan, provided AA-861 and Hoffman LaRoche provided ETYA. *N*,*O*-bis(trimethylsilyl)trifluoroacetamide was obtained from Pierce Chemical. The 5(*S*),12(*S*)-diHETE was provided by the Upjohn Company.

Mussels were collected from ponds in the Baton Rouge area, and transferred to aquaria containing aerated artificial pondwater (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, and 0.05 mM KCl). The animals were held in the laboratory for at least 1 week prior to being used. Male *Ligumia subrostrata* were used in this study.

In preliminary studies, a crude homogenate was prepared by homogenizing approx. 0.3 g wet weight of gill in 5 ml of 50 mM Tris-HCl (pH 7.6). The incubation medium consisted of 50 mM Tris-HCl (pH 7.6), 5 mM reduced glutathione and crude gill homogenate (0.6–6 mg protein) in a final volume of 1.0 ml [6]. The reaction was initiated by the addition of [^{3}H]arachidonic acid in 5 μl of ethanol and terminated by the addition of ethyl acetate. The phases were separated by centrifugation and the organic phase evaporated to dryness under reduced pressure. The residue was dissolved in methanol, concentrated under nitrogen, and applied to a TLC plate (Whatman, K5). The plate was developed in chloroform/methanol/acetic acid/water (90 : 8 : 1 : 0.8, v/v) or benzene/acetone/acetic acid/water (80.8 : 18 : 1 : 0.02, v/v). Areas corresponding to authentic 5-HETE and PGE_2 were scraped into scintillation vials, and the amount of radioactivity quantitated by liquid scintillation counting. The assay conditions for the synthesis of 5-HETE and PGE_2 were characterized with respect to assay time, protein and pH. The effect of several inhibitors of both lipoxygenase and cyclooxygenase enzymes was investigated. Although the crude homogenate assay usually gave reproducible results, we frequently encountered a loss of enzymatic activity.

The same procedures were repeated using intact isolated gills instead of crude homogenates and most of the results reported here are from these assays. Gill tissue was excised from mussels into a petri dish containing artificial pondwater. The gills were separated into the four demibranchs, and the wet weights measured (range = 0.15–0.35 g). These procedures were carried out as quickly as possible while taking care to keep the handling of the tissue to a minimum. The demibranchs were transferred to 25-ml flasks containing 5 ml of artificial pondwater. Reactions were started by the addition of 0.25–2.0 μCi of [^{3}H]arachidonic acid in 5 μl of ethanol. This volume of ethanol had no effect on arachidonate metabolism by the gill tissue. In several assays, we added the arachidonic acid to the reaction flasks and evaporated the ethanol to dryness with N_2 and the results were the same as assays containing 5 μl ethanol. In some experiments inhibitors of cyclooxygenase and lipoxygenase were added 5 min prior to the addition of substrate. Indomethacin, diphenyl disulfide, NDGA, and ETYA were added to give final concentrations of 1–500 μM, depending on the study, in 5 μl of ethanol. Propyl gallate was prepared in buffer, while AA-861 was added in 5 μl of dimethyl sulfoxide. Control tissues received 5 μl of drug vehicle.

Reactions were carried out in a shaking water bath at 20 °C, and were stopped by removing the tissues from the flasks. The flasks were gassed with N_2 and placed on ice. The contents of the flasks were brought to 20 ml with water, and passed through Sep Pak C_{18} cartridges (Waters). The cartridges were rinsed with 5 ml of water, and the lipids were then eluted with 4 ml of methanol [9]. The solvent was evaporated at 30 °C under N_2, and the metabolites redissolved in 20–50 μl of methanol.

The metabolites were separated by reverse-phase HPLC using an Altex C_{18}, 5 μm ODS column (250 × 4.6 mm). The preliminary solvent system was acetonitrile/water/acetic acid (75 : 25 : 0.1, v/v). However, to separate cycloxygenase and lipoxygenase metabolites in one step, we used a modification of Henke et al. [10], consisting of a four-step gradient of 55, 60, 70 and 100% methanol in water (pH 3.3 with acetic acid) over 105 min with changes at 30, 55 and 85 min. A flow rate of 1 ml/min was used. 1-ml fractions were collected, and radioactivity determined by liquid scintillation counting. A quench curve was used to convert cpm to dpm.

In experiments in which the incorporation of arachidonic acid into tissue lipids was determined, the tissues were transferred from the incubation flasks to tubes containing 3 vol. of $CHCl_3/CH_3OH$ (1:2). The tissues were homogenized and the lipids extracted by the method of Bligh and Dyer [11]. The lower $CHCl_3$ phase was evaporated to dryness under N_2, and the lipids redissolved in 25–50 μl of $CHCl_3/CH_3OH$ (2:1). The extracts were then applied to TLC plates (Whatman, K6) and the lipid classes separated using one of two different solvent systems: (1) chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v), or (2) hexane/diethyl ether/acetic acid (80:20:1, v/v). The first system was routinely used because it separated phospholipids ($R_F = 0.0$), free arachidonic acid ($R_F = 0.70$), and triglycerides ($R_F = 0.90$), and it allowed the determination of tissue eicosanoids (e.g. 5-HETE, $R_F = 0.55$). In general, tissue levels of eicosanoids were less than 10% of the amount extracted from the bath. The second solvent system was used to separate triacylglycerols from cholesterol esters, and confirmed the identification of triglyceride as the labeled neutral lipid. After development, the plates were dried in air and scanned with a Packard model 7230 radiochromatographic scanner. Areas corresponding to radioactive peaks were scraped into scintillation vials and the amount of radioactivity quantitated by liquid scintillation counting.

For RIA analyses the assays were performed with unlabeled arachidonic acid (100 μM). The bathing medium was passed through a Sep Pak C_{18} cartridge and the sample was fractionated by HPLC as described above. RIA analyses for the 5- and 12-HETE were performed on the column fractions (1 ml). The fractions were evaporated under N_2 (30 °C) and resuspended in phosphate-buffered saline (pH 7) containing gelatin. The RIA assays were performed in duplicate [12,13].

For gas chromatographic-mass spectrometer (GC-MS) analysis of the putative 5-HETE, we combined the Sep Pak concentrate from four to eight assays. Each assay contained one demibranch (about 0.3 g) incubated with 100 μM unlabeled arachidonic acid for 5 min. The pooled sample was then fractionated by HPLC as described above. The fractions corresponding to 5-HETE were combined and adjusted to pH 9 with NH_4OH. The solvent was evaporated to dryness and resuspended in 50–100 μl MeOH twice. This procedure reversed the spontaneous lactone formation occurring in the acidic HPLC solvent. The sample was methylated with diazomethane [14], and treated with trimethylsilyl chloride (TMS) to make the TMS derivative [15]. The sample was injected into a Hewlett Packard model 5790A gas chromatograph-mass spectrometer equipped with a 12 m × 0.2 mm high-performance capillary column (HP-1, Hewlett Packard). The injector temperature was 220 °C, the initial temperature of the column was 70 °C, and the oven temperature was increased to 210 °C at a rate of 25 C°/min. The 5-HETE methyl ester trimethylsilyl derivative eluted at 13.7 min.

Results

Gill homogenates contained enzymes capable of metabolizing [^{3}H]arachidonic acid into a variety of compounds as determined by TLC (Fig. 1). The results were similar to previously published data [6]. Using boiled homogenate, all of the radioactivity remained in the arachidonic peak (data not shown). Radioactive peaks at R_F 0.34 (CX-1) and 0.57 (5-HETE), corresponding to standards for PGE_2 and 5-HETE, respectively, were scraped from TLC plates and the metabolites extracted from the silica gel with methanol. Separation of the metabolites by HPLC using acetonitrile/water/acetic acid (75:25:0.1, v/v) gave single peaks of radioactivity, which correspond to authentic PGE_2 and 5-HETE standards (data not shown).

Fig. 2 demonstrates the optimal conditions for production of 5-HETE and PGE_2 in crude homogenates of gill tissue (20 °C). The crude homogenate demonstrates sensitivity to the cyclooxygenase inhibitor, indomethacin, which is moderately effective in reducing PGE_2 synthesis but has no effect on 5-HETE production (Table I). ETYA, propyl gallate and diphenyl disulfide are effective inhibitors of both cyclooxygenase and lipoxygenase activity in mussel-gill homogenates. However, because of the frequent loss of enzymatic activity, all subsequent studies were performed on intact isolated gills.

Using the intact isolated gill preparation, exogenous [^{3}H]arachidonic acid was rapidly taken up by gills from male *Ligumia subrostrata*, and some was incorporated

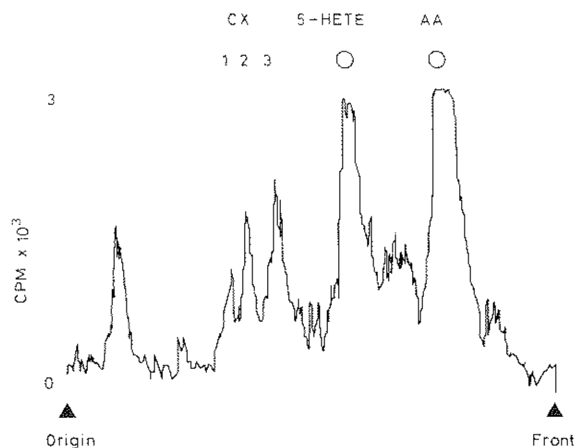

Fig. 1. Metabolism of exogenous [^{3}H]arachidonic acid (AA) by crude homogenate of gill tissues from male *L. subrostrata*. Assay conditions, extraction and TLC separation protocols are listed in Materials and Methods. The TLC plate was analyzed with a radiochromatographic scanner. Authentic standards for PGE_2 (CX 1), 5-HETE and arachidonic acid were visualized with I_2 vapors.

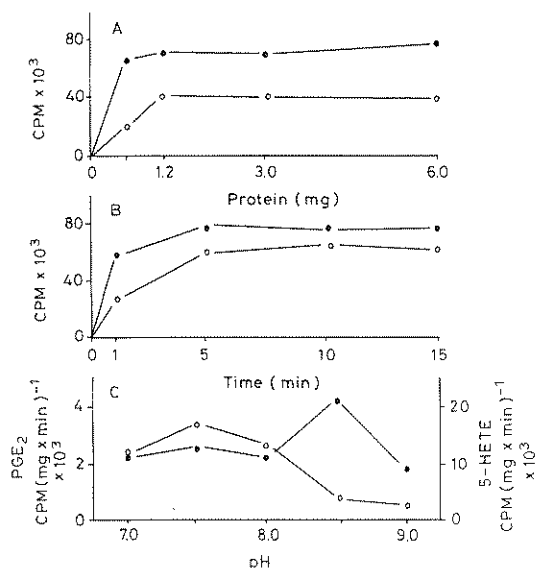

Fig. 2. Assay conditions for PGE_2 and 5-HETE synthesis from exogenous [^{3}H]arachidonic acid by crude homogenate of mussel gill. The standard assay temperature was 20 °C, pH 7.6 and 5 min duration. (A) Effect of homogenate protein concentration; (B) duration of assay; and (c) effect of pH. Symbols: ●, 5-HETE; ○, PGE_2.

TABLE I

Effect of various inhibitors of cyclooxygenase and lipoxygenases on the production of PGE_2 and 5-HETE by crude homogenates of mussel gills

The data are expressed as percentage of controls and concentration is in μM.

Treatment	Concentration	PGE_2	5-HETE
Indomethacin	20	102.7	105.4
	200	51.4	121.3
ETYA	20	85.6	80.1
	200	9.0	20.3
Propyl gallate	1	87.0	103.7
	100	7.8	21.3
Diphenyl disulfide	5	71.8	87.4
	500	36.6	56.5

into cellular lipids (Table II). The amount of label incorporated into the phospholipid and triacylglycerol fractions increased steadily with time, while the amount of free arachidonate declined slightly. After 1 min, 48

TABLE II

Incorporation of exogenous [^{3}H]arachidonic acid into tissue lipids by isolated whole gills from male L. subrostrata

Data are expressed as dpm/mg gill, and are from a representative experiment in which a gill demibranch was incubated in 5 ml of pondwater containing 0.5 μCi of substrate.

Lipid class	1 min	3 min	5 min	10 min
Phospholipid	75	140	226	332
Free Fatty Acid	63	58	54	47
Triacylglycerol	16	32	44	57

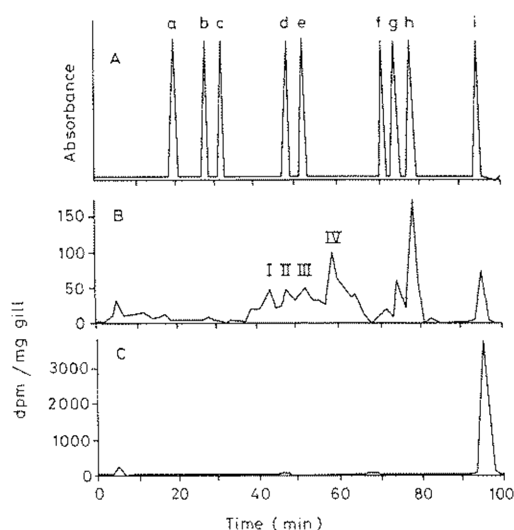

Fig. 3. Metabolism of exogenous [^{3}H]arachidonic acid by isolated whole gills from male *L. subrostrata*. Assay and extraction protocols and HPLC conditions are given in Materials and Methods. (A) Standards (a, 6-keto-$PGF_{1\alpha}$; b, PGE_2; c, $PGF_{2\alpha}$; d, 5-12-diHETE; e, LTB_4; f, 15-HETE; g, 12-HETE; h, 5-HETE; i, arachidonic acid). Prostaglandins were monitored at 207 nm, LTB_4 and 5(*S*),12(*S*)-di-HETE at 278 nm, and monoHETE at 235 nm. (B) 0.5 μCi of [^{3}H]arachidonic acid incubated in 5 ml of pondwater for 5 min at 20 °C with gill tissue. (C) 0.5 μCi of [^{3}H]arachidonic acid incubated in 5 ml of pondwater for 5 min at 20 °C without gill tissue.

and 11% of the label incorporated into tissue lipids was in the phospholipid and triglyceride fractions, respectively, while 41% remained in the free fatty acid form. After 10 min, the percentage incorporation into phospholipid had increased to 76% of the total, while the amount in the triglyceride fraction was unchanged at

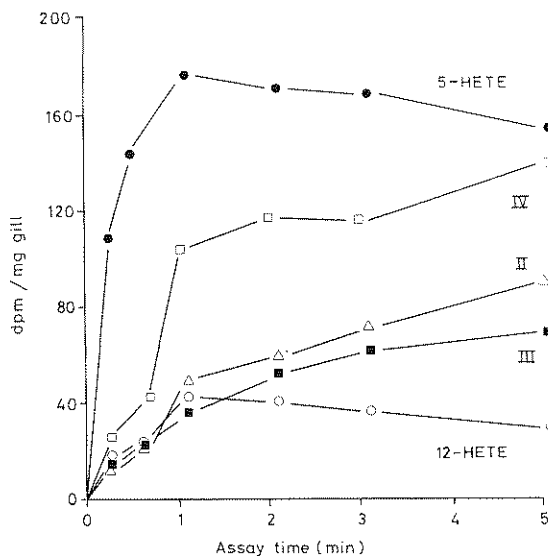

Fig. 4. Effect of assay time on the production of [^{3}H]arachidonic acid metabolites by isolated whole gills from male *L. subrostrata*. Data are expressed as dpm/mg gill, and are averages of duplicate determinations. Roman numerals refer to the peaks in Fig. 3.

12% and the amount in the free fatty acid fraction had declined to 11%. No radioactivity was observed in the prostaglandin portion of the plate.

The four-step HPLC solvent system employed in this study gave good separation of prostaglandin, dihydroxyeicosanoid, and monohydroxyeicosanoid standards (Fig. 3A). As with the crude gill homogenate, [^{3}H]arachidonic acid was metabolized into a variety of eicosanoids (Fig. 3B). Three major metabolites were observed, and two of these metabolites co-chromatographed with authentic 5- and 12-HETE standards at 74 and 79 min, respectively. The 5-HETE peak was consistently the most abundant metabolite. The other major peak (IV) eluted after LTB_4 at 59 min, and the size of this peak exhibited considerable variability. Smaller amounts of several other metabolites were produced. Two of these compounds (II and III) co-eluted with authentic 5(*S*),12(*S*)-diHETE and LTB_4 standards. A small peak of radioactivity corresponding to 15-HETE often was observed. No peaks corresponding to prostaglandins were detected. Incubation of [^{3}H]arachidonic acid without tissue resulted in a single peak that co-chromatographed with an arachidonic acid standard (Fig. 3C), indicating that the compounds were indeed tissue metabolites.

Maximum production of 5- and 12-HETE was reached in 1 min, thereafter the amounts of these two metabolites declined slowly (Fig. 4). The amounts of compounds II, III and IV also increased rapidly during the first minute. Unlike the 5- and 12-HETE, however, the quantity of these metabolites continued to increase over the next 4 min, although at slower rates than during the first min.

Several different cyclooxygenase and lipoxygenase inhibitors were tested (Table III). Indomethacin had no effect on the metabolism of arachidonic acid by mussel gills, indicating that none of the compounds were produced via the cyclooxygenase pathway. Of the compounds tested, NDGA was the most potent, inhibiting the production of all of the metabolites equally. ETYA was slightly less effective than NDGA at inhibiting arachidonic acid metabolism. AA-861 has been reported to be a specific 5-lipoxygenase inhibitor in guinea pig peritoneal polymorphonuclear leukocytes [16] and it demonstrated a similar property in mussel gill. The production of 5-HETE was most strongly inhibited, while the synthesis of 12- and 15-HETE was enhanced in the presence of AA-861. The production of compounds II, III, and IV also was inhibited, but to a smaller extent than was the production of 5-HETE. The production of compound I was inhibited only slightly.

TABLE III

Effect of various inhibitors on the production of arachidonic acid metabolites by isolated whole gills from male L. subrostrata.

Gill demibranchs were incubated for 5 min in 5 ml of pondwater containing 10 μM of the various inhibitors. 0.25 μCi of [^{3}H]arachidonic acid was then added, and the incubations continued for another 5 min. Data are expressed as percent of control, and are averages of quadruplicate determinations.

Metabolite [a]	Indomethacin	NDGA	ETYA	AA861
I	126	8	34	87
II	115	8	14	66
III	130	10	22	67
IV	105	12	18	68
15-HETE	115	12	38	174
12-HETE	97	12	36	125
5-HETE	103	10	37	46

[a] Roman numerals refer to peaks in Fig. 3.

TABLE IV

Effect of various inhibitors on the incorporation of exogenous [^{3}H]arachidonic acid into tissue lipids by isolated whole gills from male L. subrostrata

Assay conditions are described in the legend for Table 3. Data are expressed as percent of control, and are averages of quadruplicate determinations.

Lipid class	Indomethacin	NDGA	ETYA	AA861
Phospholipid	103	297	113	149
Free Fatty Acid	107	678	718	423
Triacylglycerol	174	252	220	384

In agreement with the inhibitors' effects on eicosanoid production, the amount of free fatty acid label in the tissue was unaffected by indomethacin and increased by NDGA, ETYA, and AA-861 (Table IV). The amount of arachidonic acid incorporated into phospholipids and triacylglycerols also was increased by preincubation of the tissue with these compounds. The exception was that the incorporation of arachidonate into phospholipids was unchanged in the presence of ETYA.

Since NDGA was the most effective inhibitor tested, the effect of various concentrations of NDGA on the production of the three major metabolites was examined (Fig. 5). The production of all three compounds was similarly inhibited with 50% inhibition occurring at approx. 1 μM NDGA.

Experiments with unlabeled arachidonic acid supported the identification of 5- and 12-HETE. Incubations were performed with 100 μM arachidonic acid, and the metabolites were extracted and analyzed as in the experiments with labeled substrate. Peaks co-eluting with radioactive peaks and authentic 5- and 12-HETE standards were observed by UV monitoring at 235 nm (Fig. 6). Absorbance at this wavelength is characteristic of mono-HETE. RIA analyses of the HPLC fractions further confirmed the identifications. Peaks of RIA-positive material occurred at 74 and 79 min for the 12-HETE and 5-HETE RIA analyses, respectively (Fig. 6). The total amounts of 12- and 5-HETE synthesized

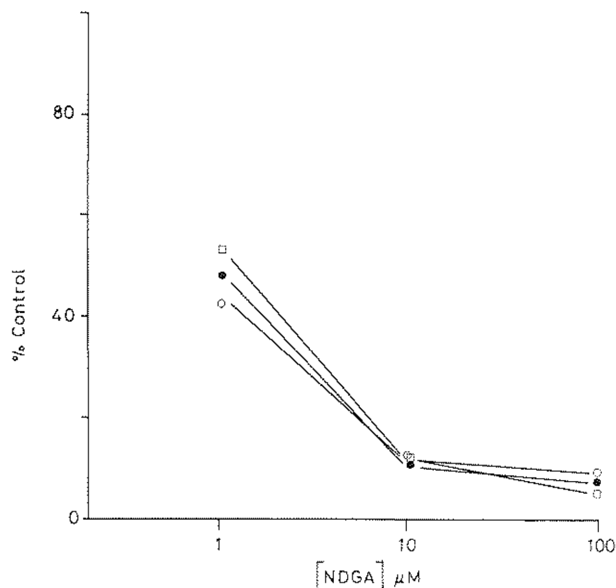

Fig. 5. Effect of NDGA on the production of compound IV (□), 5-HETE (●), and 12-HETE (○) by isolated whole gills from male *L. subrostrata*. Data are expressed as percent of control, and are averages of duplicate determinations.

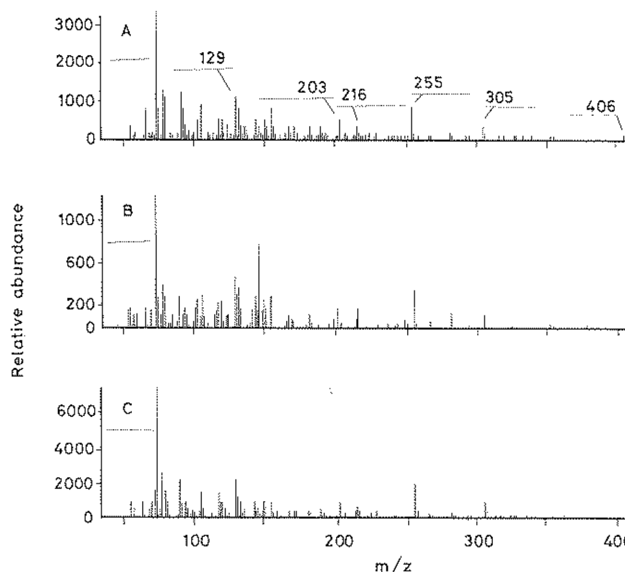

Fig. 7. Mass spectrum of the trimethylsilyl derivative of the methyl esters of 5-HETE. (A) Authentic 5-HETE standard, (B) 5-HETE from HPLC fractions of *L. subrostrata* gill incubation medium, (C) 5-HETE from HPLC fractions of *C. texasensis* gill incubation medium.

by the gill were 46 and 82 ng (g wet gill per min)$^{-1}$, respectively.

Since 5-HETE was consistently the most abundant metabolite produced by *L. subrostrata* gills, an effort was made to positively identify this compound by mass spectroscopy. Parallel reactions of four to eight demibranchs were performed using 100 μM unlabeled arachidonic acid. The reaction mixtures were combined and analyzed by reverse-phase HPLC. The column fractions corresponding to 5-HETE were collected, combined, and derivatized. The mass spectrum was identical to that of an authentic 5-HETE standard, and showed major ions of high relative intensity at mass-to-charge ratios (m/z) of 406 (M), 305 (M-101, loss of $-CH_2(CH_2)_2-CO-O-CH_3$), 255 (M-151, loss of $CH_2(CH=CH-CH_2)_2-(CH_2)_3-CH_3$), 216 and 203 (Fig. 7). It is interesting to note that another unionid bivalve, *Carunculina texasensis* also synthesizes 5-HETE as a major metabolite of arachidonic acid (Fig. 7C).

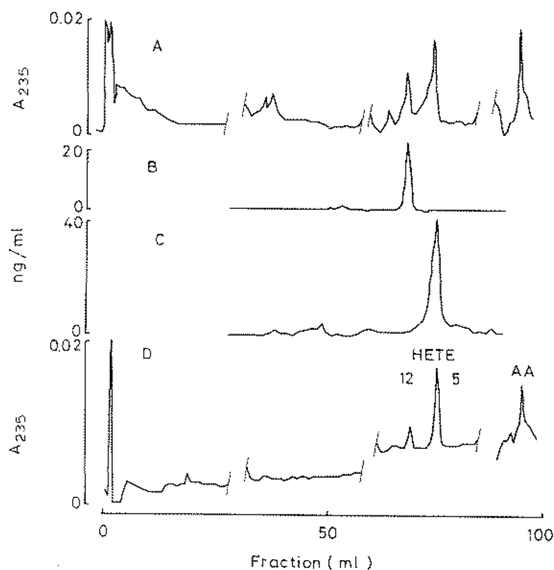

Fig. 6. Metabolism of unlabeled arachidonic acid (AA) by isolated whole gills from male *L. subrostrata*. Gills were incubated with 100 μM substrate for 5 min at 20 °C. Extraction protocol, HPLC conditions, and RIA methods are given in Materials and Methods. (A) Absorbance monitored at 235 nm for metabolites. (B) 12-HETE RIA on HPLC fractions. (C) 5-HETE RIA on HPLC fractions. (D) Absorbance monitored at 235 nm for 5- and 12-HETE standards.

Discussion

These results identify 5-HETE as the most abundant metabolite of arachidonic acid in the gills of the freshwater mussel, *Ligumia subrostrata*. This identification is based on HPLC co-chromatography with an authentic 5-HETE standard, RIA analysis of the HPLC fractions (immunochromatography), and GC-MS of the trimethylsilyl , methylester derivative. This latter piece of data is crucial for a positive identification of arachidonic acid metabolites, particularly from non-mammalian sources.

Levine and Kobayashi [17] have shown that multiple compounds from invertebrates may cross-react with mammalian-derived antibodies. Thus, without independent confirmation, a RIA analysis of tissue extracts or body fluids, using commercial antibodies, may falsely indicate the presence of particular eicosanoids. It should be noted, however, that in the case of gill tissue from *L.*

subrostrata, only single peaks of 5-HETE- and 12-HETE-positive material were observed upon immunochromatographic analyses; our antibody displayed no significant cross-reactivity; and the peaks coincided with authentic standards. The mass spectrum of the trimethylsilyl, methyl ester derivative matched that of an authentic 5-HETE standard and is in agreement with previously reported spectra for 5-HETE [15,18].

Gills from *L. subrostrata* also produced 12-HETE. This compound was identified by HPLC co-chromatography with a 12-HETE standard and by immunochromatography.

Other metabolites of arachidonic acid eluted between 40 and 65 min in our HPLC system. Their production was not inhibited by indomethacin, indicating that they are not produced by the cyclooxygenase pathway. Since 5(*S*),12(*S*)-diHETE and LTB_4 elute at 48 and 52 min, respectively, it is likely that these compounds are dihydroxyeicosatetraenoic acids. A number of such metabolites are produced both enzymatically and nonenzymatically from 5-HPETE and 5-HETE in leukocytes [19–25]. Additionally, double lipoxygenase reactions involving the 5- and 12- or the 5- and 15-lipoxygenases have been demonstrated in leukocytes [24,25]. Our data indicate that mussel gills possess 5- and 12-lipoxygenases, and possibly a 15-lipoxygenase. Thus, it would not be surprising for a variety of dihydroxy acids to be produced. However, we have not attempted to identify these metabolites.

As with the mammalian 5-lipoxygenase, the mussel-gill enzyme is at least partially inhibited by NDGA and ETYA. The enzyme also is preferentially inhibited by AA-861 as is the guinea-pig polymorphonuclear 5-lipoxygenase [16]. Preincubation of mussel gills with AA-861 (10 μM) resulted in the partial inhibition of 5-HETE production, and a concomitant increase in the production of 12- and 15-HETE. The production of eicosanoids is thought to be regulated primarily by the amount of free arachidonic acid in the cell [26]. Thus, inhibition of one pathway, in this case the 5-lipoxygenase by AA-861, will result in an increase in the flux through other pathways (i.e., the 12- and 15-lipoxygenases). The mussel gill 5-lipoxygenase is unlike most of its mammalian counterparts in that simply adding arachidonic acid to the gill results in the production of 5-HETE, whereas in polymorphonuclear leukocytes an added stimulus, such as the calcium ionophore A23187, is required. It also is not clear whether the mussel gills possess leukotriene A_4 synthase activity as does the polymorphonuclear leukocyte. The major product in mussel gill is 5-HETE, and we have not attempted to ascertain whether any of the more polar metabolites exhibit a triene UV spectrum. Thus, there is no evidence for the production of leukotriene A_4 or dihydroxyeicosatetraenoic acids in this tissue.

The intact isolated gill did not produce prostaglandins or other cyclooxygenase products from exogenous arachidonic acid. This observation differs from the results with the crude gill homogenate in which compounds that co-migrate in several different TLC systems with both PGE_2 and $PGF_{2\alpha}$ were noted [6]. The production of these putative prostaglandins was inhibited by meclofenamate, indicating that they are cyclooxygenase products. It is likely that the discrepancy can be attributed to differences in assay techniques. The enzymes for the metabolism of arachidonic acid can be shown in crude homogenates of whole gills, even though the enzymes are apparently easily denatured. Thus, we often encountered an unexplained loss of enzyme activity even though the homogenization and centrifugation were done at 4 °C. It is quite possible that the present results reflect the metabolism of arachidonic acid only by outer epithelial cells of the gill filament. PGE_2 may be produced by the epithelial cells directly involved with ion transport or by neighboring cells. Our morphological studies indicate the mitochondria-rich cells are located mostly in the central water chamber of mussel gills (Dietz, unpublished). Therefore, the exogenous [^{3}H] arachidonate may not have penetrated to these 'transport' cells in a high enough concentration for the metabolites to be detected in the bathing medium. Finally, isolated mussel gills have been shown to have stimulated sodium transport rates [27]. If PGE_2 is involved in the inhibition of sodium uptake as suggested [6,7], then PGE_2 would be expected to be produced in smaller amounts in isolated gills.

Prostaglandins and other cyclooxygenase products have been reported to occur in tissues from many species of aquatic vertebrates and invertebrates [28]. In comparison, there have been relatively few reports on the occurrence of lipoxygenase products in aquatic organisms [2]. This is due partly to the fact that most investigators have relied upon RIA to identify arachidonic acid metabolites, and only recently have RIA procedures for some of the lipoxygenase products become available. We have measured immunoreactive material reacting with leukotriene C_4 antibody but have not attempted further identification. Srivastava and Mustafa [29] investigated the metabolism of labeled arachidonic acid by tissues from the marine mussel, *Mytilus edulis*. In agreement with the present results they found that gill extracts produced at least five metabolites and that most of the metabolites separated by thin-layer chromatography had R_F values intermediate between the prostaglandins and arachidonic acid. Holland et al. [30] identified the hatching factor of the barnacle *Balanus balanoides* as 10,11,12-trihydroxy-5,8,14,17-eicosatetraenoic acid. The authors hypothesized that this compound is produced via 12-lipoxidation of eicosapentaenoic acid ($20:5(n-3)$). In barnacles, as in most marine organisms, there is a large amount of $n-3$ fatty acids but relatively low amounts

of $n-6$ fatty acids. Gills from *L. subrostrata* exhibit the opposite pattern which is characteristic of freshwater mussels [6,8]. We did not observe any radioactive peaks eluting at times which would be expected for trihydroxyeicosatetraenoic acid (i.e., between the prostaglandins and diHETE).

In summary, we have shown that gills from the freshwater mussel, *L. subrostrata*, possess an active 5-lipoxygenase. 5-HETE has been identified as the most abundant metabolite of this pathway. Immunochromatography also indicates that 12-HETE is produced but at about 50% of the level of 5-HETE. A compound co-chromatographing with 15-HETE in our HPLC system was often noted. Thus, it appears that mussel gills possess several lipoxygenase enzymes. The physiological roles of these eicosanoids are currently unknown. The possible involvement of 5- and 12-HETE in ionic regulation is under investigation.

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