Investigation of the Selectivity of Hydrogen Abstraction in the Nonenzymatic Formation of Hydroxyeicosatetraenoic Acids and Leukotrienes by Autoxidation*

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Alan R. Brash†, Arthur T. Porter, and Richard L. Maas‡
From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

The biosynthetic conversions of arachidonic acid to hydroperoxyeicosatetraenoic acids (HPETEs) and the further conversion of leukotriene epoxides are accompanied by stereoselective hydrogen abstraction from the reaction substrate. Furthermore, this hydrogen removal has always been found to occur in fixed stereochemical relationship to carbon-oxygen chiral center(s) in the substrate or product. We have used stereospecifically labeled 10-3H-substrates with 14C internal standards to investigate whether the same relationships bear in HPETE and leukotriene formation during autoxidation. After autoxidation of labeled arachidonate, both the 8(R)- and 8(S)-HPETE enantiomers (resolved as diastereomer derivatives) and the 12(RS)-HPETE were observed to retain 41–47% 3H relative to the starting material. In autoxidative formation of leukotrienes from labeled 15(S)-HPETE the four main leukotrienes, including two derived from 14,15-leukotriene A4, hydrolysis, were observed to have retained an average of 45% 3H. Primary and secondary isotope effects were found to accompany these reactions. The results prove that stereorandom hydrogen abstraction occurs in autoxidation and that the hydrogen loss bears no stereochemical relationship to chiral oxygen center(s) in the HPETE product, (8(R) or 8(S)), or the 15(S)-hydroperoxy substrate. We conclude that the chiral features of the biosynthetic reactions are a reflection of enzymatic control of stereochemistry. Nonetheless, the findings of primary and secondary isotope effects in autoxidation which are similar to those observed in the analogous biosynthetic reactions suggests that, except for stereochemical control, the autooxidative and enzymatic reactions may be mechanistically similar.

Autoxidation of polyunsaturated lipids was definitively characterized as a free radical reaction by kinetic and thermodynamic studies in the 1940s and early 1950s (reviewed in Ref. 1). When lipoxigenase enzymes were identified, their reaction mechanism was likened to the process of autoxidation, although important differences were also recognized (2). Typically, a lipoxigenase will oxygenate in only one position and in one stereospecific configuration on a fatty acid sub-

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† To whom correspondence should be addressed.

‡ Present address: Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115.

1 The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; SP- and RP-HPLC, straight-phase and reversed-phase high performance liquid chromatography; HETE, hydroxyeicosatetraenoic acid; ME, methyl ester; MC, menthoxycarbonyl; GC-MS, gas chromatography-mass spectrometry; GLC, gasliquid chromatography.

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products would retain tritium (Scheme 1, right side). If there was no fixed relationship then each of the four products would be radiolabeled, nominally at half the original activity, assuming a 50:50 chance of hydrogen or tritium abstraction. The latter alternative, in which there is no fixed relationship (Scheme 1, left side), would be compatible either with a concerted reaction without fixed chirality or with a distinct step of hydrogen abstraction followed by racemic addition of oxygen.

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Antarafacial oxygenation and hydrogen abstraction

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spectrum of each product, they were individually re-purified by SP-HPLC (see "Results" and Fig. 2) and counted.

**Controls for Hydroperoxide Epimerization during Autoxidation**

The degree of epimerization of the 15(S)-hydroxy group of 15(S)-HPETE methyl ester (50 µg) was studied in mixtures containing methyl dihomo-γ-linolenate (300 µg) as the major component under autoxidation conditions. This allowed subsequent isolation of the remaining 15(S)-HPETE methyl ester free from 20,3,6 hydroperoxide products. Autoxidation was allowed to proceed in a dry film under oxygen (57°C, α-tocopherol, 10% w/w) until UV analysis indicated 10-20% oxygenation of 20,3,6. The remaining 15 HPETE methyl ester was isolated by RP-HPLC, reduced with triphenylphosphine, and methylated. This provided the 15(S)-HPETE methyl ester (system SP-2). Following conversion to the MC derivative, oxidative ozonolysis, and re-esterification, the configuration of the original 15-carbon hydroxyl group was determined by GLC analysis of the MC derivative (Table IA).

**Autoxidation of methyl [3-14C, 10-d3-3H]arachidonate**

An autoxidation of methyl [3-14C, 10-d3-3H]arachidonate was conducted in similar fashion to verify the above results. The results (Table IB) completely support the findings from the pro-S' hydroperoxides. The 9- and 11-HETEs contained slightly less than 50% of the original tritium, owing to the secondary isotope effect centered on the 10-carbon. The 9(S)-HETE MeMC diastereomers were very highly enriched in tritium (Table IA). Notably, the methyl arachidonate recovered from the reaction was even further tritium enriched (Table IA). These results are compatible with a primary isotope effect which retards removal of the 10-δ-carbon tritium from the arachidonate in the formation of 8- and 12-HETEs. Consequently, the pool of unreacted arachidonate becomes tritium-enriched. The enrichments found in the 9- and 11-HETEs reflect the average 3H content of the arachidonate (~104.2%). Of additional interest was the finding that the 5-HETE MeMC diastereomers were very highly enriched in tritium (Table IA). This is due to a primary isotope effect involving secondary oxygens tentations centered on the 10-carbon, given 5,12-dihydroxy products.

**Autoxidative Formation of HPETEs and Leukotrienes**

Autoxidative Formation of HPETEs—Tritium loss from the 10-carbon of methyl [10-Ls-3H]arachidonate (admixed with methyl [3-14C]arachidonate as internal standard) was examined after autoxidative conversion to 8(R,S)-HPETE and other HPETE products. α-Tocopherol, included in the autoxidation mixture in relatively large amounts, had little effect on the course of the initial oxygenation, but had a marked salutatory effect on minimizing the decomposition of the resulting hydroperoxides (18). The racemic HPETE products were reduced to HETEs and purified. This was followed in some cases by preparation of the MC derivative, to allow chromatographic resolution of the R- and S-HETE enantiomers. The MC diastereomers of 8(R)- and 8(S)-HETE, followed by resolution by SP-HPLC, Fig. 1, were each found to contain 42% of the original tritium content of the methyl arachidonate. This result is compatible with stereorandom tritium loss from the 10-carbon in the formation of the 8(R)- and 8(S)-hydroperoxides. The tritium retention is less than 50% due to a secondary isotope effect which slightly slows removal of the 10-d3-protium when 3H occupies the 10-Ls position on carbon 10. Results from the corresponding experiment performed with [10-d3-3H]arachidonate support this conclusion (see below).

The 9(R,S)-HETE MeMC diastereomers contained 10S.9% of the original tritium content of the methyl arachidonate. Virtually the same tritium enrichment was found in 11-HETE, as analyzed by the MeMC diastereomers (Table IA). Notably, the methyl arachidonate recovered from the reaction was even further tritium enriched (Table IA). These results are compatible with a primary isotope effect which retards removal of the 10-Ls-tritium atom from the arachidonate in the formation of 8- and 12-HETEs. Consequently, the pool of unreacted arachidonate becomes tritium-enriched. The enrichments found in the 9- and 11-HETEs reflect the average 3H content of the arachidonate (~104.2%). Of additional interest was the finding that the 5-HETE MeMC diastereomers were very highly enriched in tritium (Table IA). This is due to a primary isotope effect involving secondary oxygens tentations centered on the 10-carbon, given 5,12-dihydroxy products.

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We also analyzed the tritium retentions from an additional autoxidation experiment conducted with [10-Ls-3H]arachidonic acid in the absence of α-tocopherol. The yields of the mono-H(P)ETEs were considerably lower in this case because the hydroperoxides were free to undergo side reactions. In spite of this constraint on precise 3H/14C determinations, the results were in good agreement with the α-tocopherol autoxidations. As expected, the 11-HETE MeMC diastereomers retained the 10-Ls-3H label while there was stereorandom 3H loss in the formation of the 8-HETE MeMC diastereomers (Table IC).

**Hemoglobin-catalyzed Autoxidation of 15(S)-HPETE to Leukotrienes**—Four 8,15-dihydroxy products were analyzed...
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Fig. 1. SP-HPLC resolution of the methyl ester menthoxy carbonyl derivatives of 8(R), 8(S), and 9(R,S)-HETEs from an autoxidation of [3-¹³C, 10-³H]arachidonate methyl ester. Chromatographic conditions: Alltech 5-µm silica column 25 × 0.46 cm, solvent 0.1% isopropanol in hexane, flow rate 0.5 ml/min, UV detection at 235 nm (left). Fractions (0.25 ml) were counted 100–250 min each.

### Table I
Tritium retentions in autoxidation of 10-³H-labeled arachidonate to HETEs (with [³14C]arachidonate as internal standard)

| Compound | Experiment |
|----------|------------|
|          | A. Autoxidation of methyl [10-³H]arachidonate with 10% (w/w) α-tocopherol | B. Autoxidation of methyl [10-³H]arachidonate with 10% (w/w) α-tocopherol | C. Autoxidation of [10-³H]arachidonic acid |
|          | H/³C ratio | % of original substrate | H/³C ratio | % of original substrate | H/³C ratio | % of original substrate |
| 20.4 (initial) | 2.368 | 100 | 1.972 | 100 | ND | 8(R,S)-HETE ⚫ |
| 20.4 (final) | 2.567 | 108.4 | 2.132 | 108.1 | ND | 8(R,S)-HETE |
| 8(R)-HETE | 1.007 | 42.5 | 0.789 | 40.5 | 0.82 | 8(R,S)-HETE ⚫ |
| 8(S)-HETE | 1.000 | 42.2 | 1.176 | 46.1 | 0.78 | 47.6% of 11(R)-HETE |
| 12(R,S)-HETE | ND | ND | 0.900 | 103.1 | ND | 12(R,S)-HETE |
| 9(R,S)-HETE | 2.508 | 105.9 | 2.034 | 102.6 | 1.64 | 12(R,S)-HETE |
| 11(R)-HETE | 2.432 | 102.7 | 2.023 | 102.6 | 1.64 | 11(R)-HETE |
| 11(S)-HETE | 2.550 | 107.7 | (R,S) | (R,S) | 1.71 | 11(S)-HETE |
| 5(R)-HETE | 3.019 | 127.5 | 2.373 | 120.3 | ND | 5(R)-HETE |
| 5(S)-HETE | 3.013 | 127.2 | (R,S) | (R,S) | ND | 5(S)-HETE |
| 15(R,S)-HETE | ND | ND | 2.380 | 119.7 | ND | 15(R,S)-HETE |

*The original sample was a mixture of 18.2 and 20.4 fatty acids and the exact ³H content of the 20.4 was not determined (see “Experimental Procedures” for details).

Contaminated with an isomer of 9-HETE as judged by the UV trace on SP-HPLC and 30% extra ¹⁴C counts compared to the 8(R)-HETE peak.

The slightly longer retention time of ³H-labeled molecules on SP-HPLC caused slight contamination of the second peak (11(S)) with 11(R)-³H label.

from this reaction, each of which must lose a hydrogen from the 10-carbon of 15(S)-HPETE. Two largely arise from conversion of 15(S)-HPETE to 14,15-LTA₄, followed by hydrolysis of the epoxide to two 8,15-dihydroxy diastereomers racemic at carbon 8. These products contain an all-trans-conjugated triene. The other two are 8,15-dihydroxy diastereomers which contain a trans,cis,trans-conjugated triene. This double bond configuration is that expected from an oxygenation reaction at carbon 8. Although this is the mechanism of formation of these compounds in platelets and leukocytes (9), analysis of incubations of 15(S)-HPETE with hemoglobin has indicated incorporation of ³⁰O from H₂¹⁸O at the 8-carbon as an additional route of formation of these trans,cis,trans-conjugated triene products (16). We have previously prepared these compounds in milligram amounts and characterized their structures by HPLC, UV, GC-MS, NMR, and static analysis of alcohols (9, 12). Several trial experiments were conducted with unlabeled 15(S)-HPETE autoxidized with
hemoglobin, and the structures reported (18) for these for major 8,15-dihydroxy products were confirmed by RP-HPLC, SP-HPLC, UV, GC-MS, and steric analysis comparisons with our standards.

Three hundred micrograms of stereospecifically labeled 15(S)-HPETE methyl ester prepared from [10-14C,10-3H]-arachidonic acid mixed with [3-14C]arachidonic acid were autotoxidized in the presence of hemoglobin. Following ether extraction and purification of the 8,15-dihydroxy compounds by RP-HPLC and SP-HPLC, the 3H/14C ratios indicated in Fig. 2 were obtained. The results from the four 8,15-dihydroxy compounds are in general agreement and in distinct contrast to the corresponding values previously determined for these compounds in platelets and leukocytes. The present results indicate that between 38–55% of the tritium in the 15(S)-HPETE substrate is retained in the products formed by autoxidation, compared with only 1–4% retention of the pro-S-3H label in the biosynthetic reactions (9). Thus, the biosynthesis is stereoselective whereas autoxidation is stereorandom.

The yield of these products was low (~0.2% each), and therefore, the determination of precise 3H/14C ratios was adversely affected by counting background. Nevertheless, there are strong grounds in support of the validity of the results. Close inspection of the 3H and 14C profiles in Fig. 2 reveals that, in all but the third peak, the 14C counts elute very slightly ahead of the 3H peaks. This is caused by the very slightly greater polarity of the 3H-labeled molecules. The same effect is apparent when larger amounts are chromatographed at high resolution on HPLC. As an example, the early elution of the 14C-labeled 8(R)- and 8(S)-HETE MeMC diastereomers is evident in Fig. 1. It can also be seen in Fig. 1 that the 3H and 14C profiles of the 9(R,S)-HETE MeMC diastereomers co-chromatograph. Close scrutiny of the chromatographic behavior of the 11-HETE MeMC diastereomers on SP-HPLC reveals that the 11(R)-diastereomer shows the separation of 3H and 14C whereas the 11(S)-diastereomer does not (not shown). These details provide a powerful argument in support of the validity of the 3H and 14C quantitations obtained with the 8,15-dihydroxy compounds. They refute the possibility that the 3H and 14C peaks are artifacts, despite the very low levels of counts over background. The fact that background readings (chromatographic base-lines) differed on the four runs in Fig. 2 adds an unpredictable element of "noise" which may wholly or partially account for the apparent differences in 3H retention determined for each compound.

The 15(S)-HETE recovered from the hemoglobin autoxidation experiment showed a 12.5% increase in 3H/14C ratio compared with the 15(S)-HPETE starting material. This is the result expected from the primary isotope effect retarding abstraction of 3H from the substrate, as noted above in the autoxidations which formed 8- and 12-HPETEs from arachidonate.

**DISCUSSION**

This study was conducted to determine whether the stereoselective hydrogen abstractions which accompany the biosynthesis of HETEs and leukotrienes are also a part of the autoxidative formation of these compounds. It is evident from our results that the hydrogen abstractions in autoxidation are stereorandom and, furthermore, that there is no chiral relationship between hydrogen removal and newly formed or pre-existing centers of chirality in the products. It is hardly surprising that hydrogen abstraction in autoxidation is stereorandom. The important new result is that the removal of one particular hydroxyl does not influence (and/or is not influenced by) other chiral features of the reaction.

Substrates labeled with one tritium atom on the 10-L,pro-S or 10-D,pro-R positions were used to study the stereoselectivity of HPETE and leukotriene formation during autoxidation. In the first set of experiments, we analyzed derivatives of the 8(R)- and 8(S)-HPETE enantiomers formed from [3-14C,10-L,pro-3H]- and [3-14C,10-D,pro-3H]-arachidonate. In principle, analysis of the 12-HETE enantiomers could also be used to investigate the hydrogen abstractions from the 10-carbon; however, the 12(R)- and 12(S)-HETE enantiomers could not be resolved by HPLC of the MeMC derivatives, thus precluding this alternative. We should also point out that control experiments were conducted with unlabeled substrates to check for possible racemization of hydroxy or hydroperoxy groups after their initial formation. Racemization was undetectable (~<5%, see "Experimental Procedures"). The hemoglobin-catalyzed autoxidation of 15(S)-HPETE was used to study the formation of 14,15-LTA, and 8(R),15(S)-

![Figure 2: Four superimposed chromatograms showing SP-HPLC resolution of four 8,15-dihydroxy products of autoxidation of stereospecifically labeled 15(S)-HPETE.](image)
and 8(S)15(S)-dihydroperoxy-5,9,11,13-Z,-E,-E,-eicosatetraenates. The latter pair of diastereomers were analyzed as their dihydroxy analogs, the reduction being effected during the reaction with hemoglobin (16). The presence of 14,15-LTA₄ was not directly demonstrated in our experiments. The transient presence of 14,15-LTA₄ was inferred from detection of the 8(R),15(S)- and 8(S),15(S)-dihydroxy-5,9,11,13-Z,-E,-E,-eicosatetraenates. This pair of diastereomers are major products of 14,15-LTA₄ hydrolysis (12), and the work of Sok and colleagues (16) has indicated their major route of synthesis in hemoglobin-catalyzed autoxidation of 15(S)-HPETE is via hydrolysis of a 14,15-LTA₄ epoxide. Recently, the formation of LT₄ during hemoglobin-catalyzed autoxidation of 5-HPETE has been directly demonstrated (19). In the chemical synthesis of leukotriene epoxides from 15(S)-HPETE (20) the 10Z-isomer of 14,15-LTA₄ is formed in approximately equimolar amounts to 14,15-LTA₄ itself (21). The two epoxides can be distinguished in a number of ways, including by analysis of their respective hydrolysis products by RP-HPLC (9). The 10Z-isomer gives rise to the same major 8,15-dihydroxy products, but different 14,15-dihydroxy hydrolysis products (9). The latter were not present on our RP-HPLC chromatograms of hemoglobin-catalyzed autoxidation, whereas the characteristic products of 14,15-LTA₄ hydrolysis were present. From this evidence we conclude that a leukotriene epoxide was almost certainly formed in our experiments and that the evidence is compatible with the epoxide being 14,15(S)-LTA₄.

Experiments using substrates stereospecifically labeled with tritium can provide more information than simply which if any hydrogen is subject to selective abstraction. It can also be inferred whether the critical hydrogen abstraction is rate-limiting and whether the hydrogen removal occurs before or after an irreversible point in the overall reaction sequence. These deductions can be made from examination of isotope effects. The slightly stronger carbon-tritium bond may promote the retention of tritium in a reaction product or it may retard the rate of reaction of the tritium-labeled molecules. The selective retention of tritium in the product signifies that the step of hydrogen abstraction occurs after the activation energy of reaction is achieved; hydrogen is removed as reaction proceeds to completion, and the relatively strong carbon-tritium bond favors ³H retention in the product. We observed this result in the so-called biomimetic chemical synthesis of leukotriene epoxides from their hydroperoxy precursors, as described in the accompanying report (14). The alternative is when hydrogen abstraction precedes or coincides with the first irreversible step in the reaction sequence. The higher activation energy required for tritium abstraction then slows the rate of reaction of ³H-labeled molecules and results in a gradual tritium enrichment of the unconverted substrate. This primary isotope effect is observed in the biosynthesis of HPETEs and leukotrienes and, as shown in this report, in their synthesis by autoxidation. This point has also been established recently by Hamberg (22).

There is also an isotope effect which is manifest in the specific activity of the product. When tritium labels the position geminal to the hydrogen targeted for abstraction (e.g. 10-N₃-tritium, 10-N₃-proton removed), a slight slowing of the reaction rate occurs, and this is measurable as an apparent deficiency of tritium in the product. This secondary isotope effect was previously observed by us in the biosynthesis of lipoxygenase products and leukotrienes (6, 9), and in the present study it was detected as a feature of autoxidation. It was evidenced by a tritium retention of only 42% (and not 50%) in the autoxidation of methyl arachidonate to 8(R)- and 8(S)-HPETEs. This observation supports the conclusion that hydrogen abstraction occurred before or during an irreversible point in the reaction sequence. If hydrogen abstraction occurred later in the reaction sequence, then the isotope effect would tend to enhance the tritium content of the products.

A summary of our findings on the tritium retentions found in the enzymatic and nonenzymatic formation of HPETEs and leukotrienes is given in Table II. The biosynthetic reactions show high stereoselectivity of hydrogen abstraction, whereas the autoxidations do not. Thus stereospecific hydrogen removal is not a necessary chemical feature of these reactions. Indeed, the finding of stereoselectivity in the biological synthesis can now be taken as strong circumstantial evidence for the enzymatic formation of HPETEs and leukotrienes in platelets and leukocytes. Be that as it may, the results do not refute the possibility of a fundamental similarity in reaction mechanisms. In this regard, it is of great interest that both qualitatively and quantitatively similarities in the isotope effects are similar in the lipoxygenase reactions, in leukotriene biosynthesis, and in autoxidation.

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