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Substrates and Products of Lipoxygenase Catalysis

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INTRODUCTION

Lipoxygenase (EC 1.13.11.12) biochemistry now spans a period of nearly 60 years. The nature of its activity and its relation to unsaturated lipids in soybeans were first recognized in 1932 by André and Hou (ref. 1). Following its discovery, the mode of action was primarily described in terms of its potency to oxidize, *i.e.* to bleach, carotenoids in flours for the baking industry in order to yield a whiter crumb. In the same decade prostaglandins and their pharmacological activities were discovered (ref. 2, and references therein) and, in addition, "a slow-reacting substance of anaphylaxis" (SRS-A), later to become known as a mixture of leukotrienes (ref. 3) was described. The connections between prostaglandin or leukotriene biosyntheses and certain unsaturated fatty acids were first described in 1964 and 1979, respectively (refs. 4-6). It appeared that prostaglandin biosynthesis and lipoxygenase catalysis have several aspects in common, *i.e.* both are dioxygenases acting upon unsaturated fatty acids and both produce compounds that have profound physiological effects.

For a long time, animal tissues were thought to be devoid of any true lipoxygenase activity (see for example: ref. 7). However, closer inspection of the product profiles found upon incubation of mammalian blood platelets with icosapolyenoic acids revealed that lipoxygenase activity occurred alongside cyclo-oxygenase activity (refs. 8-9). Lipoxygenases have since been found in numerous mammalian cell-types, and their key roles in leukotriene biosynthesis were established. Leukotriene research gained widespread interest because of the possible involvement of leukotrienes in inflammation and hypersensitivity reactions. In addition to leukotriene biosynthesis, lipoxygenases were also shown to be involved in the formation from arachidonic acid of another group of physiologically active compounds, the lipoxins (ref. 10). For reviews, covering various aspects of lipoxygenase research the reader is referred to refs. 11-39. The purpose of this chapter is to discuss recent advances in lipoxygenase-related biochemistry, with particular emphasis on the use of novel substrates, substrate analogues and product formation.

SUBSTRATES AND PRODUCTS

Dioxygenation

Free fatty acids, containing one or more 1Z,4Z-pentadiene moieties are excellent substrates for lipoxygenases. If optimal *in vitro* conditions are chosen, K_m-values of 10 µM or lower can be found and k_{cat}-values of up to 309 s^{-1} have been reported (refs. 40, 41). These parameters apply to the formation of *EZ*-conjugated monohydroperoxides from 5Z,8Z,11Z,14Z-icosatetraenoic acid (ETE, arachidonic acid, formerly eicosatetraenoic acid, ref. 40) and linoleic acid (OD, 9Z,12Z-octadecadienoic acid, ref. 41). The process of dioxygenation as catalysed by lipoxygenases has several characteristic features: *i.* one of the pro-chiral H-atoms is stereospecifically removed from the central methylene-group of a pentadiene group in the substrate, *ii.* oxygen attachment and hydrogen abstraction occur antarafacially with respect to a plane containing the five C-atoms of the original pentadiene system, *iii.* *EZ*-conjugation of the double bonds of the pentadiene system leading to a strong UV-absorption.

In Fig. 1, linoleic acid has been taken as the substrate to illustrate these features. These and other stereochemical aspects of lipoxygenase reactions have

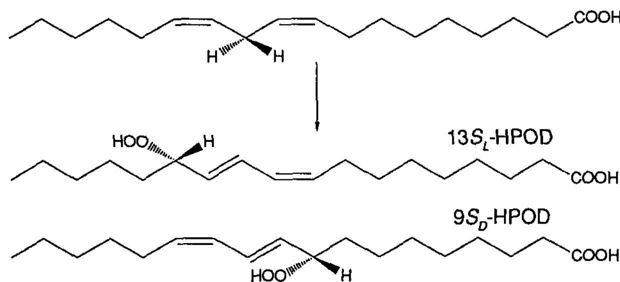


Fig.1. Lipoxygenase-catalysed dioxygenation of linoleic acid. Regio- and stereospecificities depend on the source of the enzyme and on the incubation conditions. At pH 9.0, with soybean lipoxygenase, 13*S*-HPOD is the major product [G.A. Veldink *et al.*, Biochim. Biophys. Acta 202, 198-199(1970)], whereas, at pH 6.5, corn germ lipoxygenase forms predominantly 9*S*-HPOD [H.W. Gardner, J. Lipid Res. 11, 311-321(1970)].

recently been reviewed by Kühn *et al.* (ref. 32).

Substrates containing additional *Z* double bonds in specific positions may undergo another lipoxygenase-catalysed dioxygenation reaction. Depending on the structure of the primary dioxygenation product, leukotriene-like compounds can then be formed (Fig. 2). Such 'multiple dioxygenation' of substrates has been reported for soybean lipoxygenase (refs. 40, 42-44) and for mammalian enzymes (refs. 45-47). There are two distinct modes of enzymic double dioxygenation of ETE: *i.* oxygen insertion occurs at the C-atom which is in β-position to the one from which hydrogen is abstracted. Concomitantly, conjugation of two double bonds takes place (Fig. 2A), and the resulting products have characteristic UV-spectra with a maximum at 243 nm (ref. 40, 48); *ii.* if conjugation of more than two double bonds is possible, oxygen attaches at a δ-position from the C-atom from which hydrogen was initially abstracted, thus leading to vicinal dihydroperoxides (Fig. 2B).

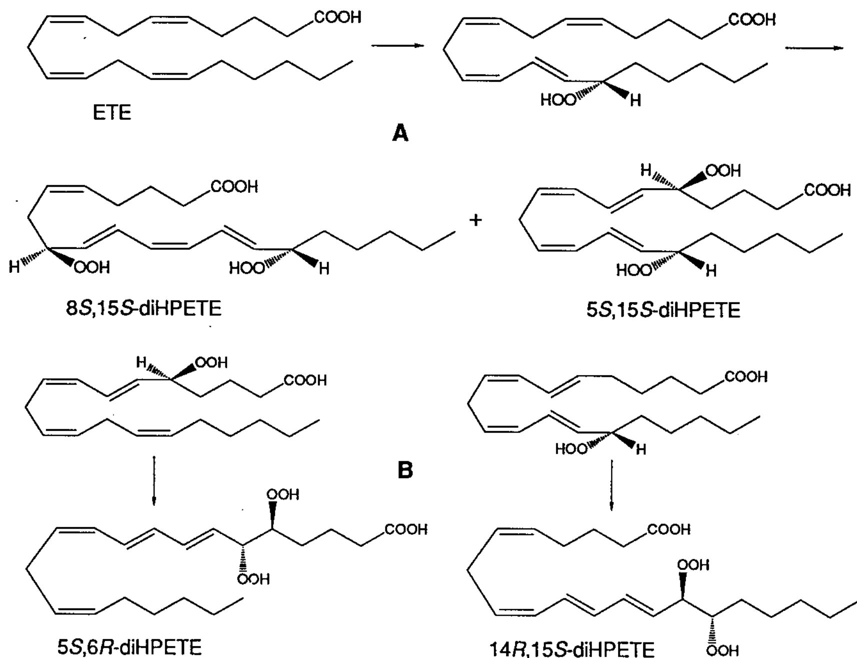


Fig. 2. (A) Double dioxygenation of arachidonic acid (ETE) by soybean lipoxygenase [G.S. Bild *et al.*, Biochem. Biophys. Res. Commun. 74, 949-954(1977); C.P.A. van Os *et al.*, Biochim. Biophys. Acta 663, 177-193 (1983)]. The main products are 8*S*,15*S*-diHPETE and 5*S*,15*S*-diHPETE. (B) Vicinal dioxygenation of 5*S*-HPETE (left) and 15*S*-HPETE (right) yields14*R*,15*S*-diHPETE and 5*S*,6*R*-diHPETE, respectively. The 14*R*-oxygenase activity is probably due to a 12-lipoxygenase [R.L. Maas & A.R. Brash, Proc. Natl. Acad. Sci. (USA), 80, 2884-2888 (1983)]. The 6*R*-oxygenase activity is thought to belong to the 5-lipoxygenase and to occur alongside LTA_4 formation [N. Ueda & S. Yamamoto, J. Biol. Chem. 263, 1937-1941(1988)]. The corresponding vicinal diols can also be formed by enzymic hydrolysis of 5,6-LTA_4 or 14,15-LTA_4. [J. Haeggström *et al.*, J. Biol. Chem. 261, 6332-6337 (1986); A. Wetterholm *et al.*, Eur. J. Biochem. 173, 531-536 (1988)].

Both modes of dioxygenation can actually be operational during the biosynthesis of lipoxins. Purified lipoxygenases from rabbit reticulocytes and from soybeans were found to produce lipoxins through three sequential dioxygenations starting from arachidonic acid (refs. 49, 50; Fig. 3). Also, bovine polymorphonuclear leukocytes were found to be capable of triply dioxygenating arachidonic acid to form lipoxins (ref. 51). Another mechanism through which lipoxins can be formed involves an epoxytetraene structure as an intermediate (*vide infra*).

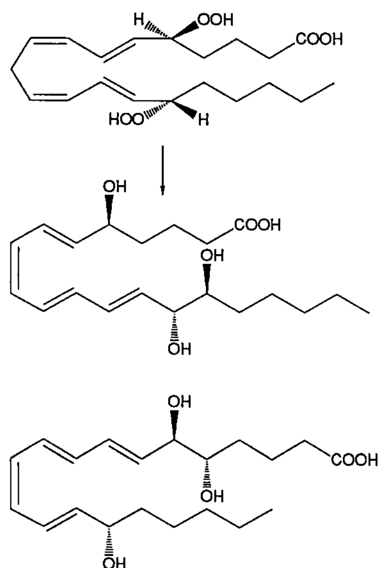


Fig. 3. Triple dioxygenation of ETE, eventually leading to lipoxins [P. Walstra *et al.*, FEBS Lett. 228, 167-171 (1988)].

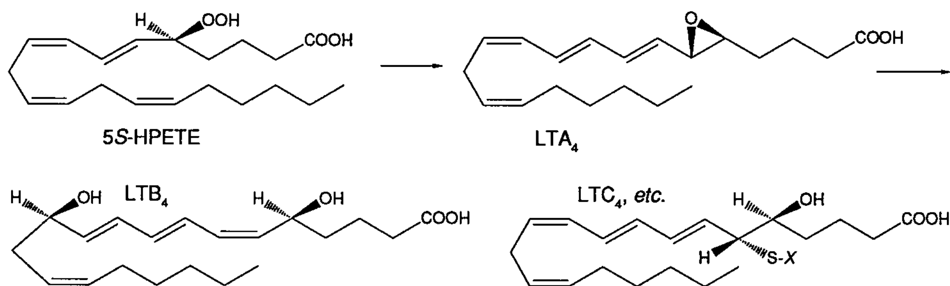


Fig. 4. LTA_4 is formed by lipoxygenase-catalysed dehydration of 5*S*-HPETE. Subsequent metabolism may lead to LTB_4 and/or sulfido-peptide leukotrienes including LTC_4, LTD_4 and LTE_4. S-*X*, glutathionyl in LTC_4; cysteinylglycyl in LTD_4; cysteinyl in LTE_4.

Dehydration of product hydroperoxides

Formation of epoxy compounds - The physiological role of fatty acid metabolites of the lipoxygenase pathway is under active investigation. In particular, in mammalian systems unsaturated lipid hydroperoxides are found to be rapidly converted into leukotrienes of either the B-series or the S-peptide series. This process involves as key intermediate leukotriene A_4 (LTA_4), a labile, unsaturated epoxy compound (ref. 52), formed from 5*S*-hydroperoxy-icosatetraenoic acid (5-HPETE) through enzymic dehydration (Fig. 4). The origins of the oxygen atoms in substituent groups are routinely determined by using $^{18}O_2$ or $^{17}O_2$ and $H_2{}^{18}O$.

Any hydroperoxide-dehydrase activity of a given enzyme preparation was always found to be closely associated with lipoxygenase activity. Improved purification procedures and, ultimately, the characterization of a 5-lipoxygenase expressed in a mammalian osteosarcoma cell line, demonstrated that dioxygenation and hydroperoxide dehydration are catalytic properties belonging to a single protein (ref. 53). Because of its reactivity, LTA_4 (Fig. 4; refs. 11, 54) constitutes a crucial structure in the metabolism of unsaturated fatty acids and the biosynthesis of the various types of leukotrienes. Enzymic and non-enzymic rehydration leads to dihydroxy compounds including LTB_4, while LTC_4-formation from LTA_4 and reduced glutathione (GSH) is controlled by a specific glutathione *S*-transferase (ref. 55).

From 15*S*-HPETE, an analogous A-leukotriene – 14,15-LTA_4 – occurs as an intermediate in the biosynthesis of 15-series leukotrienes (refs. 56-57). 12-Lipoxygenase from human platelets was found to be involved in the biosynthesis of 11,12-diHETEs which led Westlund *et al.* (ref. 57) to propose the intermediacy of 11,12-LTA_4 in the formation of 12-series leukotrienes. Biomimetically prepared 11,12-LTA_4 could be hydrolysed to yield a diastereomeric mixture of 11,12-diHETEs or 11,12-LTC_4, when incubated with rat liver glutathione *S*-transferase (ref. 58).

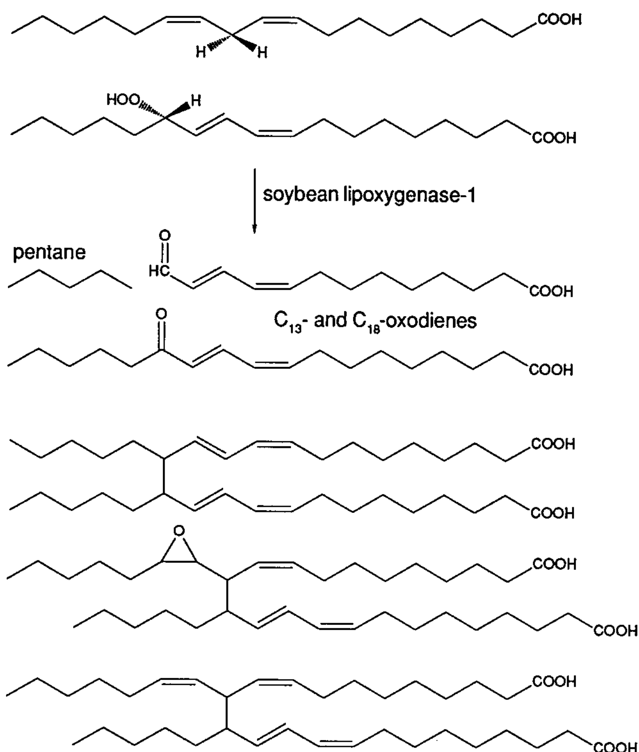


Fig. 5. An anaerobic mixture of linoleic acid and 13*S*-HPOD is converted by lipoxygenase into a complex mixture of secondary products, including oxodienes and oxygenated and non-oxygenated dimers. The reaction can be conveniently monitored spectrophotometrically at 285 nm due to the formation of oxodienes (G.J. Garssen *et al.*, ref 65).

Formation of oxo compounds - Product analysis after incubation of unsaturated fatty acids with preparations containing lipoxygenase activity often shows the presence of carbonyl compounds, including short-chain aldehydes that are probably responsible for specific flavors or off-flavors (ref. 59). In addition, oxodienoic acids are found that are directly derived from unsaturated fatty acid hydroperoxides via dehydration. The latter process can be mediated by lipoxygenase and other catalysts. Vioque and Holman (ref. 60) have described the formation of C_{18}-oxodienoates by a system containing an aqueous soybean extract, linoleic acid and oxygen. An anaerobic reaction involving linoleic acid, 13S-hydroperoxy-9*Z*,11*E*-octadecadienoate (13-HPOD) and purified soybean lipoxygenase-1 produces substantial amounts of *EZ* and *EE* 13-oxo-octadecadienoates (ref. 41).

Borthakur and Ramadoss (ref. 61) have reported the formation of this type of compounds from linoleic acid under aerobic conditions using a lipoxygenase isozyme from Bengal Gram. Two lipoxygenase isozymes purified from bush beans (*Phaseolus vulgaris*) described by Hurt and Axelrod (ref. 62) require different conditions to produce carbonyl compounds. One isozyme produced oxodiene in parallel with hydroperoxide, whereas the other one did so from linoleic acid and hydroperoxide only under strictly anaerobic conditions. Lipoxygenase isozymes from soybeans also

show marked differences in the amounts of C_{18}-oxodienes that can be produced under aerobic conditions. Lipoxygenase-1 and -2 were found to be relatively ineffective in this respect, whereas lipoxygenase-3 produced considerable amounts of C_{18}-oxodienes (ref. 63). C_{18}-oxodienes have also been reported as products from linoleate oxidation by lipoxygenases from peas (ref. 64). Reactions similar to the anaerobic reaction between soybean lipoxygenase-1, OD and 13*S*-HPOD (ref. 65) have been described for lipoxygenase from human platelets (refs. 66, 67) reacting with 12-HPETE or 15-HPETE and a suitable fatty acid, *e.g.* icosatrienoic acid (dihomo-γ-linolenic acid). Although neither C_8 nor C_5 chain fission products were characterized in these studies, a reaction scheme analogous to the one described by De Groot *et al.* (ref. 68) was proposed for the lipoxygenase-mediated formation of carbonyl compounds from 12- and 15-HPETE (ref. 67). This scheme predicts the formation of *i.a.* unsaturated C_{12} and C_{15} ω-oxo acids. Glasgow *et al.* (ref. 69) have identified such a short-chain aldehyde as a major product formed from arachidonic acid by porcine leukocytes. The compound, 12-oxo-dodeca-5*Z*,8*Z*,10*E*-trienoic acid, was found to be capable of potentiating superoxide production after initial stimulation of leukocytes with fMLP (*N*-formyl-Met-Leu-Phe). It was essential to establish the involvement of lipoxygenase from inhibition experiments with icosatetraynoic acid (ETYA) because hemoglobin also catalyzes the breakdown of lipid hydroperoxides to carbonyl compounds. The latter process has been studied in detail by Hamberg (ref. 70), using 13-HPOD as starting material. In addition to 11% C_{18}-oxodiene, he found four major metabolites, 13-HOD (25%), *threo*- and *erythro*-11-hydroxy-12,13-epoxyoctadec-9-enoate (24%) and 9-hydroxy-12,13-epoxyoctadec-10-enoate (31%). For several lipoxygenases it has been shown that the production of short-chain aldo-acids is accompanied by the formation of pentane (refs. 65, 71, 72). Pentane and other short-chain hydrocarbons can also be formed during thermal decomposition of lipid hydroperoxides (ref. 73), as well as in model systems with varying amounts of metal ions or CCl_4 and oxygen (refs. 74-76). Along with malondialdehyde, short- chain hydrocarbons like ethane and pentane can be considered as important markers of the level of lipid autoxidation *in vivo*. Once lipid hydroperoxides have been formed, they may undergo a wide variety of transformations leading to secondary decomposition products (see *e.g.* ref. 77).

Novel substrates and products

In vitro, free fatty acids with one or more 1*Z*,4*Z*-pentadiene systems are excellent substrates for lipoxygenases. This can be concluded from the rates at which this type of fatty acids are converted and from specificity features of the reaction products. In addition to free fatty acids, several other types of substrates have been tested, and were found to be less reactive or to yield a much broader, *i.e.* less specific product pattern. Most lipoxygenases show pH-optima in the range 5.5 - 7.5, with the exception of soybean lipoxygenase-1 which has its optimum at pH ≥ 9.0. A high pH

facilitates the use of free fatty acids at higher concentrations in true solutions (ref. 78). However, lipoxygenases may also be active towards emulsions of esterified fatty acid substrates or even more heterogeneous systems like intact membranes (refs. 79-81). Unfortunately, reported product characterization is mostly incomplete when non-fatty acid substrates are used. Eskola and Laakso (ref. 82) reported on the oxygenation of phosphatidylcholine (PC) from egg yolk by lipoxygenase-1 from soybeans, which was found to be specifically dependent on bile salts as an emulsifier (*cf.* 80). UV-spectra indicated that conjugation of double bonds took place during the aerobic phase of the reaction. Additional UV-absorptions after reaching anaerobiosis point to a reaction analogous to the ones described by Verhagen *et al.* (ref. 41) and Garssen *et al.* (ref. 65). 15-Lipoxygenase from human polymorphonuclear leukocytes was shown to oxygenate carbon-15 of the ETE-moiety of phosphatidylcholine, whereas 5-lipoxygenase from rat basophilic leukemia cells and 12-lipoxygenase from rabbit platelets showed no activity at all towards PC (ref. 83). The system has been characterized with respect to product profile and substrate requirements by Brash *et al.* (ref. 84). Pure 1-palmitoyl-2-arachidonyl- and 1-palmitoyl-2-linoleyl-PC were found to be excellent substrates for soybean lipoxygenase-1 at pH 9.0 in the presence of deoxycholate (*cf.* ref. 82.). Single molecular PC-species were found to be specifically converted into one product containing the monohydroperoxo derivative of the parent unsaturated fatty acid, *i.e.* the linoleic and arachidonic acid residues were converted into 13*S*-hydroperoxo-9*Z*,11*E*-octadecadienoic and 15*S*-hydroperoxo-5*Z*,8*Z*,11*Z*,13*E*-icosatetraenoic acid derivatives, respectively. Chiralities and double bond geometries thus proved to be identical to those of the products of free fatty acid oxygenation. In addition to PCs, 1-palmitoyl-2-arachidonyl-PE and 1-stearoyl-2-arachidonyl-PI were tested as substrates for soybean lipoxygenase-1. HPLC-behaviour, PLA_2-degradation and stereochemical analysis showed that the ETE-residue had been oxygenated to yield the 15*S*-HETE analogue. Like PC, these phospholipids specifically required deoxycholate to be converted. Control experiments in $H_2{}^{18}O$, showing that no ^{18}O was incorporated in the oxygenated product, excluded the possibility of hydrolysis of the phospholipid prior to oxygenation by lipoxygenase and subsequent reesterification. A set of similar experiments was conducted with the lipoxygenase from rabbit reticulocytes (ref. 85). ETE-PCs with ETE in either *sn*-1 or *sn*-2 position were found to be readily oxygenated to 15*S*-HPETE derivatives and to a much smaller extent (2-5%) to 12*S*-HPETE. Any amount of 5-HPETE appeared to be non-chiral, and completely absent if the reaction was carried out at 0°C. Incubation of reticulocyte lipoxygenase with human erythrocyte ghost containing ^3H-labelled ETE specifically yielded 15*S*- and small amounts of 12*S*-HPETEs. Reticulocyte lipoxygenase thus shows similar specificity patterns for ETE-containing phospholipid substrates and free fatty acid substrates. In the presence of deoxycholate, lipoxygenases from soybeans and rabbit reticulocytes clearly have a preferred mode of interaction with the hydrophobic methyl terminus and not with

a polar head group of any type of substrate (*cf.* ref. 86). Kühn & Brash (ref. 87) have extended the work on the 15-lipoxygenase of reticulocytes to *in vivo* experiments aimed at elucidating the physiological role of this enzyme. Reticulocyte lipoxygenase has been implicated in the maturation process of reticulocytes to erythrocytes, in particular in initiating the disappearance of mitochondria. In rabbits, augmented levels of reticulocytes were induced by a bleeding anemia. Subsequently, lipid fractions of red cell membranes were investigated for the presence of characteristic products of lipoxygenase activity, *viz.* hydroxy-octadecadienoates and/or -icosatetraenoates. On the seventh day of the bleeding scheme, reticulocytes constituted 25-40% of the red cells, and were found to contain a 20-fold increase in hydroxylated polyenoic fatty acids. No hydroperoxo derivatives were detected. The main products were 13*S*-HOD and 15*S*-HETE. On incubation of pure reticulocyte lipoxygenase with rat liver mitochondria the same major reaction products were found. These experiments constitute the first direct evidence for the involvement of reticulocyte lipoxygenase *in vivo* in biomembrane alteration.

Funk *et al.* (ref. 88) made a systematic investigation of the reactivity of *EZ*-geometric isomers of linoleate with soybean lipoxygenase-1. Isomeric linoleic acids were prepared by total synthesis. At both pH 9.0 and pH 7.0 in the presence of Tween 20 *EZ*-linoleic acids were found to be poor substrates compared to natural linoleic acid. At pH 7.0 rates amounted to 3% of linoleic acid conversion. Products were isolated from incubations at pH 7.0 and characterized with respect to the chirality of the oxygenated carbon atom and the geometries of the double bonds. Among the substrates tested, 9*E*,12*Z*-octadecadienoate was found to be converted into 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoate. Lipoxygenase-1 thus appears be able to convert the 9*E*-geometry of the substrate into 9*Z* in the product. Moreover,

TABLE 1. Relative yields of regio- and stereoisomeric hydroperoxides of lipoxygenase-catalysed dioxygenation of linoleic acid and of non-conjugated *EZ*-isomers. Soybean lipoxygenase; pH 7.0; relative rates of substrate conversion in parentheses.*

Product	13*S*,9*Z*,11*E*	13*R*,9*Z*,11*E*	13*R*/*S*,9*E*,11*E*	9*S*,10*E*,12*Z*	9*R*,10*E*,12*E*	9*R*/*S*,10*E*,12*E*
Substrate						
9*Z*,12*Z* (100)	59.2	14.8	4	14.4	3.6	4
9*Z*,12*E* (3)	15	31	18	3.4	4.6	28
9*E*,12*Z* (3)	50	5	17	7	12	9

* compiled from ref. 88.

oxygenation of C-13 is highly regio- and stereospecific. Table 1 summarizes some of the findings of this study. From Table 1 it can also be seen that the 9*Z*,11*E*-isomer is converted mainly into 13*R*-hydroperoxy-9*Z*,12*E*-octadecadienoate. Although this may seem surprising, it is, as pointed out by the authors (ref. 88), in line with the antarafaciality of hydrogen abstraction and oxygen addition with respect to a planar orientation of the 1*E*,4*Z*-pentadiene system (Fig. 6).

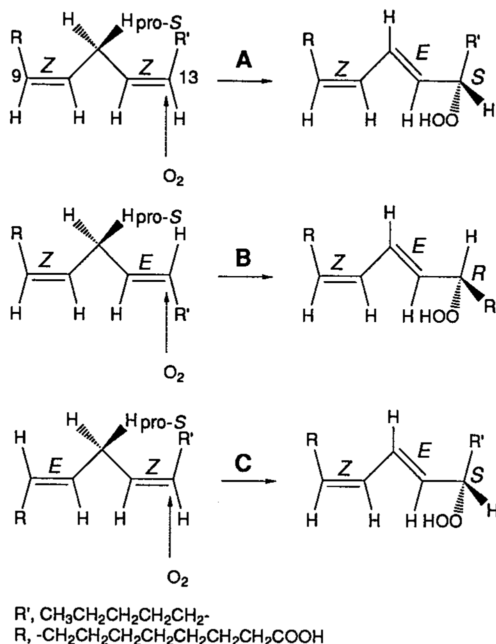


Fig. 6. Stereochemistry of lipoxygenase-catalysed dioxygenation of linoleic acid and its non-conjugated *EZ*-isomers.
(A) The natural isomer with *ZZ*-geometry is predominantly converted into the 13*S*,9*Z*,11*E* hydroperoxide. In the concurrent formation of the 9-hydroperoxide from this substrate under these conditions, the 9*S*,10*E*,12*Z*-isomer prevails. Similar results have been reported previously.
(B) The pentadiene moiety of the substrate with 9*Z*,12*E*-geometry apparently has the same spatial alignment with enzyme and oxygen as the all-*Z*-substrate thus favouring the formation of the *R*-enantiomer at C-13. However, interaction with the enzyme is suboptimal, as is apparent from the relatively low overall conversion-rate and the high content of racemic product.
(C) Product formation from the 9E,12Z-isomer, and in particular the finding that lipoxygenase catalysis restores 9*Z*-geometry, demonstrates that at the intermediate stages, the whole of the pentadiene moiety interacts with the enzyme. The interaction clearly imposes steric constraints on the mode of product formation [adapted from ref. 88].

The use of well-chosen non-standard substrates may reveal important mechanistic details of the interactions with the enzyme. An interesting type of substrate used with lipoxygenase is an unsaturated fatty acid containing a carbonyl group at the position of the central methylene group of the natural substrate (ref. 89). This study (*cf.* ref. 88) demonstrates that the presence of a 1,4-diene with all-*Z* geometry is not a strict requirement for a fatty acid to act as a substrate in lipoxygenase reactions. This particular set of substrates was, however, chosen primarily to investigate the possible influence of an electron-withdrawing functionality on the rate of lipoxygenase reactions. If in the rate-limiting step an intermediate with a carbanionic nature would occur, it might be expected that the presence of a neighbouring carbonylgroup in the substrate would facilitate proton removal and thus enhance the overall reaction rate. However, none of the enzyme-substrate

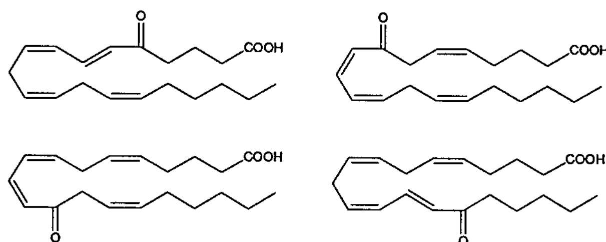


Fig. 7. Oxo-derivatives of arachidonic acid were assayed with 5-, 12- and 15-lipoxygenases from rat neutrophils, rat platelets and soybeans, respectively (ref. 89), with the objective to assess the influence of this electron-withdrawing group on the overall rate of the reaction. Besides the oxo-derivatives, the corresponding hydroxy-derivatives were tested. Results proved inconsistent with an anionic transition state in the rate-determing step.

combinations tested proved to be more efficient than the one with arachidonic acid. The authors concluded that no carbanionic transition state is generated by lipoxygenase. In this study (ref. 89), rates were measured by monitoring the disappearance of substrate while no product analyses were reported. Such analyses could, however, provide significant additional insight into the way the enzyme and the substrate interact.

Corey (ref. 28) discusses the use of various specifically designed synthetic substrates and possible inhibitors for lipoxygenases in order to probe the mechanism. The inhibition by and conversion of several dehydro-arachidonic acids (DHAs) (Fig. 8) have been described. With soybean lipoxygenase, only14,15-DHA was found to be an irreversible inhibitor in a process which was time- and oxygen-dependent. Other DHAs were found to be relatively weak, competitive, inhibitors, while the 5,6-DHA could serve as a substrate for the soybean enzyme. Incubation with tritiated 14,15-DHA produced an inactive, labeled protein, most probably containing one molecule of tritiated DHA per mole of enzyme. The results of incubating 5,6-DHA with

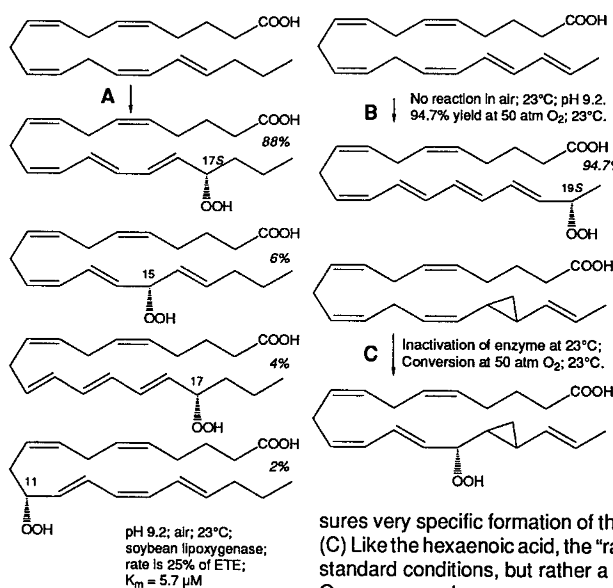


Fig. 8. (A) The presence of an additional double bond (16*E*) conjugated with the 14*Z* double bond results in a V_{max} which is only 25% of that for ETE. Absolute configuration at C-17 of the main product was found to be *S*. Hydrogen abstraction from C-13 and conjugation of 3 double bonds causes a 1,5-shift of the unpaired electron and subsequent O_2 addition at C-17. Apparently, 1,3-shifts leading to 11- and 15-hydroperoxides, are of minor importance with this substrate. The sequence of mechanistic events is basically identical to that proposed for the formation of 14,15-diHPETE by 12-lipoxygenase (ref. 45).
(B) The presence of a second extra double bond (18*E*) apparently completely blocks product formation under standard conditions. At high O_2 pressures very specific formation of the 19*S*-hydroperoxide occurs.
(C) Like the hexaenoic acid, the "radical clock" acid is not a substrate under standard conditions, but rather a time-dependent inactivator. At elevated O_2-pressures, however, conversion does take place (E.J. Corey & R. Nagata, Tetrahedron Lett. 28, 5391-5394 (1987); see also: ref. 28).

the 5-lipoxygenase from RBL-1 cells gave completely analogous results: in aerobiosis, this enzyme was irreversibly inactivated. The mechanism of the irreversible inactivation process was studied by using 7*R*-[^{2}H]-arachidonic acids as the substrate for RBL-1 and potato lipoxygenases (Fig. 9). As expected, the resulting 5S-HPETE still contained the deuterium label.

7*S*-[^{2}H]-DHA, when incubated with RBL-1 lipoxygenase, showed a rate of inactivation of 1/7 that for DHA itself. This clearly demonstrates the mechanism-based inhibition by the monoacetylenic ETE-analogue of this enzyme. Another set

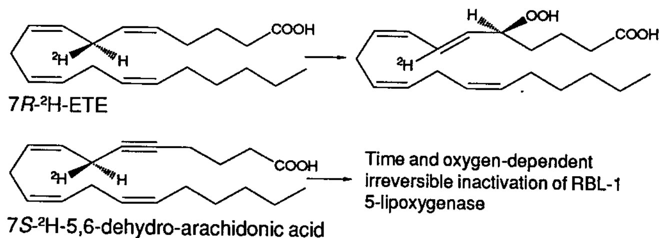


Fig. 9. Stereospecifically labeled ETE shows retention of $^{2}H_{R}$-label when incubated with RBL-1 or potato 5-lipoxygenase. Analogously, 7*S*-^{2}H-DHA irreversibly inactivates RBL-1 5-lipoxygenase at a rate of at least 1/7 that of non-labeled DHA in an oxygen-dependent fashion (ref. 28).

of substrate-analogues contained a sulfur atom instead of a methylene group between the double bond positions (Fig. 10). The 7- and 10-thiaarachidonic acids act as normal substrates for soybean lipoxygenase and are converted into the corresponding hydroperoxides. The 13-thiaarachidonic acid, however, is an oxygen- and time-dependent irreversible inhibitor for the soybean enzyme. Likewise, 7-thiaarachidonic acid proved to be an oxygen- and time-dependent inhibitor for the 5-lipoxygenase from RBL-1 cells. The absence of an abstractable hydrogen was proposed to lead to covalent attachment of the proton-acceptor, while oxygen can still be inserted between iron and the terminal C-atom of the pentadiene system (Fig. 11) (refs. 28, 90). Interestingly, the sulfoxide of the corresponding 13-thiaarachidonic acid behaved as a competitive inhibitor for soybean

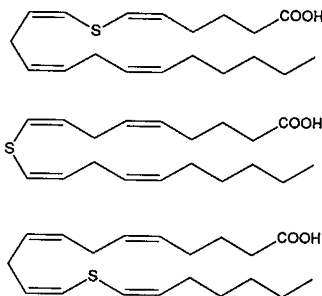


Fig. 10. 7-, 10- and 13 thia-ETEs as substrates for soybean 15-lipoxygenase. The former two are readily converted into hydroperoxides; the latter irreversibly inactivates the enzyme (ref. 28, 90).

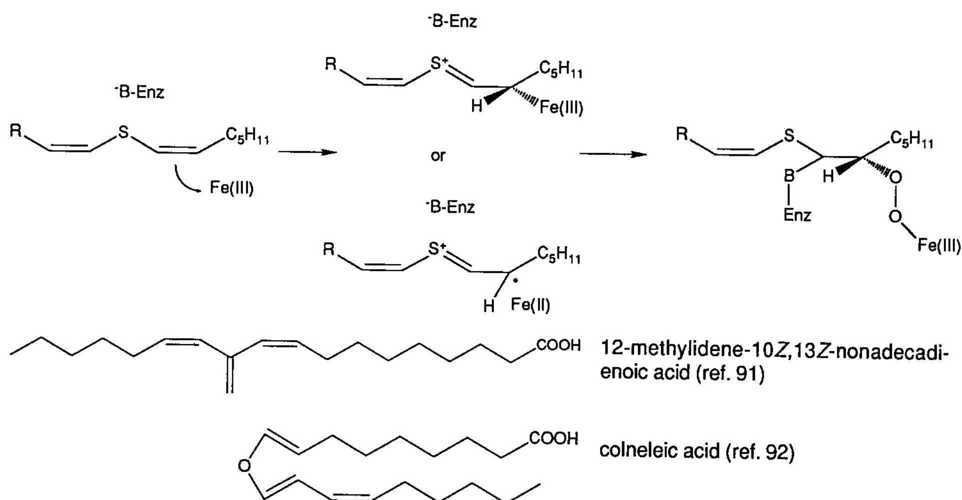


Fig. 11. Mechanistic scheme proposed by Corey *et al.* (refs. 28, 90) to explain the time- and oxygen-dependent irreversible inactivation of soybean lipoxygenase-1 by substrate-analogues not containing a lipoxygenase-abstractable hydrogen atom. Colneleic acid acts as a strong competitive inhibitor, K_i = 8μM.

lipoxygenase, K_i = 66μM. Also, colneleic acid (Fig. 11) was found to be a strong competitive inhibitor (K_i = 8μM) of potato lipoxygenase (ref. 92), whereas 12-methylidene-10Z,13Z-nonadecadienoic acid (Fig. 11; ref. 91) irreversibly inhibits soybean lipoxygenase in a fashion analogous to 13- and 7-thiaarachidonic acids with lipoxygenases from soybeans and RBL-1 cells, respectively.

The notion of an organo-iron intermediate during hydroperoxide formation or

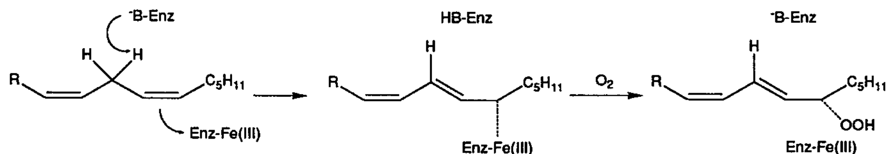


Fig.12. Organoiron-mediated pathway of lipoxygenase-catalyzed hydroperoxide formation as proposed by Corey *et al.* (refs. 93-94).

inhibition experiments with this type of substrates was further investigated by using three other substrate-analogues (ref. 93) (Fig. 8). On the basis of inactivation kinetics and oxygen pressure-dependence of product distribution in reactions with soybean lipoxygenase, a reaction mechanism involving an organo-iron intermediate was proposed (ref. 93) (Fig. 12). A biomimetic reaction for lipoxygenase, involving a strong Lewis acid (Fe(III)) was introduced by Corey and Walker (ref. 94). The model was, however, found inadequate to discriminate between π- or σ-bonding with iron. Also, it appeared impossible to demonstrate direct insertion of dioxygen into a carbon-iron bond with this model.

Corey *et al.* (ref. 95) have recently reported on the stereochemistry of LTA_4-formation from 5*S*-HPETE and the formation of novel diastereomeric diHPETEs by two types of enzymes, *i.e.* one from potato or one from the coral *Plexaura homomalla.* Incubating 5*S*-HPETE with potato lipoxygenase under 60 atm of O_2 afforded 5*S*,8*R*-diHPETE (Fig. 13A) in about 1% yield. The enzyme from the coral, which presumably produces 8*R*-HPETE from ETE (ref. 96), formed 5*S*,8*S*-diHPETE from 5*S*-HPETE in a 5% yield under 1 atm O_2 (Fig. 13B).

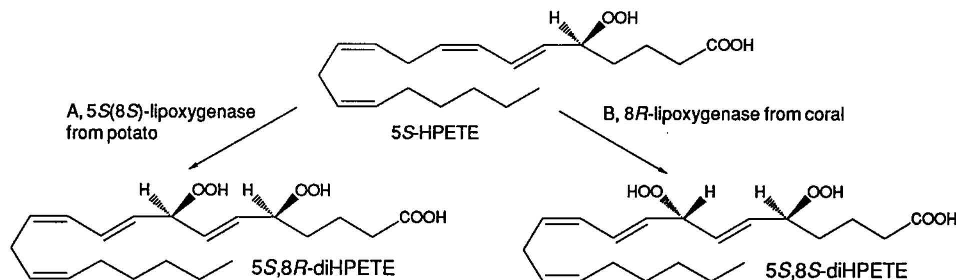


Fig. 13. Dioxygenation of 5*S*-HPETE by lipoxygenases from potato (A) and the coral *Plexaura homomalla* (B) yields diastereomeric diHPETEs. Under the experimental conditions employed (Corey *et al.*, ref. 95) the 5*S*-lipoxygenase from potato apparently acts upon 5*S*-HPETE as an 8*S*-lipoxygenase, which may explain the relatively poor yield.

The mechanism of formation of LTA_4 from 5*S*-HPETE was proposed to involve an organoiron intermediate much like the one depicted in Fig. 12 in lipoxygenase-catalysed hydroperoxide formation. This time, however, no O_2-insertion is supposed to take place, but after formation of the Fe-C6-bond, water is eliminated to eventually afford LTA_4 (Fig. 14). The finding that an *R*-lipoxygenase forms 6-*epi*-LTA_4 (ref. 95) from 5*S*-HPETE comes as no surprise, taking into account the known stereochemical

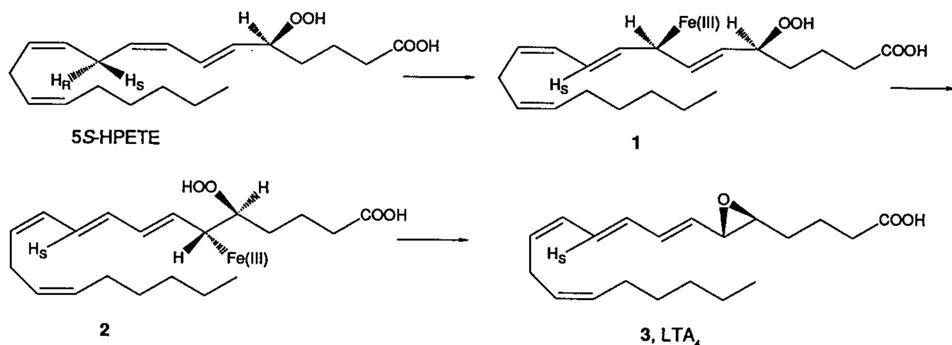
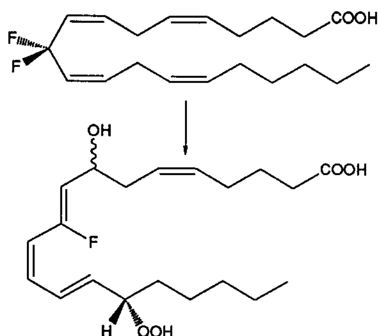


Fig. 14. According to Corey *et al.* (ref. 95), potato lipoxygenase stereospecifically removes the 10-pro*R* hydrogen from 5*S*-HPETE and forms organoiron intermediate **1**, which then rearranges to **2**. A γ-elimination step subsequently produces LTA_4.

features of LTA_4-formation and the fact that LTA_4-formation is an intrinsic property of many lipoxygenases. From intermediates **1** and **2** (Fig. 14) H_2O-elimination or O_2 insertion would either yield LTA_4 or diHPETEs, *viz.* both 5*S*,8*R*- and 5*S*,6*R*-diHPETE. Although 5*S*,8*R*-diHPETE was indeed formed, no evidence was reported for 6*R*-dioxygenation occurs under these circumstances (*cf.* ref. 46).



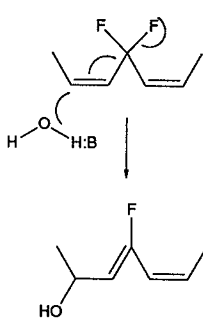


Fig. 15. 10,10-Difluoroarachidonic acid is a substrate for both soybean lipoxygenase-1 and prostaglandin endoperoxide synthase. The latter forms 10,10-difluoro-11*S*-HETE (not shown here) and 10-fluoro-8,15*S*-diHETE. No cyclization to prostaglandins was observed. Lipoxygenase forms the same mixture of diastereomeric 8,15*S*-diHETEs. Only the oxygen at C-15 of the diHETEs stems from enzymic dioxygenation (ref. 97).

Kwok *et al.* have recently investigated 10,10-difluoroarachidonic acid as a substrate for cyclooxygenase and soybean lipoxygenase (Fig. 15) (ref. 97). Soybean lipoxygenase readily formed the 15S-hydroperoxo derivative, which was in turn converted non-enzymically into a diastereomeric mixture of 8*R*- and 8S-hydroxy-10-fluoro-15S-hydroperoxoicosa-5*Z*,9*Z*,11*Z*,13*E*-tetraenoic acids. The OH at C-8 is introduced by S_N' attack of H_2O on the 15S-HP derivative, which explains the 9*Z*-

geometry in the product. In the authors' view (ref. 97), the initial formation of the difluoro-analogue of 15S-HPETE by soybean lipoxygenase supports the concept of hydrogen atom abstraction from C-13 as the initial step in the reaction sequence. Neither proton nor hydride abstraction from C-13 is likely to occur with this substrate.

Wiseman (ref. 98) has studied the secondary isotope effects for the oxidation of [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid by soybean lipoxygenase and a 5-lipoxygenase from rat peritoneal neutrophils. With both enzymes a normal isotope effect was found, *i.e.* $k_H > k_T$, which was not expected since secondary isotope effects are generally dominated by changes in hybridization. Here, this could only involve sp2→sp3 transitions at the terminal C-atoms of a pentadiene moiety of arachidonic acid, which should yield an inverse isotope effect, *i.e.* $k_H < k_T$. Although the observed effects should be interpreted with caution, Wiseman (ref. 98) concludes that these data support the notion of a carbanionic intermediate in the lipoxygenase reaction as put forward by Corey and Nagata (Fig. 12; ref. 93).

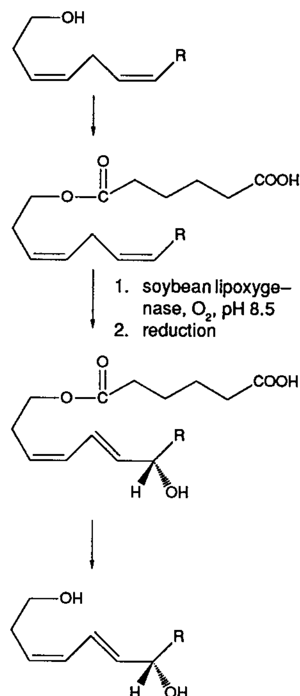


Fig. 16. Lipoxygenase as a tool in the regio- and stereoselective formation of dihydroxydienes (ref. 99).

As was recently demonstrated, soybean lipoxygenase-1 may also be used as a tool in the asymmetric hydroxylation of pentadienols (ref. 99) (Fig. 16). The starting material is first modified by attaching a C_6-prosthesis which makes it a better substrate for lipoxygenase. Next, the newly formed substrate is converted using soybean lipoxygenase, and in a final step the prosthetic group is removed. Overall yields, regioselectivity and stereoselectivity were good.

Iron-active compounds

The iron in native soybean lipoxygenase is high-spin Fe(II) and has no dioxygen coordinated. This was concluded from magnetic susceptibility measurements by Petersson *et al.* (ref. 100). The key feature of the scheme for the anaerobic and aerobic reaction mechanisms as proposed by De Groot *et al.* (ref. 68) and Petersson *et al.* (ref. 101) is the hydrogen abstraction step which is supposed to yield a Fe(II)-enzyme and a pentadienyl free radical (Fig. 17). The evidence for this mechanism however, largely stems from anaerobiosis experiments, the results of which have been extrapolated to the aerobic case. With cyclooxygenase (refs. 102, 103), evidence has been presented for the occurrence of carbon-centered free radicals in the oxygenation of arachidonic acid. De Groot *et al.* (ref. 104) have clearly shown the involvement of such radicals in the anaerobic reaction of lipoxygenase by using the water-soluble

spin trap 2-methyl-2-nitrosopropanol and deuterated substrate. Spin trapping experiments with potato lipoxygenase and 2-methyl-2-nitrosopropane have been interpreted as evidence for the occurrence of such radicals in the dioxygenation reaction (ref. 105). Kinetic experiments, in which the oxygenation of linoleic acid by soybean lipoxygenase was carried out in the presence of varying amounts of nitrosobenzene as a spin trap, have shown that the ratio $k_{oxygenation}/k_{trapped}$ was about 50 (ref. 106). This may indicate that a linoleyl radical is indeed an intermediate in lipoxygenation.

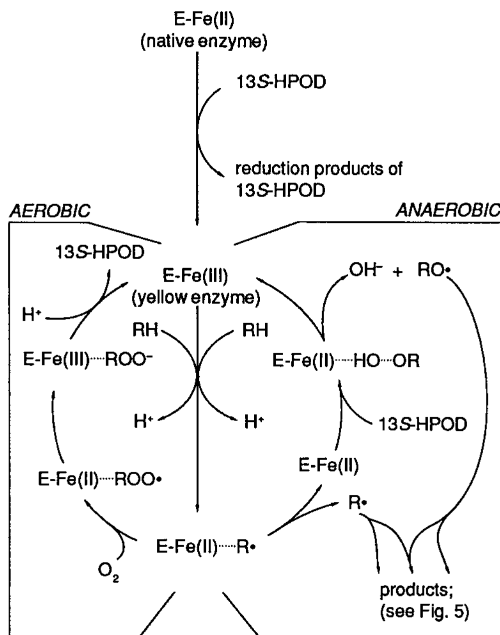


Fig. 17. Mechanistic scheme for lipoxygenase catalysis proposed by De Groot *et al.* (ref. 68) and Petersson *et al.* (ref. 100).

Gibian and Galaway (ref. 107), in reviewing the chemical aspects of lipoxygenase reactions, proposed a reaction scheme for the aerobic reaction in which hydrogen abstraction is depicted as a two-step process, *i.e.* an initial step in which Fe(III) coordinates to the π-electrons of the fatty acid double bonds and eventually one electron completely transfers to Fe(III), thus yielding an Fe(II)-enzyme

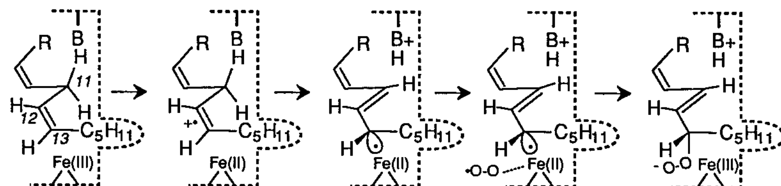


Fig. 18. Mechanistic steps in lipoxygenase catalysis as proposed by Gibian & Galaway (ref. 107).

and a radical-cationic species (Fig. 18). In this scheme, the role of Fe(III) in hydrogen abstraction is only indirect. The carbon-hydrogen bond may be weakened by the Fe(III) coordinating to the π-bond, which would obviate the need for an excessively strong base as a proton acceptor. Jefford and Cadby (ref. 108) argue that the allylic radical intermediate, as proposed by Gibian and Galaway (ref. 107) may also be regarded as a tetrahedral carbanion σ-bonded to Fe(III). According to Corey *et al.* (refs. 28, 93) molecular oxygen may be inserted here as a subsequent step in lipoxygenase-catalyzed hydroperoxide formation.

R-dioxygenation

Lipoxygenase-catalyzed dioxygenation of natural poly-unsaturated fatty acids produces hydroperoxides with *S*-configurations. Known exceptions are the formation of 9*R*-HPOD from linoleic acid by type-2 lipoxygenases from soybeans or peas (ref. 109) at high pH. Substrates with *EZ* double bond geometry may also be converted into *R*-hydroperoxides (ref. 88). The process of hydroperoxide formation involves stereospecific removal of one of the prochiral H-atoms from the central methylene group of the pentadiene system. From numerous studies, a rule of thumb has emerged that hydrogen removal and dioxygen insertion occur at opposite sides

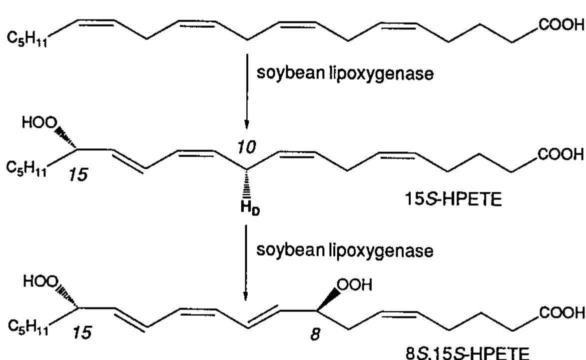


Fig. 19. Stereochemistry of the second dioxygenation step of ETE (15*S*-HPETE → 8*S*,15*S*-HPETE) catalyzed by soybean lipoxygenase. Another major product is 5*S*,15*S*-HPETE (ref. 40). 12-Lipoxygenase from porcine leukocytes also forms 8*S*,15*S*-HPETE from 15*S*-HPETE in addition to 14,15-diHPETE and 14,15-LTA_4. However, removal of the hydrogen from C-10 of 15*S*-HPETE does not occur with the same stereoselectivity as with soybean lipoxygenase. According to Brash *et al.* the results with porcine leukocytes were inconlusive in this respect (ref. 110).

of a plane in which the pentadiene system can be oriented. However, recent studies by Brash *et al.* (ref. 110) indicate that porcine leukocytes contain a lipoxygenase that fails to show such a relationship between hydrogen abstraction and dioxygen insertion. In a comparative study 15S-HPETE, stereospecifically labelled with L-^{3}H at C-10, was incubated with porcine or soybean lipoxygenase and the resulting diHPETEs were analyzed for their stereochemistry (Fig. 19). Porcine lipoxygenase is known to produce 14*R*,15*S*-diHPETE and 8*S*,15*S*-diHPETE from 15*S*-HPETE in addition to 14,15-LTA_4 and some of its decomposition products, while soybean lipoxygenase forms 8*S*,15*S*-diHPETE and 5*S*,15*S*-diHPETE from 15*S*-HPETE (ref. 40). Remarkably, the porcine lipoxygenase was found to produce 8*S*,15*S*-diHPETE while retaining the 10D ^{3}H-label for 90% in this product, whereas the antarafacial rule would predict a complete loss of this label. Such a loss of label was indeed confirmed with soybean lipoxygenase. The level of retention of a 10L-^{3}H label in 15S-HPETE varied from 50% to 250%. However, the 8,15-diHPETE formed by porcine leukocytes invariably had 8*S*,15*S*-configuration. Whether or not this intriguing observation constitutes a real exception to the antarafaciality rule requires further investigation. In addition to the well-documented examples of *R*-lipoxygenation of a *ZZ*-substrate by type-2 enzymes from soybeans and peas, some interesting observations have recently been published that might involve additional cases of *R*-lipoxygenation. Woollard (ref. 111) has reported the presence of 12*R*-HETE in

psoriatic skin lesions. Subsequent studies by Fretland *et al.* (ref. 112) have shown that 12*R*-HETE is a potent chemotactic agent for neutrophils, which may therefore account for their presence in psoriatic plaque. As yet, insufficient evidence is available to substantiate that straightforward and specific 12*R*-lipoxygenation of ETE occurs in this type of tissue. In Table 2 some recent examples of *R*-lipoxygenation have been summarized.

TABLE 2
Oxygenated fatty acids with *R*-chirality.

PRODUCT	SUBSTRATE*	ENZYME SOURCE	Ref.
9*R*-HPOD	OD	soybean, pea; pH 9.0	109
8*R*-H(P)ETE	ETE	coral	96, 113
11*R*-HPETE	ETE	sea urchin	115
12*R*-HPETE	ETE	sea urchin	115
12*R*-HETE	ETE	psoriatic skin	111

*OD, linoleic acid; ETE, arachidonic acid

Hawkins and Brash (refs. 114-115) have provided evidence for the formation of both 11*R*- and 12*R*-HETE from the corresponding hydroperoxide precursors in eggs of the sea urchin *Strongylocentrotus purpuratus*. No inhibition by cyclooxygenase inhibitors was found to occur. In addition, it was shown that biosynthesis of the HETEs did not involve 11- or 12-oxo-intermediates. It was therefore concluded that in these organisms true lipoxygenases are responsible for their formation.

Lipoxins and related compounds

Lipoxins are multiply hydroxylated derivatives of arachidonic acid (refs. 10, 27, 36), containing a conjugated tetraene chromophore. The mechanism of formation either involves multiple dioxygenation (see Fig. 3; refs. 49, 51) by different lipoxygenases or the hydrolysis of an epoxytetraene intermediate (Fig. 20; ref. 116).

Nicolaou *et al.* (ref. 117) have recently compared a large number of lipoxin isomers from enzymic or synthetic origin for their ability to contract guinea pig lung strip, in particular to evaluate the spasmogenic properties of the novel 7*Z*,11*E*-lipoxin A_4 biosynthesized by a preparation

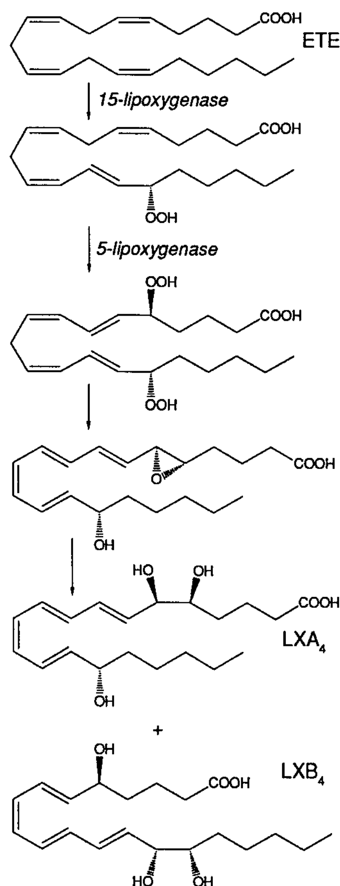


Fig. 20. Biosynthetic scheme of lipoxin A and B formation via the epoxytetraene pathway.

from human neutrophils.

The epoxytetraene intermediate is structurally analogous to LTA_4, the unstable intermediate in the formation of LTB_4 and LTC_4. This has brought Steinhilber and Roth (Fig. 21; ref. 118) to investigate the possible biosynthesis of sulfidopeptide-lipoxins by a route analogous to the biosynthesis of leukotrienes C_4, D_4 and E_4. It was found that granulocytes of human eosinophilic donors actively synthesize, from 15-HETE, the 15-hydroxy derivatives of LTC_4, D_4 and E_4, for which the trivial names lipoxins C_4, D_4 and E_4 were suggested. These novel products were identified by comparing them with 15-hydroxyderivatives of LTC_4, D_4 and E_4 obtained by incubating the authentic leukotrienes with soybean lipoxygenase and subsequent reduction of the hydroperoxo-function.

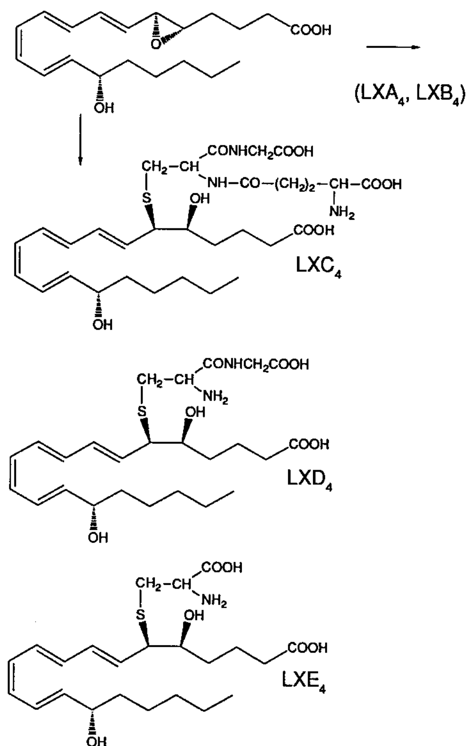
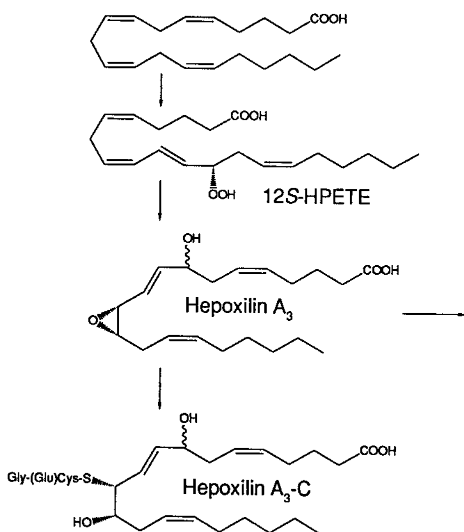


Fig. 21. Formation of sulfidopeptide lipoxins by human granulocytes (ref. 118). For this type of lipoxins, trivial names analogous to those of the leukotrienes were suggested.

Another type of oxygenated fatty acid derivative originates from the enzymic isomerization of fatty acid hydroperoxides into hydroxyepoxy compounds. Garssen *et al.* (ref. 119) reported the lipoxygenase-catalyzed isomerization of 13S-HPOD into a monoenoic 11-hydroxy-12,13-epoxy fatty acid. Falardeau *et al.* (ref. 120) then reported



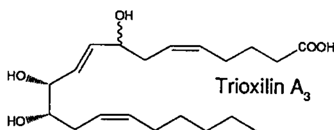


Fig. 22. The hepoxilin pathway, including the formation of glutathione conjugates by glutathione S-transferase (ref. 122).

the identification of a number of trihydroxy-icosadienoic acids after incubating human platelets with 8,11,14-icosatrienoic acid. These authors proposed a mechanism involving hydroxy-epoxides as intermediates. Conclusive evidence for the intermediacy of the latter type of compound was furnished by Pace-Asciak *et al.* (ref. 121). At a later stage, Pace-Asciak *et al.* (Fig. 22; ref. 122) have reported the conversion of this type of hydroxy-epoxides, now called *hepoxilins*, into a glutathione conjugate, catalyzed by a mixture of glutathione transferase isozymes. This conversion is closely analogous to the other two pathways described above.

The hydroperoxide isomerases from flaxseed (ref. 123, 124) and corn germs (ref. 125) produce α-ketols from the substrate fatty acid hydroperoxide. [^{18}O]-studies have demonstrated that in the reaction product only the oxygen atom of the carbonyl-group stems from the substrate hydroperoxide. Hamberg (refs. 126, 127) successfully trapped a short-lived intermediate in the conversion of 13*S*-HPOD by hydroperoxide isomerase from corn. 13*S*-HPOD is first converted into 12,13*S*-oxido-9*Z*,11-octadecadienoic acid which then spontaneously hydrolyses into 12-oxo-13-hydroxy-9*Z*-octadecenoic acid. The mechanism proposed by Hamberg is based on [^{18}O-] and [^{2}H]-labeling experiments (refs. 125, 126, 127) and revolves around the intermediate allene epoxide (Fig. 23). The allene epoxide is formed in the first step by dehydration of the substrate hydroperoxide. This short-lived compound may then undergo nucleophilic attack by H_2O. The attack is partly S_N2 and partly S_N1 in nature. The S_N2 part would explain that inversion of configuration takes place at C-13 (Fig. 23), while the S_N1 branch would account for the existence of minor amounts of γ-ketols (refs. 125, 128) and for the amount of α-ketol that is racemic at C-13. Some other pathways, including hydroperoxide lyase and cyclase, have recently been described in a review by Vick and Zimmerman (ref. 31). Another type of hydroperoxide isomerase from the oomycete *Saprolegnia parasitica* was described by Herman and Hamberg (refs. 129, 130). It was shown to convert 15*S*-HPETE into two trienoic epoxy-hydroxy com-

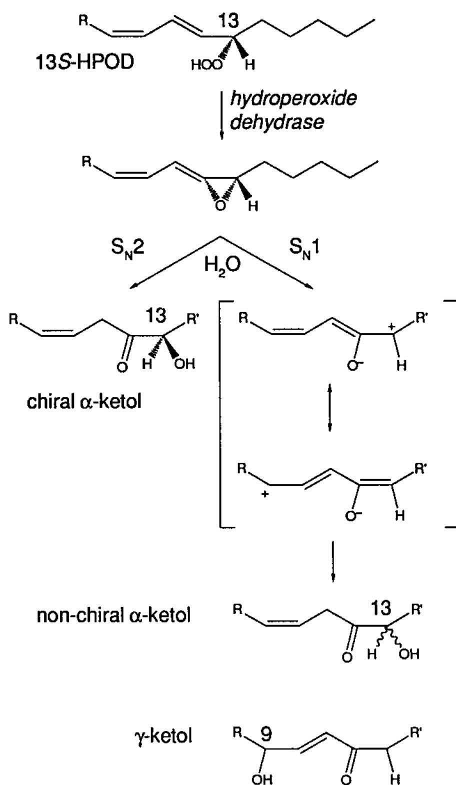


Fig. 23. Mechanism of hydroperoxide dehydrase from corn as proposed by Hamberg (ref. 126). Formerly, the enzyme was named 'hydroperoxide isomerase'.

pounds (Fig. 24). Lipoxygenase and hydroperoxide isomerase activities were found to comigrate in the various purification steps. In addition, the activities responded similarly to inhibitors. An approximate molecular mass of 145,000-150,000 was reported. An important difference between this type of isomerase and those from corn and flax was demonstrated by the use of [^{18}O]-labeled 15S-HPETE. It was found that both ^{18}O-atoms of the hydroperoxo function were retained in the epoxy-alcohols.

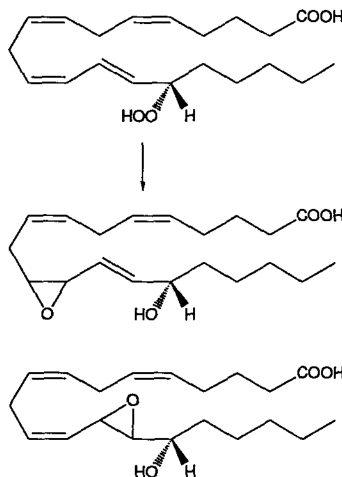


Fig. 24. A lipoxygenase from the fungus *Saprolegnia parasitica* forms 15*S*-HPETE from arachidonic acid. Subsequently, the 15*S*-HPETE is transformed into two isomeric epoxy-hydroxy-eicosatrienoates *viz.* 11*S*, 12*R*-epoxy-15*S*-hydroxy-5*Z*,8*Z*,13*E*-eicosatrienoic acid and 13*R*,14*R*-epoxy-15*S*-hydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid (ref. 129).

Manipulating the valence state of iron

In the catalytic cycle, substrate fatty acids and product hydroperoxo fatty acids change the valence state of iron in lipoxygenase (Fig. 17). The native resting enzyme contains high-spin Fe(II) that does not coordinate dioxygen. The mode of action of certain classes of inhibitors is based on the interaction with the central iron. Clapp *et al.* (ref. 131) have reported on the effect of various *N*-alkylhydroxylamines on the kinetic lag phase of soybean lipoxygenase-1. It was found that this type of compound increases the induction period, decreases the steady-state reaction velocity, and causes the *g*6 EPR-signal of Fe(III)-lipoxygenase to disappear. Optimal effects were found with *N*-octyl- and -decylamines. On the basis of the EPR-results it was concluded that the lengthening of the kinetic lag phase and the reduction of the steady-state rate are due to the reduction of Fe(III) by the alkylhydroxylamines. The effects could be reversed by adding 13-HPOD. Strasser *et al.* (ref. 132) recently developed and tested a series of synthetic hydroxylamine derivatives and other types of inhibitors, in order to evaluate their potencies as inhibitors of 5-lipoxygenases and/or cyclooxygenase. Kemal *et al.* (ref. 133) studied the effect of a number of catechols, in particular nor-dihydroguaiaretic acid (NDGA, Fig. 25) on the activity of lipoxygenase. Upon adding this compound to Fe(III)-lipoxygenase, it was found that the *g*6 EPR-signal virtually disappeared. By subsequently adding 13S-HPOD the original EPR-characteristics and full activity could be restored. In addition to this type of reversible inactivation, an irreversible inactivation occurs upon longer incubation times of NDGA and lipoxygenase. The latter inactivation could, however, be completely prevented by catalase indicating the involvement of H_2O_2, probably formed from air oxidation of NDGA. Kemal *et al.* have determined the rate constants for the reduction of Fe(III)-lipoxygenase for a number of compounds (ref. 133). These results are summarized in Table 3.

TABLE 3* Rate constants for the reduction of Fe(III)-lipoxygenase-1; 25°C, 10 μM reductant

reductant	k_{obs} (s^{-1}) (pH 8.0)	k_{obs} (s^{-1}) (pH 9.0)
nordihydroguaiaretic acid (NDGA)	5.0	81
catechol	$1.7 \cdot 10^{-1}$	
hydroquinone	$7.5 \cdot 10^{-2}$	
esculetin	$8.5 \cdot 10^{-4}$	
caffeic acid	$4.2 \cdot 10^{-4}$	
norepenephrine	$1.9 \cdot 10^{-4}$	
2,6-di-*tert*-butyl-4-methylphenol (BHT)		$1.8 \cdot 10^{-2}$
α-tocopherol		$\sim 5 \cdot 10^{-4}$
dithiothreitol (DTT)	$\sim 1.3 \cdot 10^{-4}$	$\sim 10^{-4}$

*compiled from ref. 133.

In addition to the results from kinetic methods listed above, Van der Zee *et al.* (ref. 134) utilized EPR to investigate the possible occurrence of free radical metabolites upon reaction of this type of inhibitors with Fe(III)-lipoxygenase-1. With phenidone, catechol, hydroquinone, NDGA and *p*-aminophenol the formation of free radical species was clearly demonstrated. The structures of the free radical metabolites could be derived from the hyperfine splittings and confirmed by computer simulation. These results further substantiate that the one-electron

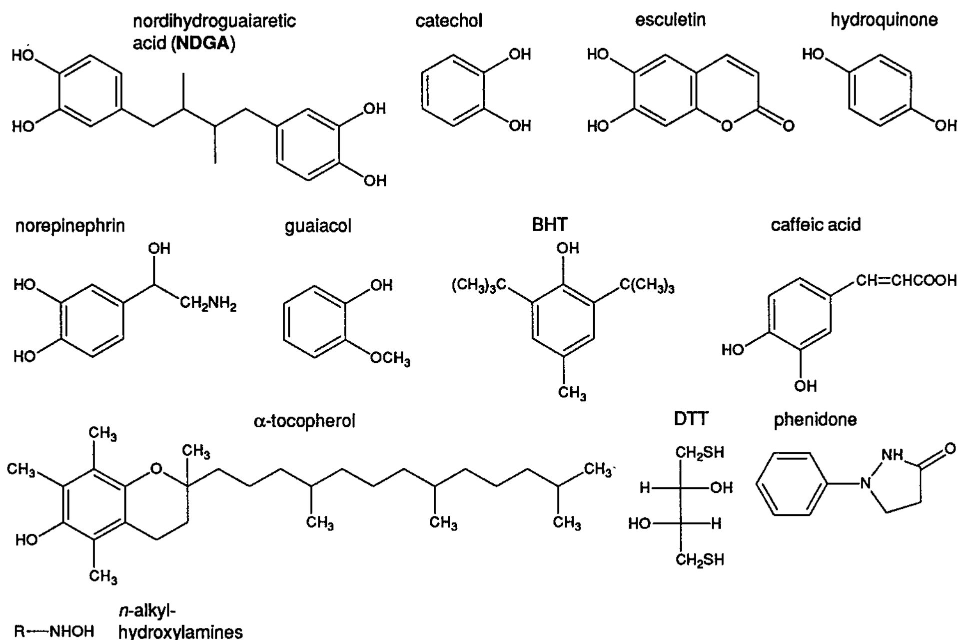


Fig. 25 Inhibitors of lipoxygenase which reduce Fe(III)-lipoxygenase-1 to Fe(II)-lipoxygenase.

reduction of Fe(III)-lipoxygenase is a key step in lipoxygenase-catalyzed hydroperoxide formation. In another paper from this laboratory (ref. 135) direct ESR-evidence was presented for the occurrence of fatty acid peroxo free radicals in the lipoxygenase reaction.

Reddanna *et al.* (ref. 136) have studied the effect of α-tocopherol on the activity of 5-lipoxygenase from potato tubers. They found a strong inhibition and presented evidence for the binding of vitamin E to a specific part of the polypeptide chain. Peterman and Siedow (ref. 137) have described the kinetics of the inhibition of soybean lipoxygenase-2 by a variety of phenolic compounds. It was clearly established that an o-dihydroxy functionality is essential for a compound to act as inhibitor of lipoxygenase-2. Another class of inhibitors, hydroxamates, do not formally reduce the Fe(III) of the enzyme, but bind and thereby inhibit strongly (refs. 137, 138) (Fig. 26). A related, neutral substrate analogue like arachidonamide (Fig. 26A) does not inhibit, but is converted into the analogue of 5S-HETE at a rate of about 7% of that of ETE. The *N*-hydroxyamide of arachidonic acid (Fig. 26B) was found to be a strong inhibitor (ref. 138), as well as salicylhydroxamic acid (SHAM, Fig. 26C; ref. 137).

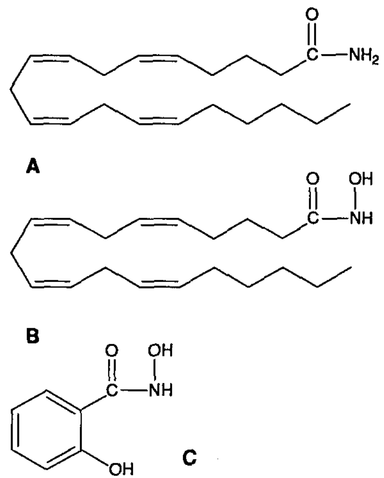


Fig. 26. Arachidonamide (**A**) is a substrate for the 5-lipoxygenase from rat basophilic leukemia cells, whereas hydroxamates **B** and **C** are strong inhibitors (refs. 137, 138).

Kristie and Thomson (ref. 139) have recently described some of the pitfalls in studying the inhibitory properties of certain compounds towards lipoxygenases, in particular if these compounds absorb strongly in the UV.

Hexaphenylhydrazone was described by Galey *et al.* as a mechanism-based inhibitor (Fig. 27; ref. 140). The molecular geometry and the redox properties of parts of the hexaphenylhydrazone are sufficiently similar to those of natural substrates for soybean lipoxygenase that processing exactly like a normal substrate does indeed occur. This eventually leads to the formation of an α-azohydroperoxide which inactivates the enzyme.

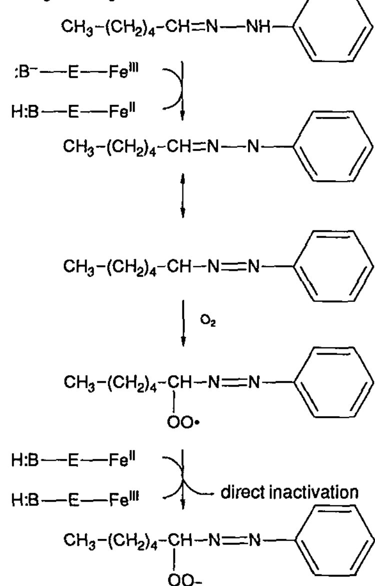
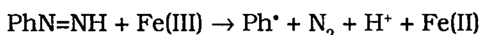


Fig. 27. Interaction of hexaphenylhydrazone with lipoxygenase. The resulting α-azohydroperoxide inactivates the enzyme (ref. 140).

Such inactivated enzyme was found to contain 1.8±0.8 residues of methionine sulfoxide (*cf.* refs. 141-143). The inactivation appears to be time- and oxygen-dependent and irreversible. Anaerobic preincubation of the protein with hexaphenylhydrazone does not affect the enzymic activity in any way. The data from aerobic experiments suggest an inactivation process involving product and enzyme. Inclusion of GSH-peroxidase in the reaction mixture prevented inactivation for up to 78%, indicating that a minor but significant part of inactivation occurs before the product is released from the enzyme (Fig. 27). This is also reflected in the results from EPR-studies in which over a wide range of concentrations a constant minimum number of 13±3 mols of hexaphenylhydrazone was found to be necessary to inactivate 1 mol of enzyme. This was calculated to correspond to 19% direct inactivation. The presence of linoleic acid protects the enzyme from inactivation, most probably by simple competition. The inactivated protein still contains one mol of iron, which is however, EPR-silent and in a high-spin state ($S = 2$) (ref. 140). A full characterization of the nature of this species will require further studies.

Inhibition of lipoxygenases and cyclooxygenase by various types of phenylhydrazones was first described by Wallach and Brown (ref. 144). Lipoxygenases from human platelets and soybeans appeared to be more sensitive to inhibition than cyclooxygenase. The inhibition was found to be non-competitive with respect to substrate. Gibian and Singh (ref. 145) have described extensive inhibition studies of soybean lipoxygenase with acetophenone phenylhydrazone and phenylhydrazine. It was concluded that in the latter case the actual inactivating species was phenyldiazene. One way of forming phenyldiazene is by autoxidation of phenylhydrazine, which also produces H_2O_2. Although some types of experiments were found hard to reproduce and/or difficult to interpret (ref. 145, and references cited therein), the evidence obtained supports the following conclusions on the mechanism. Phenylhydrazine requires O_2 to be effective as an inactivator. Autoxidation of phenylhydrazine, presumably initiated by the iron in lipoxygenase, produces phenyldiazene and H_2O_2 which subsequently inactivate the enzyme. Phenyldiazene inactivation of lipoxygenase might involve reaction with the enzyme's Fe(III) according to:



$$\mathrm{PhN{=}NH + Fe(III) \rightarrow Ph^{\bullet} + N_2 + H^+ + Fe(II)}$$

The protective effect of oleate indeed suggests that the process involves key elements at the active site of the enzyme.

Organic cofactor of soybean lipoxygenase-1

Phenylhydrazine and some of its derivatives have been applied in the determination of pyrroloquinoline quinone (PQQ) in 'quinoproteins'. This technique was also applied to establish the presence of this novel cofactor in soybean lipoxygenase-1 (ref. 146). However, methods to detect and quantitate PQQ do not seem to be very

robust, and any result requires independent confirmation (refs. 146-152). Recent X-ray structural analyses of methylamine dehydrogenase (refs. 153,154) indicate that PQQ may not exist as such when covalently bound to the protein, but rather as 'pro-PQQ' (Fig. 28). Obviously, the methodology to assess PQQ or any such cofactor in proteins, including lipoxygenase, needs further development before any realistic evaluation regarding its presence and possible role can be made (*cf.* ref. 155).

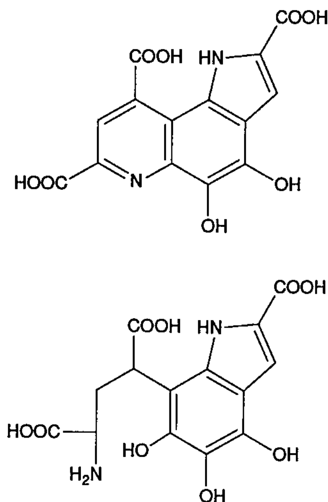


Fig. 28 Reduced forms of pyrroloquinoline quinone (PQQ, top) and 'pro-PQQ'.

pH-Dependence of product profiles

Stereo- and regiospecificities of lipoxygenase-catalyzed reactions are affected by the pH of the incubation medium. Recently, a system containing linoleate as the fatty acid substrate and soybean lipoxygenase-1 has been investigated in detail by Gardner (ref. 156). Substrates were incubated at various pH's ranging from 6.0 to 10, and products were fully characterized with respect to double bond geometry and enantiomeric composition. It was concluded that the non-ionized carboxylic group and the carboxylate anion are differentially recognized by the enzyme leading to two alternate possibilities for the substrate to bind. The presence of the carboxylic acid function apparently permits both substrate orientations which enables the formation of both 9S- and 13S-HPODs. However, the carboxylate anion may only be recognized and subsequently processed in one particular orientation eventually giving rise to 13S-HPOD. The possibility of two modes of substrate binding has been put forward also by Walstra *et al.* (ref. 157) in order to explain the observations with a 12-lipoxygenase from polymorphonuclear leukocytes, and by Kühn *et al.* (ref. 32) for the double dioxygenation mechanism of 15-lipoxygenase from reticulocytes.

Hiruta *et al.* (ref. 158) reported on the specific production of 9- and 13-hydroperoxo-γ-linolenic acids (γHPOTs) in a two-phase system consisting of an aqueous and a non-aqueous (hexane) phase. At high pH, predominantly 13-γHPOT is produced, whereas at lower pH the amount of 9-γHPOT prevails.

Xenobiotics and cooxidation

The primary products of lipoxygenase catalysis, unsaturated fatty acid hydroperoxides, are very reactive and questions regarding their metabolic fate and physiological actions are particularly relevant. In mammalian systems, substrates are dioxygenated and mostly released as mono- di- or tri hydroxy fatty acids.

Mechanistic schemes on the formation of fatty acid hydroperoxides also include reactive intermediates like alkyl or peroxo free radicals and evidence for their actual occurrence during lipoxygenase catalysis has recently been presented (refs. 103, 134, 135). Dissociation of intermediate enzyme free radical complexes of the catalytic cycle may then give rise to the release of such free radicals in solution. This may initiate random chains of radical reactions which may in turn lead to a wide variety of secondary products (for a recent review on unsaturated fatty acid free radical chemistry, see ref. 159). Cooxidation phenomena induced by lipoxygenase-catalyzed hydroperoxide formation may underly the evolution of off-flavors in foods during storage and other deteriorative processes. Lipoxygenases vary in their capacity to cause cooxidation, most probably because the stability of the enzyme-radical complex varies as well. Alkoxy free radicals may arise from the interaction of product hydroperoxide and the enzyme. Native lipoxygenase-1 from soybeans is a relatively poor cooxidation catalyst (ref. 160, 161). Compounds like carotenoids are particularly prone to cooxidative degradation (ref. 162, 163). However, other compounds may also be cooxidized, a process that may enhance the potential toxic effects of certain compounds. The role of lipid peroxides in the cooxidation of xenobiotics has been reviewed by Reed (ref. 164). The involvement of lipoxygenase in this type of cooxidation has been demonstrated by Hughes *et al.* (ref. 165) and Byczkowski & Kulkarni (ref. 166). Hughes *et al.* studied the 5- or 15-lipoxygenase-mediated conversion of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) into a diol epoxide, which is extremely carcinogenic. It was shown that this conversion is oxygen-dependent and can be inhibited by radical scavengers like butylated hydroxyanisole or NDGA.

Certain compounds, like those described in refs. 165 and 166 may gain extreme carcinogenicity upon activation by a cooxidative process involving lipoxygenase. The wide abundance of lipoxygenases in both plants and mammals emphasizes its possible role as a causative factor in chemical carcinogenesis.

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