Eggs of the Sea Urchin, Strongylocentrotus purpuratus, Contain a Prominent (11R) and (12R) Lipoxygenase Activity*

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Recent work has shown that oocytes of the starfish synthesize (8R)-hydroxyeicosatetraenoic acid and that this eicosanoid has a potent and highly specific action in induction of oocyte maturation. These striking results prompted us to examine the lipoxygenase activity of eggs of the sea urchin Strongylocentrotus purpuratus. Four hydroxyeicosanoids were formed in homogenates of sea urchin eggs; their structures and stereochemistry were characterized by high pressure liquid chromatography, UV spectroscopy, and gas chromatography-mass spectrometry. The compounds were identified as (11R)-hydroxy-5,8,12,14-ZZEZ-eicosatetraenoic acid and (12R)-hydroxy-5,8,10,14-ZZEZ-eicosatetraenoic acid (from arachidonic acid) and the corresponding (11R)- and (12R)-hydroxy analogs of eicosapentaenoic acid. The formation of these egg products was not blocked by a cyclooxygenase inhibitor, indomethacin (10 μM), and their precise structures are consistent with their formation by a lipoxygenase reaction. Eicosapentaenoic acids with a prochiral tritium label in the 10-D or 10-L position were used to investigate the mechanism of biosynthesis. The formation of (12R)-hydroxyeicosapentaenoic acid proceeded with the stereoselective abstraction of the 10-D hydrogen from the substrate. This reaction was shown to be opposite to the (12S) oxygenation catalyzed by porcine leukocyte 12-lipoxygenase. These results with S. purpuratus eggs constitute the first demonstration of (11R)- or (12R)-lipoxygenase activity in any cell type or tissue.
In artificial seawater, were routinely concentrated by centrifugation for 10 min at 1000 rpm in a Sorvall RC-3 refrigerated centrifuge. The sedimented eggs (1 ml) were homogenized on ice with a Kontes glass homogenizer in 10 volumes of 20 mM Tris, pH 7.5; added substrates and/or inhibitors were included in the homogenization buffer as appropriate. Examination of the homogenates revealed that the eggs would immediately lyse within minutes after suspension in this hypotonic buffer and that the integrity of the eggs is disrupted completely by a few passes (≤10) with the glass homogenizer pestle. Egg lysate incubations (10 ml) were terminated by the addition of 0.5 ml of a sodium formate buffer (10% formic acid adjusted to pH 3.0 with NaOH) and immediate extraction with methanol/chloroform by the Bligh and Dyer procedure (7). After phase separation, the organic phase contained 95% of added radiolabel; the aqueous phase (pH 3.5–4) was discarded.

Incubations with Porcine Leukocyte (12S)-Lipoxygenase—The 100,000 X g supernatant fraction was used as the source of enzyme (8). (12S)-HETE and (12S)-HEPE were prepared by incubation of 2 ml of supernatant with 100 μM arachidonic or eicosapentaenoic acids, respectively. After 5 min at room temperature, the incubations were terminated by acidification with sodium formate buffer and the mixtures applied to C-18 Bond Elut cartridges (Analytichem International, Harbor City, CA) preconditioned with 10 ml of methanol followed by 10 ml of water. After application of sample, columns were washed with 10 ml of water and the fatty acids eluted with 5 ml of methanol. The extracts were vortexed in 0.5 ml of methanol, centrifuged briefly, and then the MeOH mixed with an equal volume of 0.05% aqueous acetic acid and injected on the reversed-phase column. The effluent was monitored with a Hewlett-Packard 1040A diode-array detector recording at 205, 235, and 270 nm. Fractions of 0.5 ml of the eluate were collected.

Chromatography and Mass Spectrometry Analyses—Methyl esters were prepared by treatment of the compound in methanol with an excess of ethereal diazomethane for 5 min at room temperature. For catalytic hydrogenation, about 20 μg of the methyl ester derivative was dissolved in 0.1 ml of absolute ethanol, platinum oxide (1 mg) was added, and hydrogen was bubbled through the suspension for 1 min. Water was then added and the product recovered by immediate extraction with ethyl acetate. Silylation was performed by dissolving the fatty acid methyl esters in 10 μl of pyridine and 15 μl of bis(trimethylsilyl)-trifluoroacetamide. After at least 30 min at room temperature, the pyridine and bis(trimethylsilyl)-trifluoroacetamide were evaporated under nitrogen and the trimethylsilyl ether methyl ester derivatives dissolved in dodecane for GC-MS analysis.

Gas Chromatography-Mass Spectrometry Analyses—Analyses were performed using a Hewlett-Packard 5890A quadrupole instrument equipped with a 6-ft DB-1 capillary column (0.25-mm inner diameter; coating thickness, 0.25 μm) and temperature programmed from 190 to 250 °C at 5 °C/min. Mass spectra were recorded in the electron impact mode with an electron energy of 70 eV.

Steric Analysis by HPLC of Menthoxycarbonyl Derivatives—This analysis was used for 11-hydroxyeicosanoids. The methyl ester (20 μg) was converted to the (−)-menthoxycarbonyl derivative by treatment with 60 μl of (−)-menthylchloroformate (5% v/v solution in dry toluene) plus 12 μl of pyridine. After 20 min of reaction at room temperature, the solvents were evaporated under nitrogen. The sample was dissolved in dichloromethane, and pyridinium hydrochloride was removed by washing with water. The menthoxycarbonyl methyl ester derivative was isolated by RP-HPLC (Altex Ultrispheric ODS 5 S, 250 × 4 mm; solvent, methanol/water, 100:3 (v/v), elution volume, ~15 ml). The menthoxycarbonyl diastereomers were resolved by SP-HPLC using an Altichrom 5-μm silica column (250 × 4.6 mm) with a solvent system of n-hexane:2-propanol (100:0.1, v/v), with elution at 1 ml/min and UV detection at 235 nm. The absolute configuration of the two peaks corresponding to the R and S enantiomers of racemic HETE standards was established using the method of Hamberg (9) as previously described (10). Briefly, the two peaks from a racemic standard were collected separately and each subject to oxidative ozonolysis, followed by re-esterification and gas chromatographic comparison of the resulting derivative to menthoxycarbonyl methyl ester derivatives prepared from authentic R and S malic acids.

Steric Analysis Using a Chiral Phase HPLC Column—The methyl esters of 12-HETE and 12-HEPE enantiomers were resolved without further derivatization by analysis on a Biorad dinitrobenzoylphenyl glycine chiral phase HPLC column (250 × 4.6 mm) using a solvent system of n-hexane:2-propanol (100:0.5, v/v) and a flow rate of 0.5 ml/min. The R and S assignments were established using a HETE standard of known chirality, e.g. use of platelet or leukocyte-derived (12S)-HETE.

RESULTS

Identification of Products—Four products of polyunsaturated fatty acid oxygenation were formed from endogenous substrates during the incubation of homogenized eggs at 22–24 °C. On RP-HPLC analysis these appeared as two pairs of peaks with strong absorbance at 235 nm, and they were designated as Compounds I, II, III, and IV in order of elution. When incubations were carried out in the presence of 100 μM [14C]eicosapentaenoic acid, Compounds I and II were radio-labeled and the amounts were increased 3-5-fold. Incubation with 100 μM [14C]arachidonic acid resulted in incorporation of radioactivity in Compounds III and IV and an increase in their relative abundance (Fig. 1). The preponderance of the two products within each pair was within a 4:1 to 1:2 ratio of peak heights in 10 separate experiments. Typically, about 50% of the radiolabel was recovered in these products; the remainder being unmetabolized substrate and polar lipids. No products were synthesized in the presence of calcium-free artificial sea water containing 2 mM EGTA. The enzymatic activity was calcium dependent, and it was not stimulated by addition of 1 mM ATP. The cyclooxygenase inhibitor indo-
methacin (10 μM) did not affect the production of Compounds I-IV.

The UV spectrum of each product exhibited the characteristic chromophore of a conjugated diene, \( \lambda_{\text{max}} = 237 \text{ nm} \) for Compounds I, II, and IV and 235 nm for Compound III. Comparison of the UV spectra of authentic standards and further analyses on RP-HPLC and SP-HPLC indicated the probable structures of the four products as 11-HEPE, 12-HEPE, 11-HETE, and 12-HETE. Each product and corresponding standard were analyzed by GC-MS as the methyl ester (Me3Si ether derivative), before and after catalytic hydrogenation of the double bonds. Positive ion electron impact spectra of the Me3Si derivative of Compounds I and II are shown in Fig. 2. The spectra were indistinguishable from the corresponding spectra of 11-hydroxy-5,8,12,14,17-eicosapentaenoate (11-HEPE) and 12-hydroxy-5,8,10,14,17-eicosapentaenoate (12-HEPE), respectively. In each case the high mass ions are found with the expected shift of 2 atomic mass units relative to the well characterized spectra of their eicosatetraenoate analogs (11). The position of the hydroxyl group was confirmed by analysis of the Me3Si derivatives of the hydroxylated compounds; the mass spectra of the saturated products were essentially identical to the published spectra of 11-hydroxy- and 12-hydroxyeicosanoates (11). The mass spectrum of the Me3Si derivative of the arachidonate product, Compound III, exhibited significant ions at \( m/z \) 406 (M+), 391 (M-15), 316 (M-90), and 225 (loss of C15-C16) and was indistinguishable from the spectrum of authentic 11-HETE (Me3Si). The Me3Si derivative of Compound IV exhibited significant ions at \( m/z \) 406 (M+), 391 (M-15), 375 (M-31), 316 (M-90), and 295 (loss of C13-C14) and was identified as 12-HETE by comparison to the authentic standard.

The position and configuration of the double bonds in Compounds I-IV appear to correspond to a cis/trans-conjugated diene unit allylic to the hydroxyl group, with the other bonds in their original positions and retaining the cis configuration. This is the most likely result on biochemical grounds, and it is supported by the UV and HPLC data. (i) The UV spectra are identical to the authentic standards prepared by controlled autoxidation (5) in agreement with the cis/trans nature of the conjugated system; (ii) products and standards co-chromatograph on RP-HPLC, SP-HPLC, gas-liquid chromatography, dinitrobenzoylphenyl glycine chiral phase HPLC (vide infra), and, significantly, on a silver-loaded cation exchange HPLC column used with normal phase solvent systems (12). Silver-loaded normal phase systems are especially sensitive to the cis or trans configuration of double bonds (13, 14), and, therefore, the co-chromatography supports the result one might anticipate, namely that the bonds remote from the site of oxygenation remain unaffected by the metabolic transformation.

Steric Analyses—The chirality of the 11-hydroxy products from the eggs was analyzed by SP-HPLC of the methyl ester (-)-menthoxycarbonyl derivatives (10). The racemic 11-hydroxy standards resolved as two menthoxycarbonyl diastereomers with elution in the order (11R) and (11S), as previously established by oxidative ozonolysis of the individual compounds and assignment of stereochirality by comparison with derivatives of authentic R and S malic acid (10). The methyl ester menthoxycarbonyl derivative of Compound

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![Fig. 2. Mass spectra of Me3Si derivatives of Compounds I (top) and II.](image)

![Fig. 3. Steric analysis of 11-HETE from sea urchin eggs. Diastereomers of 11-HETE methyl ester menthoxycarbonyl derivatives were separated on an Alltech 5-μm silica column (250 x 4.6 mm) eluted at 1 mL/min with hexane/isopropyl alcohol, 100:0.1 (v/v) with UV detection at 235 nm. Elution volumes: (11R) at 16.6 mL; (11S) at 17.6 mL. The natural product co-chromatographed with the (11R) standard (with a 3% abundance of (11S) enantiomer).](image)

III (11-HETE) migrated as a single symmetrical peak which co-chromatographed with the earlier eluting (11R) diastereomer of the racemate (Fig. 3). Similarly, the (-)-menthoxycarbonyl derivative of the egg 11-HETE methyl ester co-chromatographed with the first of the two diastereomeric menthoxycarbonyl derivatives prepared from racemic 11-HETE methyl ester (not shown).

A dinitrobenzoylphenyl glycine chiral phase HPLC column was used for the assignment of chirality of the 12-hydroxy products. Elution volumes were compared with those of racemic standards and with enantiomers of the (12S) configuration prepared using the well characterized (12S)-lipoygen-
ase of porcine leukocytes (15). The results established that in each case the egg products were of the (12R) configuration (Fig. 4).

Experiments with [10-3H]Eicosapentaenoic Acids—In the course of (12S)-HETE synthesis in platelets and porcine leukocytes, the (12S)-lipoxygenase catalyzes stereospecific removal of the 10-L-hydrogen from the substrate (15, 16). Because the 12-HETE and 12-HEPE formed by the eggs were of the R configuration, it was of great interest to examine the stereochemistry of hydrogen abstraction in this reaction. Separate incubations of egg homogenates were conducted with [10-d-3H]eicosapentaenoic acid and [10-L-3H]eicosapentaenoic acid (one experiment with each enantiomer); [3-14C]eicosapentaenoic acid served as internal standard for measurement of tritium retention. The HEPE products were purified by RP- and SP-HPLC and then the 3H/14C ratio determined by liquid scintillation counting.

In the 10-d-3H experiment, the 12-HEPE retained 7% of the tritium in the original substrate. In contrast, there was 82% retention of tritium in the 12-HEPE from the incubation with [10-L-3H]eicosapentaenoic acid. In both experiments the specific activity of the 11-HEPE product was unchanged (100% and 106% of the original substrate, respectively).

For direct comparison, the [10-L-3H]eicosapentaenoic acid was reacted with the (12S)-lipoxygenase of porcine leukocytes. As found before using arachidonic acid (15), the leukocyte lipoxygenase catalyzed (12S)-oxygenation with removal of the 10-L-hydrogen (only 7% retention of tritium in the (12S)-HEPE product). Thus, the chiralities of hydrogen abstraction and stereospecific oxygenation are mirrored in the (12S)-HEPE product. Therefore, the chiralities of hydrogen abstraction and stereospecific oxygenation are mirrored in the (12S)-HEPE product. Thus, the chiralities of hydrogen abstraction and stereospecific oxygenation are mirrored in the (12S)-HEPE product. Therefore, the chiralities of hydrogen abstraction and stereospecific oxygenation are mirrored in the (12S)-HEPE product. Thus, the chiralities of hydrogen abstraction and stereospecific oxygenation are mirrored in the (12S)-HEPE product.

An additional characteristic of lipoxygenases is that stereoselective removal of a prochiral tritium atom is associated with a primary isotope effect measured in the unreacted substrate (16–19). However, in the incubations of sea urchin eggs the measured changes were too slight to be meaningful. Less than 50% of the eicosapentaenoic acids were metabolized in these particular incubations; the incomplete reaction, together with the competing 11-lipoxygenase (which accounted for the larger portion of the total lipoxygenase activity), resulted in relatively trivial changes in the specific activity of the [10-d-3H]eicosapentaenoic acid during the incubation (134% of the original value, compared with 120% for the [10-L-3H]). Interestingly, the incomplete reaction was associated with a marked secondary isotope effect in the 10-L-3H experiment; the (12R)-HEPE product showed only 82% (not +90%) tritium retention, in line with similar findings with platelet (12S)-lipoxygenase and the enantiomeric 10-n-labeled substrate (20).

**DISCUSSION**

In this study we have established that the eggs of the sea urchin, S. purpuratus, contain a prominent (11R)- and (12R)-lipoxygenase activity. In the absence of added substrates, products were formed from endogenous arachidonic and eicosapentaenoic acids, implying that these fatty acids may be natural substrates of the enzyme(s). It has been noted before that eggs of S. purpuratus undergo a burst of oxygenase activity with formation of an "HETE-like" product upon fertilization (4). However, this property is not shared by the eggs of all species of sea urchin (3). We confirmed that eggs of the sea urchin Arbacia punctulata contain no detectable lipoxygenase activity. Nevertheless, the biosynthesis and biological activity of (8R)-HETE in starfish oocytes sets an important precedent for a specific function for monohydroxyeicosanoids (2). Identification of the lipoxygenase products of S. purpuratus eggs will enable detailed investigation of the role of these oxidized unsaturated fatty acids in fertilization and/or maturation processes.

11-Hydroxyeicosanoids have been reported before both as lipoxygenase (21) and as cyclooxygenase products (22–26). When 11,14-eicosadienoic acid is used as a cyclooxygenase substrate, formation of an endoperoxide is not possible and the predominant product is the (11R)-hydroxy-12,14-eicosa- dienoic acid (22). This evidence was used in support of other data which established that prostaglandin biosynthesis is initiated by (11R) oxygenation of the substrate (23). Interestingly, the chirality of 11-HETE formed as a by-product of prostaglandin synthesis has not been reported. Our colleague Dr. Douglass Taber questioned whether the monohydroxy product was eliminated because it is of the "wrong" (i.e. (11S)) configuration for cyclization to an endoperoxide? To test this hypothesis we determined the chirality of the 11-hydroxy-8,12,14-eicosatrienoic acid which is formed as a by-product of prostaglandin E synthesis in ram seminal vesicle microsomes. Our results (Fig. 5) showed that this by-product was the (11R)-hydroxyeicosanentiomer, thus disproving this intriguing idea. Our experiments with sea urchin eggs are the first study showing the chirality of 11-hydroxy products of a lipoxygenase enzyme.

It has generally been assumed that the stereoselectivity of (12S)-HETE biosynthesis in platelets and leukocytes would prevail in the synthesis of 12-HETE by other cells and tissues. Recently it was shown that the 12-HETE in human proriotic scales is of the R configuration; it is not yet established whether a lipoxygenase, P-450, or other enzyme is involved in the biosynthesis (27). Mouse epidermis is known to form 12-HETE (28), and we have examined the chirality of the
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Fig. 5. The (11R) configuration of the cyclooxygenase product 11-hydroxy-8,12,14-eicosaatrienoic acid. The compound was isolated as a by-product of prostaglandin E, synthesis from [3-14C]eicosaatrienoic acid in sheep seminal vesicle microsomes. The identity was confirmed by GC-MS of the Me,Si derivative. Steric analysis was carried out on the methyl ester derivative exactly as described in the legend to Fig. 4.

Fig. 6. The (12S) configuration of mouse skin 12-HETE. The 12-HETE was isolated as the major radiolabeled product from incubation of the 10,000 g supernatant fraction of mouse (strain C5BL/6) epidermal homogenate with 6.5 nM [1-14C]arachidonic acid in 20 mM Tris, pH 7.3 (37°C). The identity of the product was confirmed by GC-MS of the Me,Si derivative. Steric analysis was carried out on the methyl ester derivative exactly as described in the legend to Fig. 4.

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