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Fatty Acid Oxidation In Isolated Chloroplasts From The

Tropical Marine Chlorophyte, *Anadyomene stellata*

by

Debra Lynn Bemis

Doctor of Philosophy

in

Biology

Committee Members
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December 1998
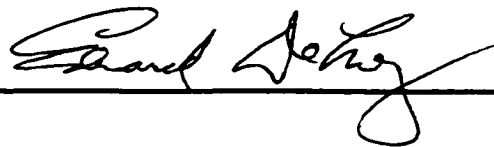
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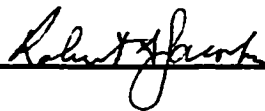
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DEDICATION

This dissertation is unquestionably dedicated to my parents, Jerry and Kay Bemis. They have always been right there, cheering me on, through whatever obstacle fell before me. I am extremely fortunate to be blessed with such loving parents. and am eternally grateful for their support. I will always be their biggest fan.

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1993   Bemis. D.L.. Brown. M.R.. and Jacobs. R.S. Effects of Topsentin. Pseudopterosin A - Methyl Ether, and Pseudopterosin E on LTC_4 and PGE_2 Release From Stimulated Mouse Macrophages.

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1993   American Society for Pharmacology and Experimental Therapeutics. Experimental Biology Meeting. San Francisco. California.

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ABSTRACT

Fatty Acid Oxidation in Isolated Chloroplasts from the Tropical Marine

Chlorophyte. *Anadyomene stellata*

by

Debra Lynn Bemis

The marine Chlorophyte. *Anadyomene stellata*. was utilized as a model to study fatty acid oxidation and resultant oxylipin production. Two oxidation reactions were observed. Initially. we identified the presence of five conjugated tetraene containing fatty acids by UV spectrophotometry and GC/MS suggesting fatty acid oxidase activity. Structural analyses of two novel compounds. determined by NMR experiments. led to identification of bosseopentaenoic acid (20:5) and stellaheptaenoic acid (22:7). a previously undescribed 22 carbon fatty acid. Further work pointed to the chloroplast as the subcellular organelle with high enzymatic activity largely responsible for the formation of these unique metabolites. Biosynthesis studies with six fatty acid substrates (palmitoleic. 6.9.12.15-octadecatetraenoic. arachidonic. eicosapentaenoic. 7.19.13.16-docosatetraenoic. and 4.7.10.13.16.19-docosahexaenoic acids) were carried out with the isolated chloroplast preparation. and revealed the capability of all substrates to support conjugated tetraene synthesis. Interestingly. the 22 carbon

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substrates were the only substrates able to support the biosynthesis of all five conjugated tetraenes identified. Kinetic analyses with all substrates revealed the enzyme preparation had the highest affinity towards arachidonic acid (20:4), but the greatest V_{max} was observed with 4,7,10,13,16,19-docosahexaenoic acid (22:6).

A semi-purified enzyme preparation was obtained following anion exchange chromatograph and gel filtration that showed high lipoxygenase activity, as measured by an increase in absorbance at 234 nm following the addition of linoleic acid. TLC analyses of the reaction products suggested the presence of 9-HODE based on comparison of R_f values obtained from standards. Furthermore, products from chloroplasts incubated with arachidonic acid were derivatized with 9-anthryldiazomethane (ADAM). These derivatives were analyzed by LC/MS (APcI⁻), and resulted in the identification of a compound with a molecular weight consistent with that of a dihydroperoxy eicosatrienoic acid. The presence of these oxygenated metabolites are indicative of lipoxygenase catalysis of the substrate.

Endogenous fatty acid concentrations in the chloroplasts were examined by GC/MS to determine the endogenous PUFAs present as potential substrates to these oxidative pathways. Chloroplasts isolated from algae collected in both the Florida Keys and the Mediterranean were analyzed and their resultant fatty acid profiles were compared. Interestingly, all samples contained high levels of 20

carbon PUFAs, comprising approximately 18 – 20% of total fatty acids. This is a unique finding for a Chlorophyte which typically have levels closer to 5% of total fatty acids. Significant differences were noted between algae collected at different sites, which may result from environmental factors.

To examine the relationship between these fatty acid oxidative pathways and their physiological role, arachidonic acid was added to the sea water in a controlled environment while oxygen flux from the thalli were measured. In both light and dark conditions, oxygen flux was significantly reduced suggesting that this fatty acid was able to enter the algal cells and elicit an effect. The order of magnitude of this effect was consistent with the theoretical net uptake of oxygen required for the various fatty acid oxidation reactions described.

This work has addressed a few key factors involved in the elucidation of fatty acid metabolic pathways in the chloroplasts of *A. stellata* resulting in unique oxylipins with apparent physiological significance.

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1.0 INTRODUCTION

1.1 *Anadyomene stellata*

Anadyomene stellata (Wulfen) C. Agardh is a macrophytic marine Chlorophyte found growing in dense clusters or forming a foliose, ruffled turf up to 8 cm tall (Littler and Littler, 1991) (Figure 1). These algae are generally found in shallow waters from the low intertidal to subtidal regions of tropical and subtropical waters of the Western Atlantic and Mediterranean (Littler and Littler, 1991). However, some species have been identified in waters as deep as 91.5 m. Dense clusters are typically found in intertidal habitats. While in the protected waters of subtidal areas, thalli are often seen in single, upright sheets. Morphologically, the *Anadyomene* thallus can be described as a thin blade or cluster of blades that are one to two cell layers thick with polychotomously branched veins arranged in a lacy pattern. The branched veins are not believed to be used for nutrient transport, as implied by the classical definition of this term, but rather as structural support. Interstitial spaces contain small cells whose arrangement is one factor considered in species differentiation. Intertwined rhizoids form a stipe that attaches the blades to the substrate.

Algae cannot be considered a monophyletic group with a common linear evolutionary sequence (Chapman, 1992). Due to the significant evolutionary distance between different classes of algae, a broad spectrum of

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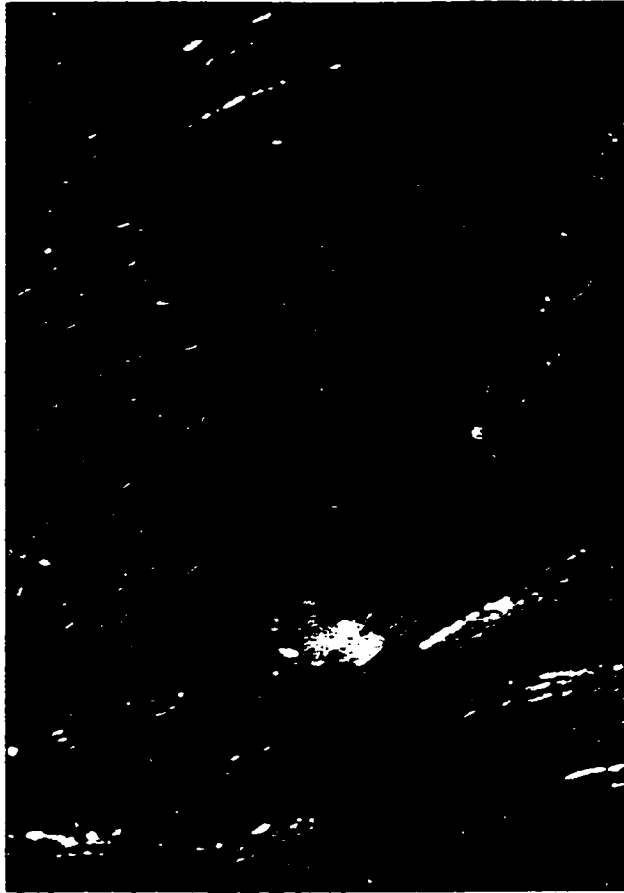
Figure 1. *Anadyomene stellata* (100x magnification)

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biochemical strategies enabling the algae to interact with and survive in their environment are observed. Because of the extreme diversity of this group of organisms, one must be extremely prudent when drawing conclusions based on observations from algae of different classes. In fact, some researchers have suggested that most algal groups may be more closely related to other protists than with each other (Kumar, 1990). Hence, many classification schemes have been presented for algae with differences depending on what guidelines and techniques were used. One of the most common systems proposed divides algae into six divisions: *Cyanophyta, Glaucophyta, Chromophyta, Rhodophyta, Chlorophyta,* and *Charophyta* (Lee, 1989). The division *Charophyta* is often included in the division *Chlorophyta,* and will be considered as one division for this discussion. A generally accepted classification scheme of Chlorophyta, or green algae, is its division into five classes based on ultrastructure studies: Pedinophyceae, Prasinophyceae, Ulvophyceae, Chlorophyceae, and Charophyceae (Sluiman, 1992). The first two classes comprise the more primitive, flagellate members of the phylum, while the last three include the more advanced macroalgae. Land plants are believed to have arisen from the evolution of the Charophyceae lineage. In regards to the biochemistry of subcellular organelles, Ulvophyceae and Chlorophyceae are quite similar. Distinct differences between these two classes and the Charophyceae group exist, with glycolate metabolism being a strong example. *A. stellata* belongs to the

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class Ulvophyceae. It is interesting to note that the ordinal assignment for this algae is still an issue of dispute amongst algal taxonomists. Some feel it fits more appropriately with the Cladophorales (van den Hoek, 1984; Wynne, 1986), others place it in the Siphonocladales (Bold and Wynn, 1978), and yet others believe it merges into both (O'Kelly and Floyd, 1984).

In field studies, we have observed that *A. stellata* is often found growing sympatrically with *A. saldanhae*. However, the two species can be easily separated by comparison of the interstitial cells under a light microscope. The interstial cells of *A. stellata* appear as small laterally stacked bars that are parallel to each other at vein bases, while those of *A. saldanhae* are randomly arranged (Littler and Littler, 1991). Furthermore, *A. stellata* thalli are typically lighter green and more undulating that those of *A. saldanhae* which also have a more leathery texture.

The life history of *A. stellata* involves the alteration of isomorphic generations between the diploid sporophyte and haploid gametophyte (Enomoto and Hirose, 1970; Mayhoub, 1975). Zoospores are quadriflagellates and gametes are biflagellate. As typical of other members of the order Siphonocladales, gametes are morophologically and physiologically indistinguishable (isogamous), and are referred to as (+) and (-) mating types (Lee, 1989). Flagellates (swarmers) are formed in localized regions on any portion of the thallus, excluding the base, in either veins or interstitial cells (Littler and Littler,

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1991). Flagellate formation occurs in approximately 10% of the thallus, and release is via a conical opening in the cell.

Interestingly, *A. stellata* is unique in that it is found in a broader variety of habitats than the eight other species of this genus, even habitats characterized by high herbivory (Littler and Littler, 1991). Other species appear to be restricted to regions with low herbivore populations. Such habitats include areas of high carnivorous predation in combination with a lack of protective shelter, areas that do not support herbivore existence, or areas with limited access to herbivores. The presence of a chemical defense system in *A. stellata* allowing it to inhabit such environments has been postulated (Targett and Mitsui, 1979; Littler and Littler, 1991). Two bioassays, fish erythrocyte hemolysis and fish mortality, were performed by Targett and Mitsui (1979) on 19 tropical marine macroalgae to detect the presence of bioactive substances that may confer chemical defense. *A. stellata* was ranked as the most toxic algal species in the fish mortality assay with an LD_{50} 10-11 fold lower than all other species. Results from the fish erythrocyte hemolysis assay demonstrated that *A. stellata* was the second most effective speices in the group tested, with *Dictyota dichotoma* having the most toxic effect. From these results, it would appear that *A. stellata* could be chemically defended.

Field observations of *A. stellata* provide supporting evidence for the presence of a potent chemical defense system. We, along with other researchers,

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have observed that *A. stellata* commonly grows sympatrically with *A. saldanhae*. A potential plant - plant defense association has been proposed for this close association suggesting that *A. saldanhae.* which is possibly a quite palatable species to herbivores. may select for refuge amongst the potentially toxic *A. stellata* (Littler and Littler. 1986: Targett and Mitsui. 1979). Another observation that we have made in agreement with other workers. is the high concentration of crustacean epifauna living amonst the ruffled thalli of this algae. A study conducted in the Gulf of Mexico reported significantly more crustaceans finding habitat within *A. stellata* than in any of the other macroalgae and seagrass considered (Lewis. 1987). Littler and Littler also suggest that this observation is not explained by predation of the crustaceans on the algae. but is an advantage gained by organisms coexisting amongst this potentially toxic algae (1991).

1.2 Fatty Acid Biochemistry in Algae

Because this research considers certain aspects of fatty acid biochemistry in an Ulvophycean alga, a brief description of the biochemical roles of organelles involved in these reactions is now given. These organelles include chloroplasts. peroxisomes. and mitochondria. When class-specific data is unknown. generalizations relating to algae and / or plants will be noted.

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1.2.1 Chloroplasts

In all Chlorophyta, chloroplasts are characterized by having only a double membrane with no chloroplast endoplasmic reticulum. and by storing starch in the chloroplasts rather than the cytoplasm (Kumar. 1990). Chloroplasts of Rhodophycean and Charophycean algae. as well as those of higher plants. are also characterized by having only a double membrane. However. most other eukaryotic algal chloroplasts are surrounded by an additional one or two membranes of the chloroplast endoplasmic reticulum. Beyond the obvious role chloroplasts have in photosynthesis. these organelles are also known as the principle site of fatty acid synthesis (primarily palmitic and oleic) in plants and algae. The chloroplasts provide and ideal environment for this anabolic process since there is a high $[NADPH]/[NADP+]$ ratio which provides the reducing environment required for fatty acid synthesis (Lehninger er al.. 1993). The high concentration of reducing equivalents is generated by non-cyclic electron transport.

1.2.2 Peroxisomes

Peroxisomes are defined as organelles with the structural features of microbodies containing at least one characteristic enzyme of the microbody

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family (Stabenau, 1992). In plants, these enzymes include catalase, enzymes involved in the degradation of purines and fatty acids, and enzymes for glycolate metabolism and the glyoxylate cycle. Most of these enzymes are also found in other cell compartments in algae, but if found in microbodies then the organelle is termed a peroxisome (Stabenau, 1992). The current thought regarding peroxisomes is that they are multifunctional organelles that can adapt to different metabolic requirements by acquiring or loosing the enzymes listed above (Stabenau, 1992).

Although the size, shape, and enzyme content of algal peroxisomes differs, a few characteristics are consistent within algal classes. This includes the presence of enzymes for glycolate metabolism, glyoxylate cycle, and B-oxidation of fatty acids. From an accumulation of data from all classes of algae, the general trends in green algae suggest that peroxisomes of Ulvophyceae and Chlorophyceaen algae contain enzymes for B-oxidation but not for glycolate metabolism (Stabenau, 1992). Peroxisomes of Charophyceaen algae, however, contain enzymes for glycolate metabolism and B-oxidation. None of the green algae utilize peroxisomes for the glyoxylate cycle, while catalase appears to be a consistent peroxisomal component in all Chlorophytes. Another distinguishing point between both the Ulvophyceae and Chlorophyceae and the Charophyceae is the main enzyme utilized for glycolate metabolism. In the Charophyceae algae, glycolate oxidase located in the peroxisomes is utilized, while

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mitochondrial glycolate dehydrogenase is found in algae of the Ulvophyceae and Chlorophyceae classes.

The role of fatty acid B-oxidation in peroxisomes of plants and algae is to provide biosynthetic precursors from lipid stores. Unlike animals. B-oxidation is not a significant source of metabolic energy in plants (Gerhardt. 1992). Furthermore. lipids are not stored in significant amounts to be available as energy reserves in green algae. except during certain stress conditions (Thompson Jr.. 1996). But. this pathway is useful in protecting the organism from the harmful effects of free fatty acids and is likely involved in carbon recycling during senescence (Gerhardt. 1992). During the B-oxidative pathway. fatty acids are released from triacylglycerols via lipases and activated to their coenzyme A derivatives prior to being oxidized. The final end product of this oxidation is acetyl-CoA which is then exported. Reducing equivalents produced in peroxisomes following B-oxidation are exported to the cytosol and eventually to the mitochondria.

1.2.3 Mitochondria

The mitochondria is often the site of many reductive reactions because of the high [NADH]/[NAD$^-$] ratio formed from the flow of electrons into NAD$^-$ from the oxidation of amino acids. pyruvate. and acetyl-CoA (Lehninger *et al.*.

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1993). Plant and algal mitochondria do not contain enzymes for B-oxidation. unlike animal mitochondria. Acetate production for fatty acid synthesis occurs in this organelle. This association between the mitochondria and chloroplast regarding fatty acid synthesis will be discussed in the next section.

1.2.4 Fatty acid Synthesis and Desaturation

The main site for fatty acid synthesis in plant and algal cells is the chloroplast. Interestingly. the plant fatty acid synthetase enzymes (PFAS) are of a prokaryotic type with non-associated proteins. unlike that seen in animal systems (Stumpf et al.. 1982). Fatty acid synthesis in the chloroplasts begins with the formation of acetyl-CoA from acetate and culminates in the production of palmitic (16:0) and oleic (18:1) acids. Acetyl-CoA is synthesized via the pyruvate dehydrogenase complex (PDC) located in the plant mitochondria (Stumpf et al.. 1982). The substrate for this complex. pyruvate. is produced in the cytosol from dihydroxyacetone phosphate which is formed during CO_2 fixation in the chloroplast. Following synthesis. the acetyl-CoA can proceed in two directions: (1) it can be hydrolyzed to acetate which diffuses into the cytosol. or (2) it can be oxidized via the TCA cycle and result in ATP formation. Once in the cytosol. acetate can diffuse into the chloroplast where it is synthesized into acetyl-CoA via acetyl-CoA synthetase. and is utilized for fatty acid synthesis.

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Thus, fatty acid synthesis involves biosynthetic coupling between chloroplasts and mitochondria.

Usually, the fatty acid content of green algae is similar to that of the vegetative tissues of higher plants, containing mainly a variety of unsaturated C16 and C18 fatty acids (Thompson Jr., 1996). The fatty acid compositions of non-Chlorophyte algal species differs in that PUFAs with chain lengths of 20 carbons are more frequently found (Pohl and Zurheide, 1979).

Desaturation of 16:0 and 18:1 has been reported to occur via two main pathways involving lipid-linked desaturases in the endoplasmic reticulum and the chloroplast in plants (reviewed in Browse and Sommerville, 1991). The respective pathways of PUFA synthesis in algae are believed to be similar to those characterized in plants, at least for PUFAs of up to 18 carbon chain lengths (Norman *et al.*, 1985). These reactions have been referred to as the prokaryotic and eukaryotic pathways. The diverse distribution of fatty acids on glycolipid species suggests simultaneous occurrence of both pathways is occurring. The prokaryotic pathway proceeds entirely in the chloroplasts and leads to the synthesis of glycolipids containing 18 carbon fatty acyl residues at the *sn*-1 position and 16 carbon residues at the *sn*-2 position. In this pathways, 18:1 is stepwise desaturated to 18:3 (n-3) while linked to a galactolipid. The eukaryotic pathway involves the transport of 18:1 from the chloroplast to the cytoplasm where it is acylated to phosphatidylcholine (PC). After acylation, it is

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desaturated to 18:2 and 18:3 (n-3). PC containing 18:2 is converted to diacylglycerol (DAG) species which are transported back into the chloroplasts. Once in the chloroplast. DAG is galactosylated and further desaturated. Glycolipid species produced via this pathway contain 16 or 18 carbon fatty acyl moieties at the sn-1 position and 18 carbon residues at the sn-2 position. A complete listing of all desaturases and elongases present in algae to account for the various fatty acid and lipid species observed has not yet been compiled. However. it has been noted that in photosynthetically active green algal species. lipids of the prokarytoic pathway comprise over half of the whole cell lipids (Thompson. Jr.. 1996).

1.2.5 Lipid Composition

Most fatty acids in both plants and animals do not occur in the free state. but rather bound to complex lipids such as phospholipids and glycolipids. The fatty acids comprising the phospholipids and glycolipids of both plants and animals have the cis- configuration. The one exception known to occur in nature is the trans-hexadec-3-enoic acid found in at the sn-2 position of phosphatidylglycerol (PG) in the chloroplast envelope and thylakoid membranes of photosynthetic eukaryotes (Wood. 1974: Haverkate and van Deenen. 1967).

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Interestingly, this phospholipid species is absent from envelope membranes purified from non-photosynthetic tissues (Alban *et al.*, 1988).

In addition to the presence of PG with *trans*-16:1, other differences between chloroplast and extraplastidal lipids exist. Phospholipids are more predominantly located in the extraplastidal membranes, while galactolipids (monogalacto- and digalactodiacylglycerol: MGDG, DGDG) and sulpholipids are found almost exclusively in the chloroplasts (Thompson Jr., 1996). While galactolipids typically compose the largest portion of chloroplast lipids, ratios of MDGD / DGDG in envelope and thylakoid membranes could represent species specific trends. PG is typically the most abundant phospholipid found in chloroplasts. Phosphatidylethanolamine (PE), on the other hand, has been suggested not to be a chloroplast membrane constituent, and its presence in membrane preparations could reflect contamination (Mazliak, P., 1977). Green algae are reported to have roughly the same classes and proportions of galactolipids, phospholipids and sulpholipids as higher plants, with the exception of the possible presence of the non-phosphorous containing polar lipid diacylglycerol trimethylhomoserine (DGTS) not found in higher plants.

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1.3 Eicosanoids / Oxylipins in Marine Organisms

Oxidative metaobiltes of arachidonic acid (AA). termed eicosanoids. are of intense interest to researches for their well known effects in signaling a variety of fundamental physiological processes in mammals. They act locally to mediate such processes as the inflammatory cascade. production of pain and fever. regulation of blood pressure. initiation of blood clotting. smooth muscle contraction involved in labor. and the regulation of sleep / wake cycles (Voet and Voet. 1990). Biosynthesis of these compounds has been fairly well characterized in mammals. and involves the release and subsequent oxidation of AA found esterified to the sn-2 position on the glycerol backbone of phosphatidylinositol and other phospholipids. Upon membrane injury or hydrolysis by phospholipase A_2. AA is cleaved from the phospholipid and metabolized by at least three main enzymatic mechanisms: lipoxygenase. cyclooxygenase. and cytochrome P_{450} monoxygenase pathways. A simplified scheme of these pathways and the metabolites produced is illustrated in Figure 2.

Given the importance of these molecules. it is of no surprise that the identification of prostaglandin A_2 (PGA$_2$) in the soft coral *Plexaura homomalla* by Spraggins and Weinheimer in 1969 initiated an exciting new field of study focused on eicosanoid biosynthesis in marine organisms. Avid research in this field has demonstrate that these compounds represent a structurally diverse group of natural products that are found in virtually every class of marine organisms

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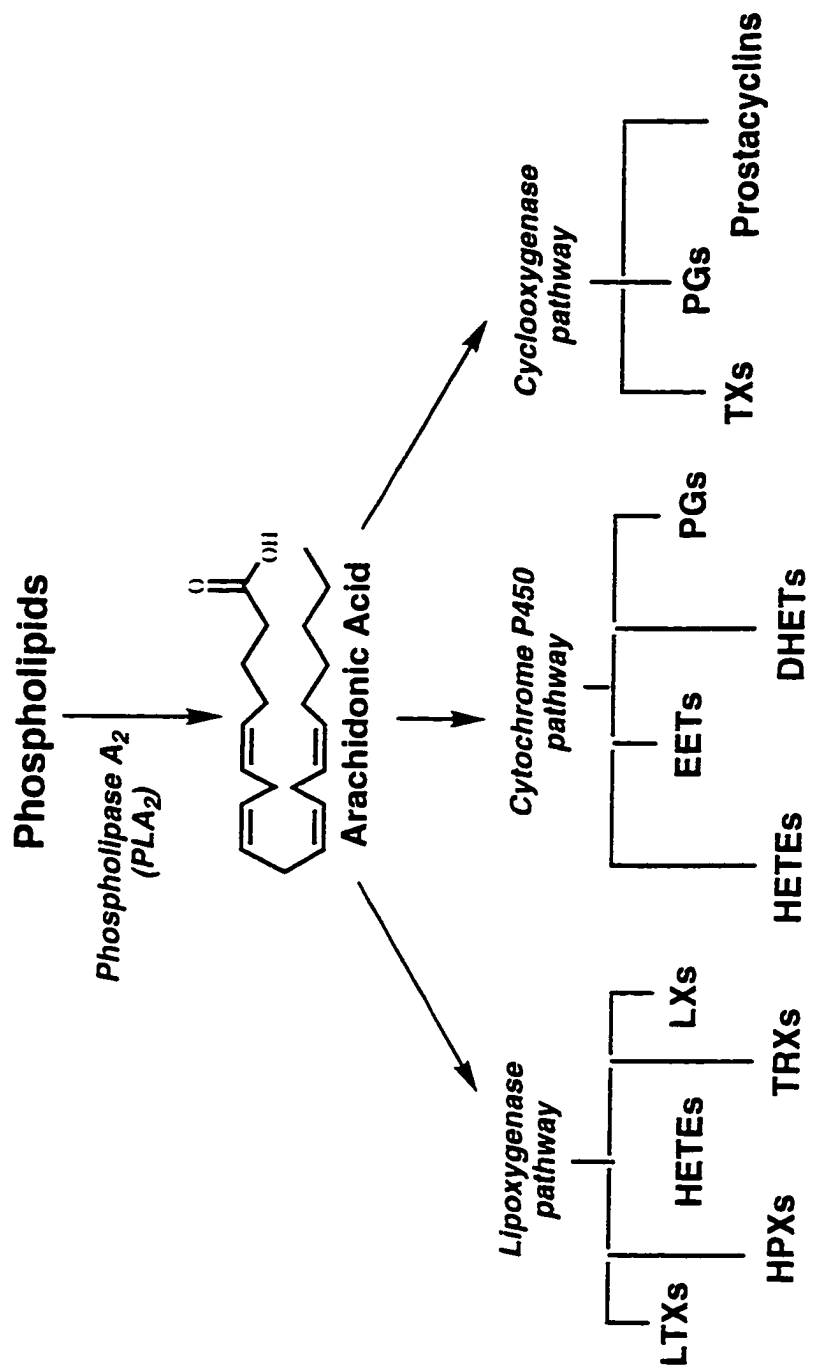
Figure 2. Arachidonic acid cascade as defined in mammalian systems. Abbrev.: LTXs, leukotrienes; HPXs, hepoxilins; HETEs, hydroxyeicosatetraenoic acids; TRXs, trioxylins; LXs, lipoxins; EETs, epoxyeicosatrienoic acids; DHETs, dihydroxyeicosatrienoic acids; PGs, prostaglandins; TXs, thromboxanes.

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(Gerwick *et al.*, 1993; Gerwick, 1994; Rychnovsky *et al.*, 1992; Jacobs *et al.*, 1993). In fact, marine organisms have been considered as the richest source for structural diversity in this class of compounds. Since the term 'eicosanoid' (eikosi = 20, Greek) suggests an oxidative metabolite containing a 20 carbon chain length, need for a more encompassing term quickly arose with the continual discovery of novel structures containing varying chain lengths. To address this issue, Gerwick proposed the term 'oxylipin' to represent fatty acid metabolites of varying chain lengths formed by reactions involving at least one step of mono- or dioxygenase-dependent oxidation (Gerwick *et al.*, 1991).

Among the wide variety of marine algal species found to be capable of oxylipin biosynthesis, a few generalizations have been compiled. Cyanobacteria typically appear to metabolize shorter-chain fatty acids (C14-C18) resulting in only hydroxy fatty acids (Gerwick and Bernart, 1993). No carbocyclic or oxycyclic metabolites have been noted for this group. Rhodophytes are typically seen to utilize 12-lipoxygenase-mediated oxidation of C20 fatty acids, while oxidation of C18 and C20 fatty acids mediated by lipoxygenases with n-6 specificity has been observed in algae of the class Phaeophyceae (Gerwick, 1994). Oxylipin biosynthesis in algal species of the class Chlorophyceae has generally been observed to occur via oxidation at C9 and C13 of C18 fatty acids (Gerwick, 1994). However, with the constant discovery of unique oxylipin structures, these observations are continually challenged.

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1.4 Algal Oxylipin Biosynthesis

Because eicosanoids play such crucial roles in mammalian physiology. researchers' interest in gaining new perspectives on modulation of mammalian pathways and potential elucidation of new schemes has led to increased attention towards understanding oxylipin biosynthesis in other organisms. Marine organisms have been found to be rich in PUFAs of which many can serve as appropriate substrates for a variety of lipoxygenase enzymes (Gerwick. 1996). Gerwick and Bernart have reported numerous marine oxylipins which they suggest have been derived from the activities of 5-. 8-. 9-. 11-. 12-. 15-lipoxygenases (Gerwick. 1994; Gerwick and Bernart. 1993). Oxygenation catalyzed by LO activity on PUFAs resulting in hydroperoxy fatty acid formation appears to be a common first step in the biosynthesis of many marine oxylipins (Gerwick and Bernart. 1993). Ensuing steps appear to involve enzymes of the jasmonic acid pathway. or more generally known as the octadecanoid biosynthetic pathway.

1.4.1 Lipoxygenases in Marine Organisms

Lipoxygenases are enzymes that catalyze the double oxygenation of polyunsaturated fatty acids containing a (Z.Z)-1.4-pentadiene configuration to a conjugated (E.Z)-2.4-diene hydroperoxide (Gaffney *et al.*. 1995). This family of

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enzymes are non-heme, non-sulfur iron containing enzymes that insert molecular oxygen via hydrogen abstraction and peroxide addition (Figure 3). The primary mammalian lipoxygenases recognized insert peroxide into AA at carbons 5, 12, and 15. Hence, these enzymes are known as 5-, 12, and 15- LO's. Plant LOs appear to be similar to mammalian LO's, except that the preferred substrate in plants is linoleic acid and 13- or 9-hydroperoxides are formed from this substrate. A variety of LO's in marine organisms have been implicated based on product analysis (Gerwick, 1994; Gerwick and Bernart, 1993). However, most LO plant genes isolated so far can be grouped into one of two gene families based on overall sequence similarity: LOX-1- and LOX-2-type enzymes (Voros *et al.*, 1998). At a biochemical level, LO's have been characterized by comparisons of product formation, substrate specificity, pH optimum, molecular mass and enzymatic stability (Axelrod *et al.*, 1981). In regards to the aquatic environment, direct LO activity has been observed in fresh water cyanobacteria *Oscillatoria* (Beneytout *et al.*, 1989), unicellular green algae (Zimmerman and Vick, 1973), and macrophytic green alga *Enteromorpha intestinalis* (Kuo *et al.*, 1996).

Enzymes of the lipoxygenase family act in a stereoselective manner that appears to be somewhat dependent on the organism. All characterized mammalian lipoxygenases form hydroperoxides of the *S*-configuration (Brash *et al.*, 1996). Lipoxygenases with *R* stereochemistry are observed in a variety of marine invertebrates (Corey *et al.*, 1987; Brash *et al.*, 1987; De Petrocellis and

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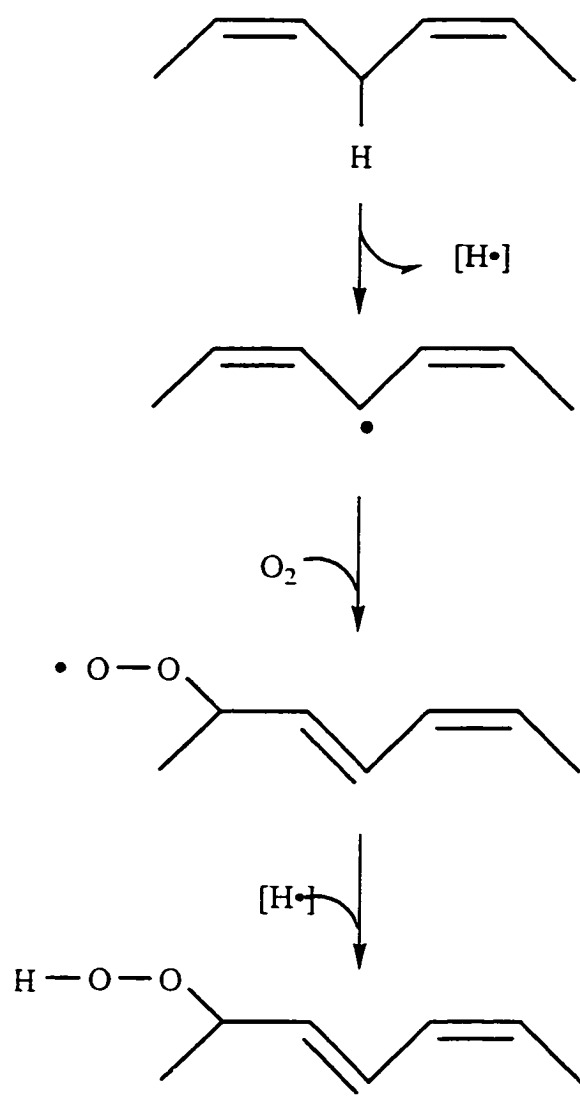
Figure 3. Enzymatic mechanism of lipoxygenase enzymes (Voet and Voet. 1990).

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Di Marzo. 1994). Interestingly. prostaglandin synthesis in gorgonians. such as *P. homomalla*, has been demonstrated to occur via *R*-LO activity (Bundy. 1985). This is a completely different enzymatic mechanism than observed in prostaglandin production in mammals which is mediated by cyclooxygenase or monooxygenase pathways. LO's with *S*-stereospecificity are predominant in the plant kingdom. however prostaglandin formation observed in some marine algae and higher plants has also been proposed to be catalyzed by *R*-LO activity (Bundy. 1985: Gerwick. 1994). Surprisingly. a few examples of *R*-LO products have been isolated in mammalian tissues such as skin and cornea. however their biosynthesis has previously been proposed to involve a cytochrome P_{450} rather than a *R*-LO (Baer *et al.*. 1991: Schwartzman *et al.*. 1987: Woolard. 1986: Holtzman *et al.*. 1989). Brash and co-workers have recently suggested that *R*-LO should be considered a potential source of these *R*-configuration hydroperoxides (Brash *et al.*. 1996).

While LO products are known to be protent mediators of inflammatory responses in mammals. they have important roles in plants. invertebrates. and algae as well. Oxylipin biosynthesis involving LO dioxygenation as a first step are thought to be involved in the signaling of growth and senescence. wound response. and response against pathogen attack (Rosahl. 1996: Creelman and Mullet. 1997). These compounds have also been identified as hatching factors in a variety of barnacles (Hollald *et al.*. 1985; Hill *et al.*. 1988). chemical defenders

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in the opisthobranch mollusc *Tethys fimbria* (Marin *et al.*, 1991), and also as potential chemical defenders in *P. homomalla* (Gerhardt, 1991). Numerous prostaglandins and hydroxy fatty acids identified in various algal species are thought to be derived from biosynthetic pathways involving *R*-LO activity (Gerwick, 1994; Gerwick and Bernart, 1993). Physiological significance of these metabolites is still quite unclear. However, roles in osmoregulation by ATPase ion pump modulation, mediation of the synchronous development of gametes and their release in red algae, wound response, chemical defense, and regulation of growth and development have been suggested (reviewed in Lamacka and Sajibidor, 1995).

The important role of oxidative fatty acid metabolites in plants is illustrated by a consideration of hydroperoxide metabolism and octadecanoid biosynthesis. This pathway beginning with hydroperoxides produced from LO-mediated dioxygenation of α-linolenic acid has previously been thought to culminate in the production of jasmonic acid, and was named the jasmonic acid pathway (Vick and Zimmerman, 1984). However, it has recently been determined that other cyclic fatty acid metabolites produced in this pathway also have distinct biological roles in plants. The global term 'octadecanoids' has now been adopted. This family of compounds has been demonstrated to be senescence promoters (Ueda and Kato, 1981; Parthier, 1991), signal transducers in chemical defense mechanisms (Farmer and Ryan, 1990; Gundlach *et al.*,

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1992), and mediators in mechanotransduction (Falkenstein *et al.*, 1991; Weiler *et al.*, 1993). Further research is likely to uncover even more roles for these potent mediators.

The octadecanoid biosynthetic capability of other organisms is a new area of research that has already revealed interesting data. This group of enzymes, including both the allene oxide synthases and the hydroperoxide lyases, are quite common in plants, but are so far believed to not exist in animals (Brash *et al.*, 1991). Allene oxide synthase and hydroperoxide lyase activities were detected and their products isolated in starfish oocytes (Brash *et al.*, 1991). Additionally, all enzymes involved in jasmonic acid synthesis were identified in the unicellular green algae *Chlorella pyrenoidosa*, with the exception of allene oxide cyclase which was not investigated (Vick and Zimmerman, 1989). The presence of these biosynthetic pathways in both higher plants and *Chlorella* suggests that these mechanisms of fatty acids oxidation are well-conserved (Vick and Zimmerman, 1989). However, much work is needed to gain a broader understanding of the distribution of octadecanoid biosynthesis.

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1.5 *A. stellata* as a Model for Studies of PUFA Metabolism and Resultant Oxylipin Production

We found *A. stellata* to be an ideal species for studies of PUFA metabolism for a variety of reasons. Since this alga is relatively abundant and is readily accessible. supplies were not a concern. and repeated qualitative observations of the collecting sites suggested minimal environmental impact. The large uniserate veins and characteristic organization of the interstitial cells allowed for relatively facile identification of the algae. Unlike plants. the lack of an integrated transport system in algae creates a unique situation requiring each cell to respond independently to the environment. This characteristic in conjunction with the relatively large size of the algal cells renders them ideal for observation and experimentation. Finally. previous work by other researchers suggests the presence of biologically active compounds that may be used as a means of chemical defense for this alga (Targett and Mitsui. 1979). These observations present *A. stellata* as an ideal model for physiological and biochemical studies.

The purpose of the work presented here was to use *A. stellata* as a model to study fatty acid oxidation and eicosanoid / oxylipin production. The chloroplast of this alga was discovered to be a site of synthesis for numerous unique oxidative metabolites of fatty acids. Complete characterization of the metabolic system leading to the production of these fatty acid metabolites is

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beyond the scope of this project. However, key factors were addressed towards this end. Enzymatic activities leading to oxylipin production, including potential oxidase and lipoxygenase reactions were detected. Furthermore, these metabolites were identified in isolated chloroplasts following stimulation of biosynthetic pathways with exogenous PUFA substrates. The pool of endogenous fatty acid precursors located in chloroplast membranes available for enzymatic oxidation was also analyzed. Lastly, a pronounced physiological response of the algae to AA exposure was detected, offering clues towards elucidation of potential roles for these interesting metabolites. Although a diversity of oxylipin structures has been identified in marine algae, an understanding of their biosynthetic pathways and physiological significance is still lacking. This work offers important data that will add to that of others, and eventually lead to uncovering these mysteries.

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2.0 MATERIALS AND METHODS

2.1 Algae Collections

2.1.1 Florida Keys

Anadyomene was collected 1 – 2 miles off the northwest coast of Long Key. Florida in approximately 0.3 to 2.0 meters of water. Water temperatures ranged from 14 – 31.5 °C, while average salinity was 36 g/L. Actual temperature and salinity measurements are listed in Table 1. Immediately following collection. the algae was put into sea water and transported back to the Keys Marine Laboratory. Long Key. Florida. Upon arrival. it was cleaned of infauna, epizooa. and epiphytes in a constant flow sea water table. Identification of the algae was readily accomplished using a light microscope. and resulted in the identification of two species. *stellata* and *saldanhae*, growing sympatrically. Based on the work of Littler and Littler (1991), the two species of *Anadyomene* were distinguished from each other on the basis of the branching pattern of the uniserate veins and organization of the interstitial cells of their unistratose blades. Following sorting. the algae were damped dried. placed in 1 gallon size freezer bags, and flash frozen in liquid nitrogen prior to storage in a −80°C freezer. Taxonomic voucher specimen of the species studies are deposited in the Algal Collection. U.S. National

Table 1. Water Temperature and Salinity in the Florida Bay (KML)		
Collection Date	Temperature (°C)	Salinity (g/l)
3/24/94	27.5	35
3/25/94	28	35
3/26/94	28	35
3/27/94	28.5	37
3/28/94	29	37
6/28/94	31.5	36
9/8/94	29.5	36
9/9/94	29	37
9/10/94	29	36
6/21/95	27.5	34
6/22/95	27	35
3/11/96	15	37
3/12/96	14	37
9/12/96	30	36
9/13/96	30	36
6/10/97	27	39
6/11/97	27	38

Herbarium, National Museum of Natural History, Smithsonian Institution (Washington D.C.).

Experiments utilizing live algae (not previously frozen) were conducted at either the Keys Marine Laboratory or at the Smithsonian Marine Station at Link Port (SMSLP), Fort Pierce, Florida. Since *Anadyomene* is not found off the coast of mid-Florida, it was necessary to transport the algae from the KML to SMSLP for these experiments. Ice chests equipped with continuous air supply and fresh sea water obtained near the collection site were used to insure the health of the algae during the transportation. Fresh sea water was brought back to SMS, filtered through a 0.2 μ filtration system, and used for all experiments.

2.1.2 Bahamas

A. stellata distribution was also considered throughout the Bahamas during research expeditions that took place for a three week period each summer from 1993-1996. The trips were supported by an NSF grant awarded to W. Fenical and R.S. Jacobs. Waters around the following islands were explored: Little San Salvador, Chubb Cay, Andros Island, Eleuthera Island, Exuma Cays, Acklins Island, and Crooked Island. *A. stellata* was found in the greatest abundance and physical state in the lagoon on Little San Salvador Island in 0.5 to 2 meters of water on a rocky substrate underneath an overhanging ledge.

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Average thalli diameter was between 3-4 cm. Additionally. we surveyed the waters around Lee Stocking Island during a exploratory visit to the Caribbean Marine Research Center (CMRC) located on the island. The project was funded by CMRC with intent of surveying the facilities to determine if it would be an appropriate marine research station for our needs. *A. stellata* was found in very small quantities at only one of seven locations observed near the island. This site is named the `Waterfall` and is located at the mouth of a channel that empties into the surrounding ocean.

2.1.3 Mediterranean Sea

Three distinct sites in the Mediterranean Sea were chosen for studies involving the comparison of chloroplast fatty acids. The sites were Daskalio and Ftelios located on the coast of Greece mainland. and Vagias Beach on the coast of the Greek Island. Aegina. *A. stellata* was the predominant *Anadyomene* species at all three sites.

The collection site at Daskalio is a small bay with a rocky shoreline. This was the highest energy site of all three Mediterranean sites in terms of surge and wave action. *A. stellata* was found growing on rock substrate in 0.5 to 2 m of water. The average thallus size was approximately 1.25 cm in diameter. The abundance and physical condition of the algae collected at this location were

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superior to the other two collection sights. Algae were collected in October of 1997 with a water temperature of 17 °C and a salinity of 36.34 g/L.

Ftelios, a protected cove, was the lowest energy site regarding wave action and surge. *A. stellata* was found growing on an unidentified rod-like sponge, the brown algae *Cystoseira*, and rock substrate in 0.5 m of water. The alga was much less abundant at this site than Daskalio, but the average thallus diameter was 2.0 cm. A collection at this site was made during end of October, 1997 with a water temperature of 16.5 °C and a salinity of 36.07 g/L.

The last of the three sites, Vagias Beach on Aegina Island, is similarly characterized by large rock slabs entering the water. The distribution and scarcity of *A. stellata* at this site was quite unique. It was found growing only on one small rock located about 7 m off shore. The algae were covered in a thin layer of sediment. No sightings of *A. stellata* were made on the rock substrate lining the shore. The quantity present was by far the least of all three sites and the physical condition of the algae was poorest. Average thallus size was 1 – 1.5 cm in diameter. Algae were collected in November, 1997 with a water temperature 19 °C and salinity at 37.64 g/L.

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2.2 Conjugated Tetraene PUFA Identifiation in Algae Homogenates

2.2.1 Lipid Extraction

Approximately 100 g of frozen algae were ground with a mortar and pestle in liquid nitrogen, and homogenized at 0 °C with a Tekmar (Cincinnati, OH) homogenizer in chloroform / methanol (2:1). A Buchner funnel fitted with Whatman (Clifton, NJ) filter paper #1 was used to filter the homogenate which was then purified further by the addition of distilled water with 1 M NaCl in a 1:10 final volume. The organic phase was dried with anhydrous Na_2SO_4, filtered and reduced *in vacuo*. One milliliter of hexanes was used to re-suspend the dry sample.

2.2.2 Diazald Methylation

Next, the sample was methylated with diazomethane gas as described by Lombardi (1990). Briefly, 5 grams of Diazald (Aldrich Chemical Co., Milwaukee, WI) was dissolved in 75 mls ethanol. Diazomethane gas was generated upon addition of 10 N NaOH into this reaction flask. The sample was dissolved in a separate round bottom flask with 100 mls of dichloromethane and then subjected to the diazomethane gas produced in the reaction flask. Following

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the methylation reaction, Na_2SO_4 was added to the methyl ester preparation to insure complete dehydration.

2.2.3 Vacuum Liquid Chromatography and UV Spectrophotometry

Methyl esters were chromatographed over TLC grade silica gel under vacuum with increasing percentages of ethyl acetate in hexane from 0 to 100% ethyl acetate. Fractions were then analyzed by UV spectrophotometry by scanning the samples in the UV range against a blank of hexanes with either an IBM (Danbury, CT) 9430 UV-VIS scanning spectrophotometer, a Shimadzu UV-265 UV-VIS Recording Spectrophotometer (Shimadzu, Kyoto, Japan), or a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer. The presence of conjugated tetraene containing compounds is readily identified by this method based on the characteristic absorption pattern observed for these compounds with λmax at 294, 306, and 322 nm.

2.2.4 HPLC Separation of Conjugated Tetraene PUFAs

Fractions identified to contain conjugated tetraenes were further purified via straight-phase HPLC using either a Hitachi (Mountain View, CA) 4200 or a Waters (Milford, MA) M-6000 A pump with a Waters Lambda Max 480 UV

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detector. Two 4 mm x 30 cm Alltech (Deerfield, IL) 10 μ silica columns were connected in series and an isocratic solvent system of ethyl acetate / hexanes (1:99) was used with a 3 ml/min flow rate. UV detection was set at 328 nm.

2.2.5 Structural Analysis – GC/MS and NMR

Gas chromatography / mass spectrometry (GC/MS) analyses of HPLC purified fractions were accomplished in collaboration with Dr. William Gerwick of the Department of Pharmacy, Oregon State University using instrumentation in his laboratory (Hewlett-Packard Model 5890 Series II gas chromatograph and an HP5971A quadrupole mass selective detector interfaced with a Hewlett-Packard Chemstation using G1034B software for data analysis). The GC column used was an 11.5 m Ultra 1 (100% dimethylpolysiloxane) capillary column and was operated in the splitless injection mode. The GC protocol was set at an injector temperature of 250 °C and an oven temperature of 100 °C with a 10 °C / min temperature ramp to a final temperature of 240 °C. The carrier gas was helium (11 psi).

NMR experiments were also conducted in collaboration with Dr. William Gerwick on either Bruker ACP 300 MHz or AM 400 MHz instruments at Oregon State University.

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2.3 Isolated Chloroplast Studies

2.3.1 Chloroplast Isolation

Chloroplasts were isolated via a protocol modified from that of Gegenheimer (1990). The algal preparation was kept on ice throughout the isolation procedure. Fifty grams of frozen *A. stellata* thalli were first minced with scissors. The algae were then ground to a fine pulp in a mortar and pestle using purified sand (Fisher Scientific, Pittsburgh, PA) and 200 mls of grinding buffer (330 mM sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol (DTT) and 1mM phenylmethylsulphonyl fluoride (PMSF), pH 7.8). DTT and PMSF were added to the buffer the day of the experiment to avoid degradation. Next, the preparation was filtered through 6 layers of cheesecloth and 1 layer of Miracloth (Calbiochem, La Jolla, CA). Approximately 1 g of polyvinylpolypyrrolidone (Sigma, St. Louis, MO) / 35 mls of algae filtrate was added to the preparation prior to centrifugation at 400 x g for 1 min at 4 °C. The pellet was discarded, and the supernatant was filtered through 1 layer of Miracloth and centrifuged at 1000 x g for 5 min. at 4 °C. The resultant pellet was brought up in 12 mls of grinding buffer using a #3 paintbrush to avoid harsh treatment of chloroplasts and centrifuged at 6000 x g for 30 sec. at 4 °C. The supernatant was discarded and the pellet was re-suspended in 12 mls of grinding buffer prior to addition to a continuous Percoll (Sigma, St. Louis, MO) gradient. The gradient

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was formed by centrifuging a 50% Percoll solution in grinding buffer without DTT and PMSF for 1 hour at 20,000 x g (4 ct. 30 ml Corex tubes with 22 mls of the 50% Percoll solution). After the crude chloroplast preparation was carefully added to the gradient (6 mls of prep to 22 mls of Percoll), it was centrifuged at 7000 x g for 20 minutes. In comparison to a control tube containing Sigma Density Marker Beads, the isolated chloroplasts migrated in a defined band at approximately 1.054 g/ml with the broken chloroplasts and mitochondrial contaminants separating out and migrating in a diffuse, light green band above the dark green chloroplasts (Gegenheimer, 1990). The dark green chloroplast band was removed from the gradient, brought up in 4 volumes of ice cold grinding buffer, and centrifuged at 8000 x g for 1 minute to separate the chloroplasts from the Percoll solution. The pellet of isolated chloroplasts was brought up in 5 mls of grinding buffer with a #3 oval artist's brush.

The efficiency of the isolation protocol was determined by analyzing 40 μl aliquots of the preparation with both light and fluorescent microscopy at a magnification of 400x (Figure 4). Samples were collected as follows: 1) after initial filtration through 6 layers of cheesecloth and one layer of miracloth. 2) following the 400 x g spin and filtration through miracloth. 3) prior to addition to the percoll gradient, and 4) directly from the final preparation. The use of both

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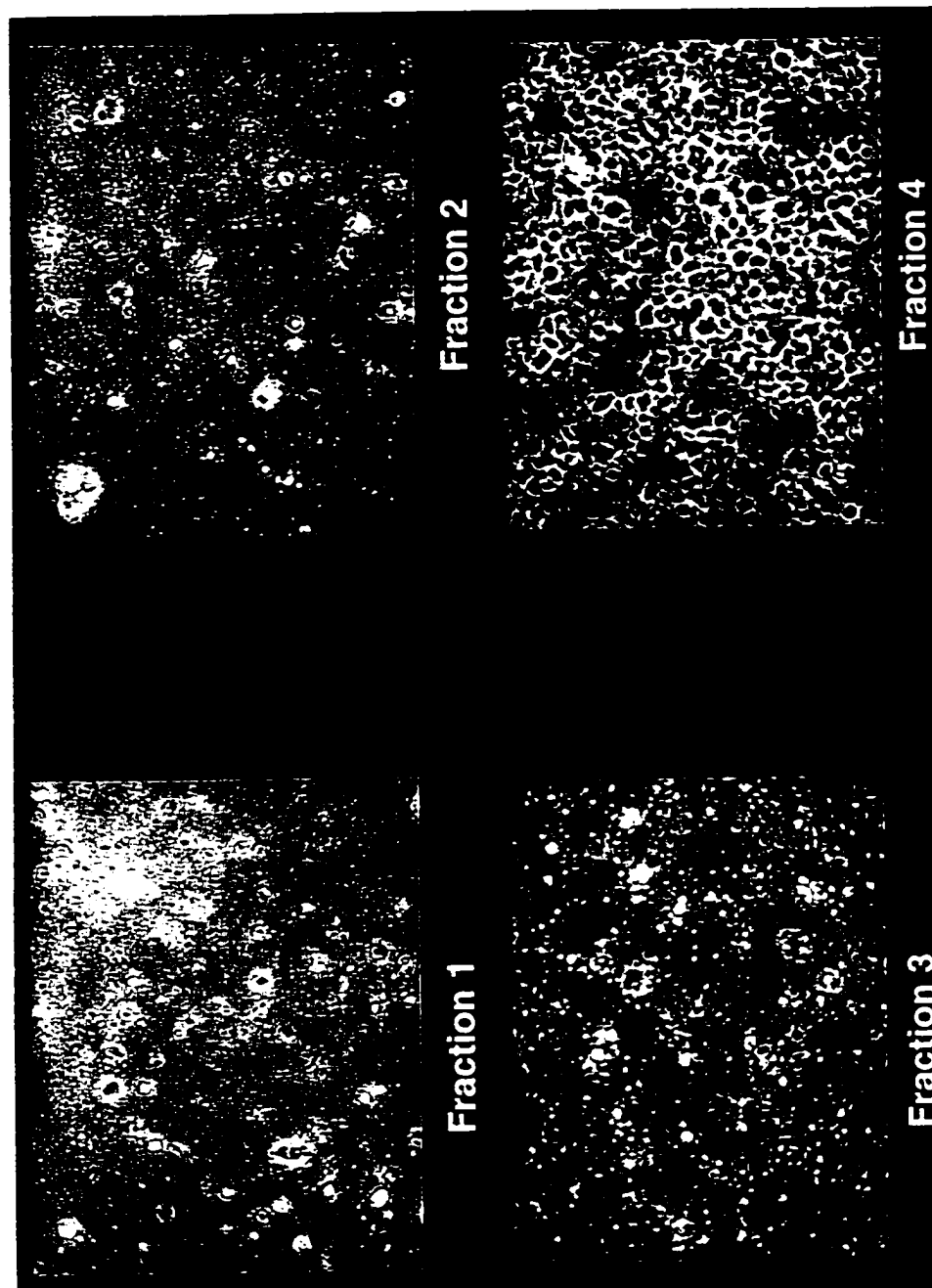
Figure 4a. Light microscopy samples of chloroplast isolation (400x) taken at four points throughout the isolation protocol, as described in the Materials and Methods section.

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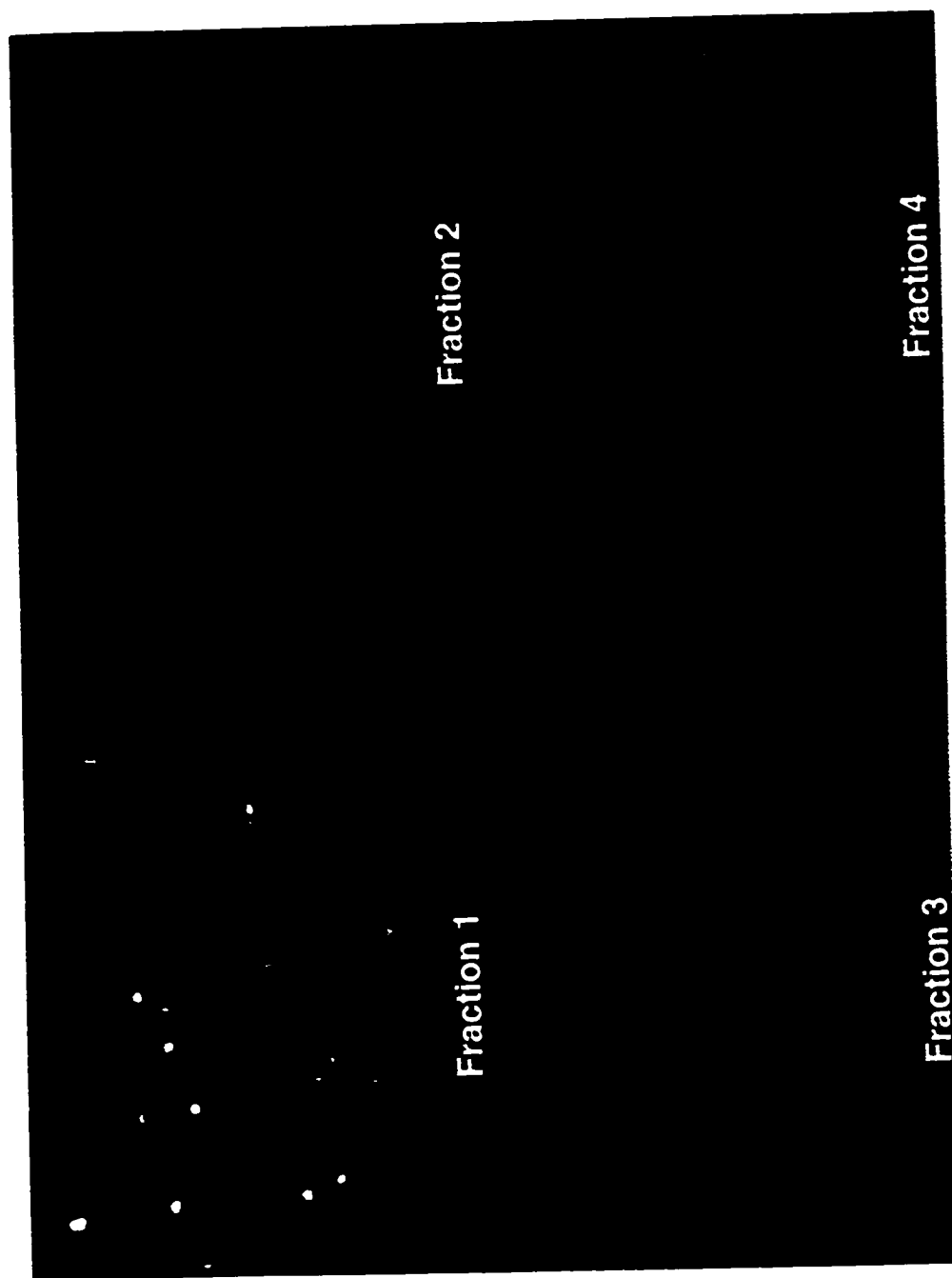
Figure 4b. Fluorescent microscopy of samples from Figure 4a.

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light and fluorescent microscopy allowed for the qualitative analysis of contaminants.

2.3.2 Protein Concentration and Chlorophyll Content

Total protein concentration of the isolated chloroplasts was determined by the method of Bradford (1976) and was typically between 2.0 – 3.0 mg/ml. Chlorophyll concentration of the isolated chloroplast preparations was determined by methods described by Gegenheimer. (1990) and involved the addition of 80% of acetone in water to pelleted chloroplasts followed by measurement of the absorbance at 645 and 663 nm. Following spectrophotometric measurements. chlorophyll concentrations in μg/ml were calculated using the following equation and correcting for the initial dilution: $20.2(A_{645}) + 8.02(A_{663})$. Typical chlorophyll concentrations ranged from 21.5 to 22 μg/ml.

2.3.3 Biosynthesis Studies with Fatty Acid Substrates

The six fatty acid substrates chosen for the biosynthesis studies ranged in carbon chain length from 16 to 22 carbons and did not contain any conjugated double bonds. These included 9Z-hexadecenoic (palmitoleic); 6Z.9Z.12Z.15Z-octadecatetraenoic: 5Z.8Z.11Z.14Z-eicosatetraenoic (arachidonic):

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5Z,8Z,11Z,14Z,17Z-eicosapentaenoic; 7Z,10Z,13Z,16Z-docosatetraenoic; and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acids (Sigma, St. Louis, MO). Isolated chloroplasts were diluted to a final concentration of 60 μg/ml of which 500 μl aliquots were prepared in triplicate for each substrate. Substrates were added in the desired concentrations (substrates prepared in ethanol) and incubations were mixed continuously for the desired time at room temperature (*ca.* 22 °C). Incubations were terminated and products extracted by the addition of 3 mls hexanes, followed by centrifugation at 1000 x g for 5 minutes. The organic fractions were removed and saved, while the aqueous fractions were re-extracted. Correlating organic fractions were combined and all samples dried under a constant stream of nitrogen gas followed by reconstitution in small volumes of hexanes. Appropriate controls for substrates and incubation times were treated as described for other samples.

2.3.4   Detection of Products by GC/MS

As described for the whole algae work, incubation products were methylated by diazomethane gas, and analyzed by UV spectrophotmetry and GC/MS. UV spectrophotometry was conducted on either an IBM (Danbury, CT) 9430 UV-VIS scanning spectrophotometer or a Shimadzu UV-265 UV-VIS Recording Spectrophotometer (Shimadzu, Kyoto, Japan). GC/MS for these

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samples was performed on a modified HP 5985B instrument. Samples were loaded onto a solvent-free GC injector (dropping needle type; Ray Allen Associates. Boulder CO) connected to a bonded phase silica capillary column with the following specifics: DB-1. 15 m, 0.26 mm i.d., film thickness 0.25 μM (J&W Scientific. Folsom. CA). The GC protocol was set with the injector temperature at 250 °C and the oven temperature at 80 °C for one minute ramping up to 300 °C at a rate of 7 °C / min. The carrier gas used was helium.

2.4 Derivatization with 9-Anthryldiazomethane (ADAM)

2.4.1 Derivatization

Ten milliliters of isolated chloroplasts. diluted to 60 μg protein / ml in grinding buffer (Section 2.3.1). were incubated with 200 μM AA (in ethanol) for 30 minutes at room temperature and extracted in hexanes (Section 2.3.3). Final hexane extracts were combined. dried to completion under nitrogen gas. and immediately reconstituted in 0.5 ml methanol. Using a modified method from the work of Yamaki and Oh-ishi (1992). 0.5 ml of ADAM (2 mg/ml in ethyl acetate) was added to the preparation which was then incubated in the dark for 12 hours at room temperature. To insure the effectiveness of labeling, AA was

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incubated with ADAM in the absence of the chloroplast preparation, as described.

2.4.2 Extraction

Following derivatization, the samples were chromatographed separately over a silica Sep-Pak (Waters Associates, Milford, MA) that had been equilibrated in chloroform : toluene (1:1). Two solvent systems were utilized to elute off the AA derivatives. First, 5 mls of chloroform : toluene (1:1) were used to elute any HETEs and unmetabolized AA that were derivatized with ADAM (Fraction 1). Next, any ADAM derivatives of prostaglandins and leukotrienes contained in the sample were eluted in acetonitrile : methanol (4:1) (Fraction 2). Both fractions were then dried to completion under a stream of nitrogen gas and then re-constituted in methanol : ethyl acetate (1:1) for reverse phase or chloroform for straight phase HPLC separation prior to analysis by liquid chromatography / mass spectroscopy.

2.4.3 Analysis of ADAM Derivatives – LC/MS (APcI⁻)

LC/MS was conducted on both fractions from the silica Sep Pak separation described above. HPLC separation was conducted with a C18 (5 μ,

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100 Å) Microsorb – MV (Rainin Instrument Co. Inc., Emeryville. CA) column at a flow rate of 700 ml / min and UV detection at 254 nm. Solvent systems used were modified from those described by Yamaki and Oh-ishi (1992) by removal of the H_3PO_4 since phosphate ions dampen the mass spectroscopy signal and leave a residue inside the instrument. A step wise profile starting with acetonitrile: $H_2O + 0.1\%$ trifluoroacetic acid (85:15) for 10 minutes and then increasing to acetonitrile:$H_2O + 0.1\%$ trifluoroacetic acid (95:5) over a 30 minute period and then maintaining for the remainder of the run was utilized for Fraction 1. While an isocratic system of acetonitrile:H_2O (70:30) was used with Fraction 2. Effluent from the column was directed straight into the atmospheric pressure chemical ionization (APcI) probe set in the positive ion mode of a Fisons VG Platform II single quadrupole mass spectrometer (Micromass Ltd., Altrincham. UK) to analyze ADAM derivatives present. Source temperature of the mass spectrometer was set at 150 °C and the probe temperature was 400 °C. Cone voltage was set at 30 V.

Figure 5 illustrates the mass spectrum obtained from the AA standard. Both derivatized and underivatized AA were identified. The ion found at m/z 495 is consistent with the molecular weight of AA derivatized with one ADAM molecule. and the underivatized AA ion is observed at m/z 305. The peaks at m/z 381 and m/z 685 could likely be an effect of ion-molecule reactions occurring within the mass spectrum source (J.G. Pavlovich. personal communication). A

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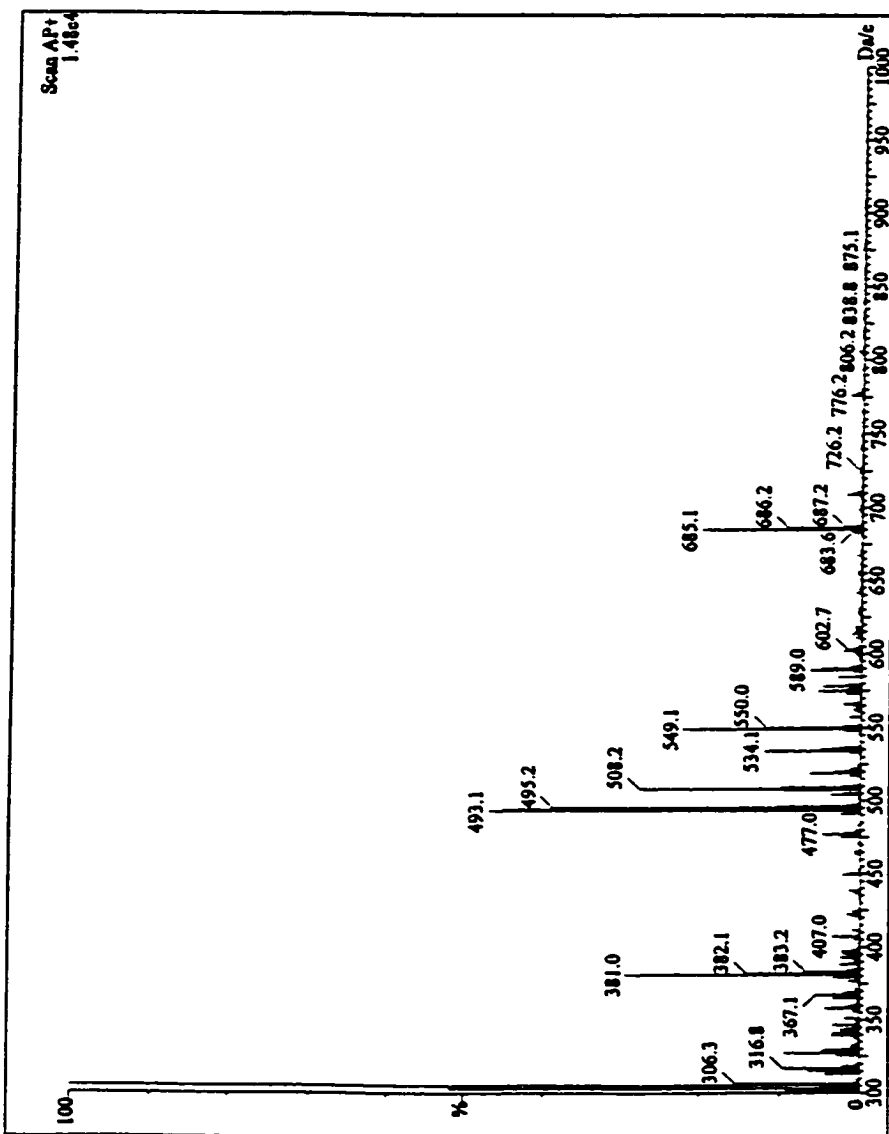
Figure 5. APcI positive-ion mass spectra of arachidonic acid derivatized with ADAM.

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pairing of two ADAM molecules would result with a peak at *m/z* 381. while *m/z* 685 is consistent with the molecular weight of two molecules of ADAM and AA present as a potassium salt.

2.5 Crude Protein Preparation

2.5.1. Chloroplast Lysate

Chloroplasts. either freshly prepared or frozen. were lysed via hypotonic lysis in a buffer composed of 10 mM HEPES – KOH (pH 8.0). 1 mM EDTA. 1 mM $MgCl_2$. 1 mM $MnCl_2$. 1 mM DTT. and 1 mM PMSF. First. 5-7 volumes of cold lysis buffer were added to the chloroplasts and stirred on ice for 15 minutes. Then. the preparation was probe sonicated on ice for 6 short bursts. and centrifuged for 20 minutes at 20.000 x g. The pellet was re-suspended in 3-4 mls of 25 mM Tris buffer. 0.2 mM $CaCl_2$. and 1% TX-100. and stirred on iced for 15 minutes.

2.5.2 Anion Exchange Column

An anion exchange column was utilized in this next step of the purification. Macro-Prep High Q (Bio-Rad Laboratories, Hercules. CA) was

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utilized as the column matrix in a buffer of 25 mM Tris, 0.2 mM $CaCl_2$, 0.05% TX-100, pH 8.0. The typical bed volume was 88 cm^3 which is appropriate for protein amounts up to 130 mg when using a salt gradient elution protocol (Scopes, 1987). The chloroplast lysate was added to the equilibrated column and eluted with 200 mls of each of the following NaCl solutions prepared in the column buffer listed above: buffer without salt, 0.3 M NaCl, 0.35 M NaCl, 0.4 M NaCl, and 0.45 M NaCl. Initial runs of the column started with a wider range of NaCl concentrations to identify the regions in which the protein was eluting. The column flow rate was approximately 17.5 mls / hr and fractions were collected by an automated fraction collector and then stored on ice. All fractions were analyzed for total protein concentration and potential lipoxygenase activity as detailed below.

2.5.3   Protein Concentration

Fractions containing high levels of protein were concentrated in an Amicon ultrafiltration cell (Amicon, Danvers, MA) under nitrogen gas, 30 psi, using a membrane with a 10,000 Da molecular weight cut off. Typically, active fractions would elute in a diffuse manner over approximately 10 fractions. Therefore, a series of 10 fractions were concentrated together from a total volume of 58 mls to approximately 5 mls.

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2.5.4 Gel Filtration Column

The concentrated preparation was then added to a gel filtration column using Sephacryl S-300 HR (Pharmacia Biotech, Uppsala, Sweden) as a matrix. The column was equilibrated in 25 mM Tris buffer with 0.15 M NaCl, pH 7.5. Column bed volume was typically 53 cm^3 and flow rate was 18.6 mls / hr. Fractions were collected by an automated fraction collector.

2.5.5 Protein Quantification

The presence of protein in column fractions was identified by monitoring the UV absorbance at 280 nm (Freifelder, 1982). Concentrations were calculated by the Beer-Lambert relationship, assuming an extinction coefficient of 5600 $M^{-1}cm^{-1}$ for tryptophan at 280 nm.

2.5.6 Lipoxygenase Activity Assay

Lipoxygenase activity was assayed for by measuring the increase in absorbance at 234 nm over 3 minutes in the presence of linoleic acid (Sigma, St. Louis, MO). Data was collected on an Ultraspec 2000 UV/Vis spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Linoleic acid substrate was prepared by the "Low Ethanol" method according to the

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recommendations of the manufacturer to avoid reduction in enzyme activity due to high ethanol levels. 50 µl of linoleic acid neat was added to 50 µl of ethanol (200 proof) in a 50 ml volumetric flask and mixed. The volume was slowly brought up to 50 mls with H_2O while continuously stirring to form an emulsion. Five milliliters of this preparation was added to 25 mls of 0.05 M potassium phosphate buffer with 0.2 mM $CaCl_2$, pH 7.0. for use in activity assays. Each assay was conducted in a 1.5 ml quartz cuvette with 300 µl 0.05 M potassium phosphate buffer. 0.2 mM $CaCl_2$ (pH 7.0), 200 µl enzyme preparation. and 1 ml of linoleic acid dilution for a final concentration of 353 µM linoleic acid. Absorbances were monitored either continuously or every 15 seconds over the three minute incubation period.

2.5.7 Biosynthesis Studies

Further studies of lipoxygenase activity involved incubations of the semi-purified enzyme preparation with linoleic acid (Sigma. St. Louis. MO). Incubations were prepared as described above for the A_{234} assay. Tubes were capped and mixed continuously for 30 minutes at room temperature. Incubations were stopped by the addition of 2 drops of 1 N HCl to acidify the reaction to pH 3.

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Products were extracted over C18 Amprep minicolumns (Amersham International, Arlington Heights, IL) modified from the methods of MacPherson *et al.* (1996). The minicolumns were first washed with methanol and equilibrated with H_2O. Each sample was loaded onto an equilibrated minicolumn and fractions eluted as follows: 5 mls of H_2O, 5 mls of 15% ethanol, and 10 mls of ethyl acetate. Polar compounds and salts were removed from the sample with the ethanol and H_2O washes, respectively. The ethyl acetate fractions containing all PUFAs present were concentrated under N_2 to a final volume of 0.2 mls. Benzene was added during this last step to aid in the removal of any aqueous solvents remaining. Fractions were then frozen at –80 °C over night and analyzed the next day. All solvents used were HPLC grade.

2.5.8 Detection of Products by TLC

TLC was conducted on Whatman Al Sil G/UV (F_{254}) 250 μ silica plates in a solvent system of ethyl acetate : hexanes (25:75). Short wave UV detection was utilized to visualize compounds containing a conjugated double bond, followed by spraying with the lipid- and hydroxy fatty acid- specific indicator phosphomolybdic acid (Sigma, Chemical, St. Louis) and heating. R_f values were compared to those of the following standards: ± 9-HODE, ± 13-HODE (Cayman Chemical, Ann Arbor, MI), and linoleic acid (Sigma, St. Louis).

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2.6 Oxygen Electrode Measurements

To determine if exposure of arachidonic acid administered directly into the sea water environment could have a physiological effect on whole algal thalli, oxygen flux was measured using custom made 1.2 L chambers fitted with Endeco pulsed oxygen electrodes (YSI Inc., Yellow Springs, OH) and shielded magnetic stir bars. Electrodes were standardized as described by Johnson (1972). Eight chambers were used per experiment: seven contained an individual thallus of *A. stellata* each and the eighth contained only pre-conditioned seawater to serve as a control. Neutral density filters were used to adjust the incident light levels to 30-60% ambient. Oxygen levels were monitored continuously. Following a 1 hour conditioning period, data was collected 10 minutes prior to and after AA (Sigma, St. Louis, MO) administration. Daytime experiments were conducted between the hours of 0800 and 1700 EST, and nightime trials were between 1900 and 2400 EST. During the nightime experiments, chambers were wrapped with foil and the water baths were completely covered with black plastic sheeting to insure total darkness. Incubation specifications in regards to ratios of thallus weight : water : incubation time followed the recommendations of Littler and Arnold (1985). Wet weights of individual plants were determined prior to each experiment after damp drying in soft paper towels.

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For each experiment, AA was prepared by adding a 1 ml dilution in ethanol to 4 ml of sea water and sonicating in a cold bath for 5 minutes. These preparations were injected into the chambers containing the algae to yield final concentrations of 3.13, 6.25, 12.5, 25, 50 or 100 μM for daytime experiments. Nightime experiments were conducted with 100 μM AA only.

2.7 Fatty Acid Analyses of Isolated Chloroplasts

2.7.1. Formation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMEs) of isolated chloroplasts were prepared by the methods of Sasser (1997) and were carried out as follows. The mixture was first saponified by addition of 4 mls of a 3.75 M NaOH solution in methanol / H_2O (1:1) to 2 mls of isolated chloroplasts. This solution was heated at 100°C for 30 minutes in a pyrex tube capped with a PTFE-lined cap. After allowing this solution to cool to room temperature, 6 mls of the methylation reagent (65 ml of 6N HCl in 55 mls of methanol) were added to the tube and boiled at 85°C for 10 minutes. Once again the solution was allowed to cool to room temperature prior to the addition of 3 mls of the extraction solvent (hexanes : dichloromethane, 1:1). The tube was then inverted continuously for 3 mins and centrifuged at 1000 x g for 5 minutes. The lower aqueous phase was removed.

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Lastly, 3 mls of the base wash (0.3 M NaOH in H_2O) was added to the organic fraction and inverted for 10 mins continuously. Once again, the solution was centrifuged at 1000 x g for 5 minutes. The organic layer was removed and concentrated under a constant stream of nitrogen gas prior to analysis by GC/MS.

2.7.2 GC/MS Analysis of FAMEs

Mass spectra of samples prepared from algae collected off Long Key, Florida were obtained on Hewlett-Packard 5790 gas chromatograph in line with a VG 70SE double focusing mass spectrometer operating on EI mode and fitted with a DB -MS column, 30m x 0.25 μ film thickness. Initial temperature of the column was 50 °C for 1 min, then increased to 128 °C at 18 °C/min, and finally 255 °C at 3 °C/min. FAMEs eluted off the column within 45 min and were identified by comparison of retention times with those obtained from a standard mixture supplied by Matreya (Pleasant Gap, PA) run under identical conditions and/or compared with known spectra (NIST library).

The GC/MS data collected for the samples prepared in Dr. Vassilios Roussis' laboratory in the Department of Pharmacy, Division of Pharmacognosy, University of Athens, Greece were obtained from a Hewlett Packard 5973-6890 GC/MS system operating on EI mode (equipped with a HP 5MS capillary column, 30m x 0.25μ film thickness). The GC protocol was set with the initial

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temperature of the column at 60 °C and then was heated to 280 °C with a 3 °C/min rate. All FAMEs were off the column within 45 minutes. The identification of the chemical constituents was based on comparison of the R_t values and mass spectra with those obtained from authentic samples and/or the NIST/NBS library spectra. Isomers were identified from one another by comparison to ECL data. Five independent preparations from each site were analyzed, except from Vagias Beach due to the limited amount of algae collected. In this case, only three samples were prepared.

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3.0 RESULTS

3.1 Conjugated Tetraene Production in Homogenates of *Anadyomene stellata*

The UV absorbance spectrum of lipid extracts from whole thalli incubated with AA and from homogenized *A. stellata* thalli showed λmax at 294, 306, and 322 nm indicating the presence of conjugated tetraene containing compounds (Figure 6) (Borgeat *et al.*, 1990). Preliminary UV spectrophotometric analysis of these compounds suggested that the crude extracts were unstable. To address this problem, methyl ester extracts were prepared that were markedly more stable, thus allowing for further analysis and identification of the fatty acids present. No degradation of these derivatives was detected by TLC after one month of storage under nitrogen gas at −70 °C.

Vacuum liquid chromatography using mixtures of ethyl acetate in hexanes ranging from 0 - 100% ethyl acetate was employed to begin separation of these compounds. Following elution off the silica, fractions were analyzed by UV spectrophotometry for the presence of the conjugated tetraenes. These PUFAs were found to elute off the column in ethyl acetate : hexanes (1:99). This fraction was concentrated under nitrogen and further separated by straight phase

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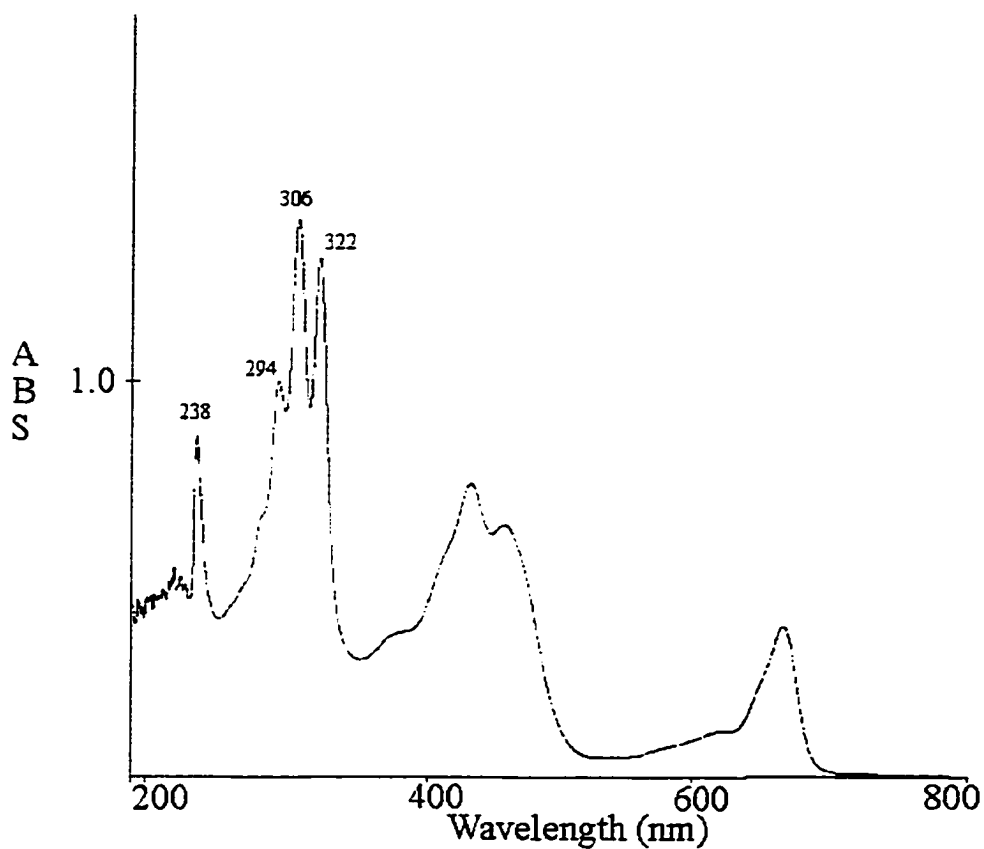
Figure 6. UV Spectrophotometric analysis of organic extracts from *A. stellata*. The characteristic absorbance pattern for the presence of a conjugated tetraene functionality is illustrated with maxima at 294, 306, and 322 nm. Absorbance maximum at 238 nm is typical of a conjugated diene functionality.

HPLC. Three peaks eluting at 42, 48, and 54 mls showed significant absorption at 328 nm, and were collected for further analysis. Following concentration under nitrogen gas, fractions were scanned in the UV range, as described earlier. All three fractions showed λmax at 294, 306, and 322 nm. Samples were then analyzed by GC/MS, and NMR to identify the mass and structure of the conjugated tetraenes present.

GC/MS AND NMR analyses of these three fractions were conducted in Dr. W. Gerwick's laboratory at the Department of Pharmacy, Oregon State University. The NMR analyses summarized below were detailed in a joint publication (Mikhailova et al., 1995). GC/MS analysis of the three fractions resulted in the detection of five compounds having molecular ions at $m:z$ 260, 290, 314, 316, and 340 which are consistent with the molecular weight of methyl esters of C16:5, C18:4, C20:5, C20:6, and C22:7. These structures are illustrated in Figure 7 with their conjugated tetraene functionalities delineated. Rigorous NMR analysis was completed for the C20:5 and C22:7 compounds. Following ^{1}H NMR and additional proton decoupling experiments, the unsaturation pattern of the C20:5 compound was clearly shown to be 5Z, 8Z, 10E, 12E, 14Z, as previously described for bosseopentaenoic acid by Burgess et al. (1991). The isolate with a molecular ion consistent to the molecular formula of $C_{23}H_{32}O_2$ was thoroughly analyzed by ^{1}H NMR, ^{13}C distortionless enhancement by polarization transfer, and ^{1}H-^{1}H COSY experiments. All structural possibilities were

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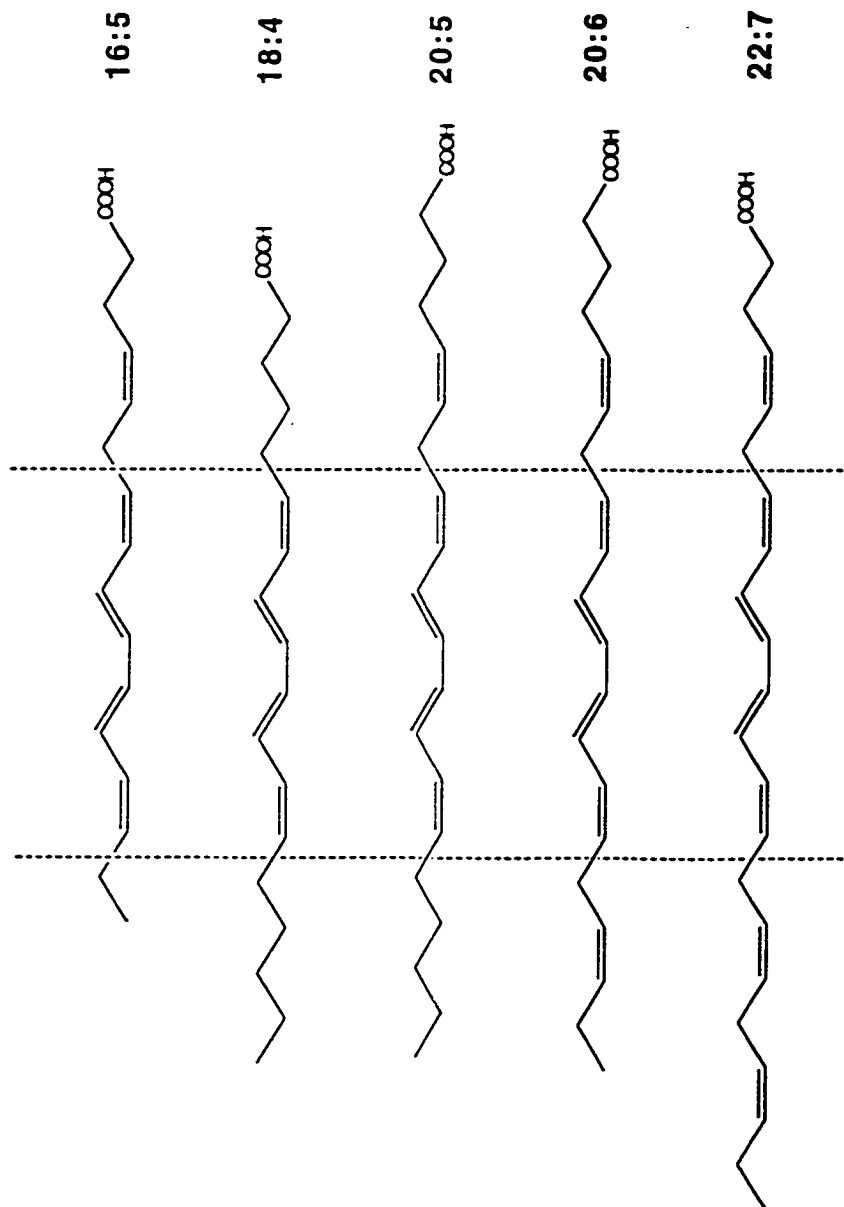
Figure 7. Conjugated tetraene PUFAs isolated from *A. stellata*. Dotted lines delineate the conjugated tetraene functionality in each molecule.

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narrowed down by these experiments to two regioisomers: 4,7,9,11,13,16,19-docosaheptaenoic acid or 4,7,10,12,14,16,19-docosaheptaenoic acid. The structure was resolved to be the former with the conjugated tetraene functionality occurring at carbons 7,9,11,13 by a NOESY experiment. The relative stereochemistry of the seven olefinic groups was determined to be 4Z, 7Z, 9E, 11E, 13Z, 16Z, 19Z based on NMR data and the precedence of Z,E,E,Z orientations observed in other known conjugated tetraene systems (Tulloch, 1982; Hamberg, 1992) . Interestingly, 4,7,9,11,13,16,19-docosaheptaenoic acid is a previously undescribed molecule of the C22 PUFAs. Therefore, we proposed the common name stellaheptaenoic acid.

It is worth noting in Figure 7 that the conjugated tetraene functionality did not occur at a fixed carbon position relative to the terminal carboxyl group. This raises the possibility that the catalytic site on the oxidizing enzymes was highly stereospecific with respect to its substrate binding site. Conjugated double bond formation was also independent of the carbon chain length between 16 and 22 carbons.

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3.2 Conjugated Tetraene Production in Isolated Chloroplasts

3.2.1 Initial Observations

After identification of the conjugated tetraene containing PUFAs in the algal preparations and preliminary observations of high enzymatic activity in the chloroplast fractions. we focused our studies on this fatty acid oxidative pathway in isolated chloroplasts. Characteristic absorbance patterns of conjugated tetraenes with λmax at 294. 306. and 322 nm were seen in initial UV scans of chloroplast extracted in hexanes. In addition. another peak at 238 nm was consistently observed (Figure 6). The λmax = 238 nm resultant from the organic extraction protocol used is indicative of a conjugated diene containing molecule (Smith and Lands. 1972). In chloroplast preparations. the ratio of the presence of conjugated diene containing products to conjugated tetraene PUFAs was on average 4.8:1. assuming E = 24.000 $M^{-1}cm^{-1}$ for conjugated dienes (Smith and Lands. 1972) and E = 72.000 $M^{-1}cm^{-1}$ for conjugated tetraenes (de Carvahlo and Jacobs. 1991). Initial studies were directed towards the identification of the conjugated tetraenes. while later studies focused on the elucidation of the compound(s) present containing the 234 nm absorbing chromophore. This λmax is based on LO products containing a conjugated diene (Surrey. 1964).

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3.2.2 Enzymatic Activity

To effectively study the enzymatic oxidation kinetics occurring in the production of the conjugated tetraene PUFAs, the optimal incubation times, substrate specificity, and substrate concentrations were determined. Since fatty acid oxidation can also occur by non-enzymatic mechanisms, we had to confirm the existence of an enzymatically driven system and illustrate its involvement in the production of the conjugated tetraene PUFAs. To accomplish this, a triplicate series of isolated chloroplasts diluted to 60 μg/ml protein and aliquoted out into 0.5 ml volumes was boiled for 15 minutes. The 22:6 PUFA substrate was added to triplicates of both boiled and un-boiled chloroplast preparations at a final concentration of 200 μM. Additionally, a series of control tubes were included which contained the chloroplast preparation without the substrate. All tubes were incubated for 1 hour at room temperature, extracted, and absorbance determined at 306 nm. The mean absorbance values $\pm$ SEM between the boiled preparation (0.225 $\pm$ 0.003) and the control tubes (0.236 $\pm$ 0.008) were not significantly different. In the unboiled preparations, absorbance at 306 nm increased to 0.532 $\pm$ 0.015, a greater than two-fold increase over control. This elevated activity was statistically significant ($P < 0.01$) and consistent with enzymatic catalysis involved in the formation of conjugated tetraene containing products.

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3.2.3 Optimization of Reaction Conditions with Arachidonic Acid as a Substrate

Kinetic optimization of the chloroplast enzyme preparation following AA incubations was accomplished by monitoring the absorbance at 306 nm while varying the incubation time. The extraction procedure and UV spectrophotometric analyses of these samples were the same as employed for the detection of products following incubation of isolated chloroplasts with different PUFA substrates. As seen in Figure 8, oxidative product formation appeared to approximate pseudo first order kinetics at less than 12 minutes with nearly a two-fold increase above control levels occurring at the 12 minute time point. The rate of product formation reached saturation within 30 minutes as evidenced by only an 11% increase in product formation between 30 and 60 minutes. An estimate of the optimal incubation time for tetraene formation from AA was interpolated to be 10 minutes. The crude enzyme preparation was calculated to have a specific activity of 6.7×10^{-4} μg $min^{-1} mg^{-1}$ protein, using the Beer-Lambert relationship and assuming an $E = 72.000$ $M^{-1} cm^{-1}$ (deCarvahlo and Jacobs, 1991).

The optimal concentration for AA was determined by varying the concentrations while keeping both the protein concentration (60 $\mu g/ml$) and the incubation time (10 min) constant. AA bench solutions were prepared in ethanol at the appropriate concentrations to achieve the final substrate concentrations of 50, 100, 200, 300, 400 μM while keeping the dose volume constant. Reactions

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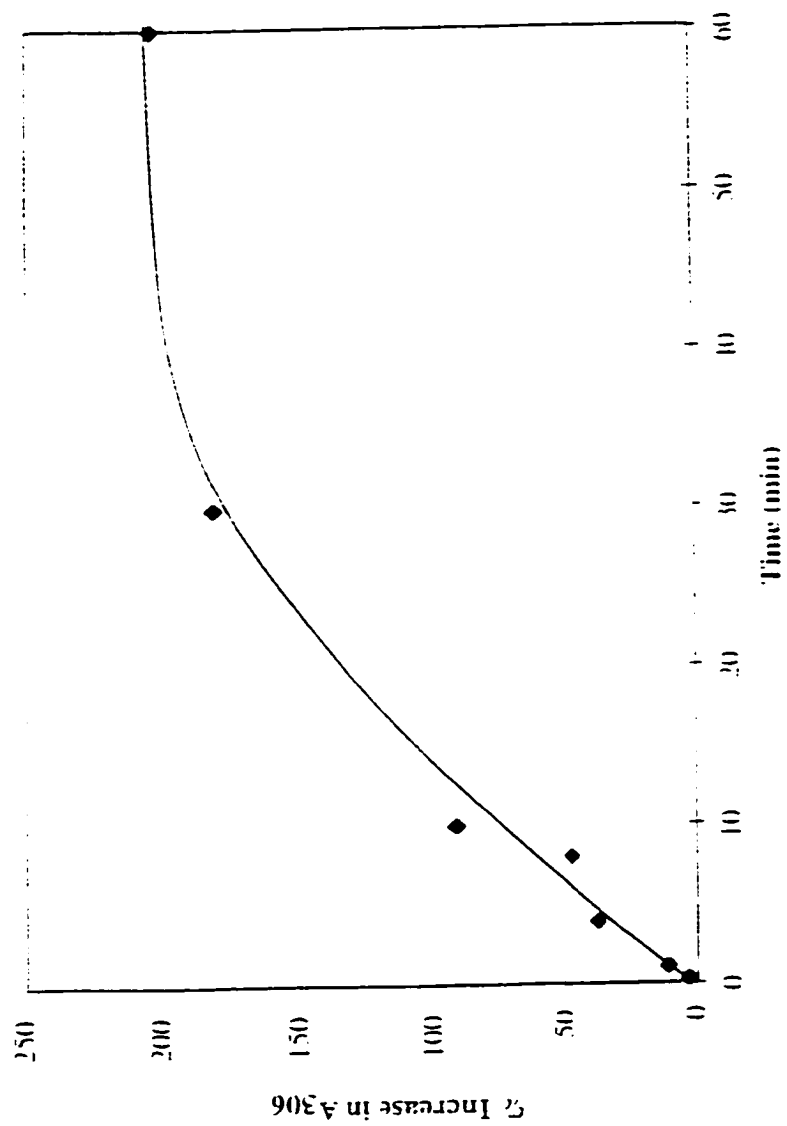
Figure 8. Conjugated tetraene product formation as a function of time. Incubations prepared with 0.5 ml isolated chloroplasts (60 µg/ml) incubated with 100 µM AA.

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for each AA concentration were prepared in triplicate at 0.5 ml each. As seen in Figure 9. a typical substrate concentration dependent curve was observed with a linear increase in product formation occurring at less than 100 μM AA and reaction velocity saturating at concentrations greater than 200 μM. The apparent V_{max} was graphically estimated to be 6.76 μM min^{-1} mg^{-1} at 200 μM AA. The apparent K_m was also empirically determined and found to be 60 μM AA. Michaelis-Menton kinetics while apparently present were not assumed because of the possibility of a mixture of enzymes present.

In consideration of the potential for some of the AA to be shunted towards other enzymatic pathways present. such as that initially observed leading to the production of putative conjugated diene containing compounds. the ratio of conjugated dienes to conjugate tetraenes was calculated for each AA dose. Ratios were calculated using the average concentrations of both compounds present. In controls. the ratio of dienes to tetraenes was found to be 4.8:1. However. with an increase in AA concentration. the ratio decreased to 2.5:1 at 50 μM AA and 2.1:1 at 100 μM AA. Increases in AA concentration to 200 μM resulted in further decreases in the ratio to 1.69:1. However. the 300 and 400 μM AA incubations did not result in further decreases in this ratio (1.32:1 at 300 μM and 1.31:1 at 400 μM). The lack of further changes in the ratio with increases in the AA concentration is indicative of the substrate is being shunted mainly towards conjugated tetraene formation as it supports the saturation in product

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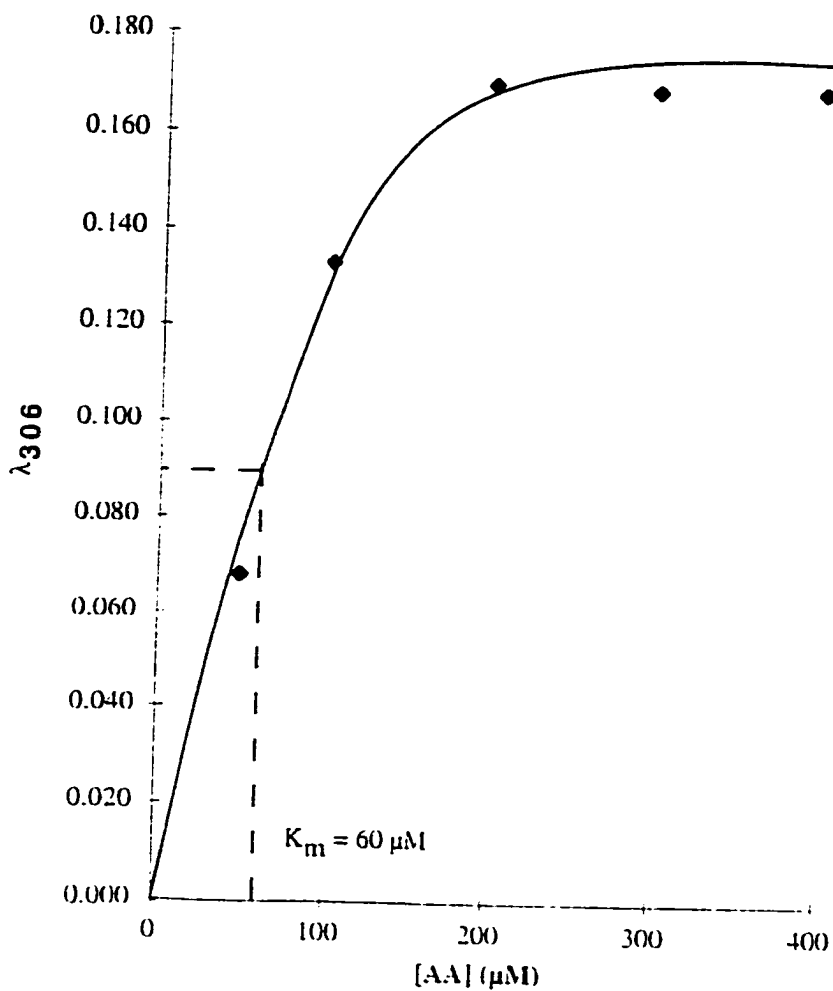
Figure 9. Conjugated tetraene product formation as a function of arachidonic acid concentration. V_{max} observed with 200 μM AA, K_m = 60 μM.

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formation observed in the studies described above. Furthermore. concentrations of putative conjugated diene containing products present remained relatively constant from control levels of 33.4 µM. Absorbance at 234 nm was 33.9 µM at 50 µM AA. 35.6 µM at 100 µM AA. 35.5 µM at 200 µM AA. 36.21 µM at 300 µM AA. and 24.38 µM at 400 µM AA. Thus. the increased tetraene formation following AA incubations was independent of concentrations of dienes present.

3.2.4 Substrate Specificity and Kinetic Analyses

To analyze the nature of substrate binding to the enzyme. a series of biosynthesis experiments were conducted using a variety of PUFA substrates which included three n-3 polyunsaturates. two n-6 polyunsaturates. and one n-7 monounsaturated fatty acid. These substrates were 9Z-hexadecaenoic acid (16:1, palmitoleic): 6Z. 9Z. 12Z. 15Z-octadecatetraenoic (18:4): 5Z .8Z. 11Z. 14Z-eicosatetraenoic (20:4. arachidonic); 5Z. 8Z. 11Z. 14Z. 17Z-eicosapentaenoic (20:5. eicosapentaenoic): 7Z. 10Z. 13Z. 16Z-docosatetraenoic (22:4); and 4Z. 7Z. 10Z. 13Z. 16Z. 19Z-docosahexaenoic (22:6) acids. Incubations were conducted with a fixed protein concentration of 60 µg/ml for a time period of 30 minutes. All fatty acids proved to be substrates for the chloroplast enzyme preparation, as indicated by an increase in production of the characteristic conjugated tetraene chromophore (λ_{max} 294. 306. and 322).

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Kinetic analyses were performed using the same six fatty acid substrates as described above. Substrate concentrations used were 50, 100, 200, 300, and 400 μM. Protein concentrations and other reaction conditions were also consistent with those already described. Although the enzyme preparation under study was a crude preparation, comparison of empirically determined K_m and V_{max} values offers useful information regarding the preference of the preparation to each substrate studied. Two of the six substrates, 18:4 and 20:4, displayed saturation kinetics in product formation within the dose range utilized. Therefore, these data sets were plotted and the apparent K_m and V_{max} values were estimated graphically. Because saturation in product formation was not reached within the dose range utilized for the 20:5, 22:4, and 22:6 studies, K_m^{app} and V_{max}^{app} values were estimated from a Lineweaver-Burk plot by least squares linear regression analysis. All apparent K_m and V_{max} values were determined graphically and are presented in Table 2. Arachidonic acid, an n-6 fatty acid, appeared to have to lowest K_m^{app} value (55 μM), indicating the greatest affinity for the chloroplast enzyme(s). Intermediate K_m^{app} values were observed for the 22:6, 20:5, and 22:4 substrates which possess n-3, n-3, n-6 olefins, respectively. Interestingly, no clear trend was observed in apparent K_m values between the n-3 and the n-6 fatty acids. V_{max}^{app} was greatest for the 22:6 substrate, followed by 20:5.

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Table 2. Kinetic analyses with various fatty acid substrates

Substrate	K_m^{app} (μM)	V_{max}^{app} (μM min^{-1} mg^{-1})	V_{max}/K_m
16:1	*	*	*
18:4	180	4.321	0.024
20:4	55	4.167	0.076
20:5	118	6.944	0.059
22:4	122	2.145	0.018
22:6	98	8.380	0.086

* denotes lack of any dose-response relationship

Intermediate V_{max} values were observed for the 20:4 and 18:4 fatty acids. The 22:4 substrate gave the lowest V_{max} value. Palmitoleic acid. the n-7 monunsaturate. proved to be the poorest substrate in terms of the insignificant levels of product formation. and the lack of any sort of concentration dependence. Hence. apparent K_m and V_{max} values were unable to be estimated.

High catalytic efficiencies (V_{max}/K_m) were demonstrated for all substrates. except palmitoleic acid (16:1). All V_{max}/K_m values were within the same order of magnitude. however. 22:6 had greatest value, 0.086. followed by AA with an efficiency value of 0.076. The lowest catalytic efficiency was observed for 22:4 (V_{max}/K_m = 0.018). with 18:4 having only a slightly higher efficiency value of 0.024.

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3.2.5 Identification of Conjugated Tetraene PUFAs

Table 3 illustrates the conjugated tetraene containing fatty acids extracted from isolated chloroplast preparations following incubation with the same six fatty acid substrates used for the above studies: palmitoleic, 6.9.12.15-octadecatetraenoic, arachidonic, eicosapentaenoic, 7,10,13,16-docosatetraenoic, and 4,7,10,13,16,19-docosahexaenoic acids. Product identification was based on comparison of R_t and mass spectra with those in the NIST database. Fatty acids present in samples incubated with 6.9.12.15-octadecatetraenoic acid were determined to be 16:5, 18:4, 20:5, and 20:6. Incubation of the chloroplast preparations with AA resulted only in the production of fatty acids with 20 carbon chain lengths. However, 16:5, 18:4, 20:5, and 20:6 were detected in assays in which eicosapentaenoic acid was added. Samples incubated with both of the 22 carbon substrates demonstrated the presence of all five conjugated tetraene containing PUFAs. In contrast, the only conjugated tetraene identified in the samples incubated with palmitoleic acid was the 20:6 compound. Which of these extracted compounds represent conjugated tetraenes as unmetabolized PUFAs was not determined by the methods utilized in this study. Thus, the high V_{max} observed for 22:6 may reflect either a degree of pleomorphism expressed by this chloroplast enzyme(s), or the collective affinity of this substrate for a group of enzymes.

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Table 3. Conjugated tetraene fatty acids identified following biosynthesis experiments with isolated chloroplasts

Fatty Acid Substrate	Conjugated Tetraene Metabolites
(16:1) cis-9-hexadecaenoic acid (palmitoleic)	20:6
(18:4) cis-6,9,12,15-octadecatetraenoic acid	16:5, 18:4, 20:5, 20:6
(20:4) cis-5,8,11,14-eicosatetraenoic acid (AA)	20:5, 20:6
(20:5) cis-eicosapentaenoic acid	16:5, 18:4, 20:5, 20:6
(22:4) cis-7,10,13,16-docosatetraenoic acid	16:5, 18:4, 20:5, 20:6, 22:7
(22:6) cis-4,7,10,13,16,19-docosahexaenoic acid	16:5, 18:4, 20:5, 20:6, 22:7

3.3 Hydroperoxy Fatty Acids in *A. stellata* Chloroplasts

3.3.1 9-Anthryldiazomethane Derivatization and LC/MS (APcI⁺) Detection

The probe 9-anthryldiazomethane (ADAM) was utilized in the biosynthesis studies with the isolated chloroplasts since the formation of the ester-linked derivative results in a product that is readily detectable by both UV (A_{254}) and fluorescent analysis (excitation at 365 nm, flourescent at 412 nm). Furthermore, derivatization with the diazomethane analog theoretically increases the stability of PUFA metabolites. Following incubation of the chloroplast

fraction with AA (200 μM final concentration), extraction, derivatization with ADAM, and chromatographic separation over silica Sep Paks, the fractions were analyzed by LC/MS (APcI⁻). Data collected suggested the biosynthesis of metabolites, presumably oxylipin-like molecules. As can be seen in UV and TIC chromatograms of the control and treated samples illustrated in Figure 10, A_{254} absorbing compounds are seen in the treated samples that do not appear in the controls. Of greatest interest to us, was the compound with a peak in the TIC of the treated samples at 9.17 min. This peak observed at m/z 637 is consistent with the molecular weight of a dihydroxy hydroperoxy eicosatrienoic acid (mw 370) present as a potassium salt and derivatized with ADAM. The mass spectrum is illustrated in Figure 11. The presence of the oxylipin in the salt form is not unexpected when working with biological buffers. Further support of this compound is found in an additional set of peaks with retention time of approximately 18.7 minutes observed at m/z 409 (M + H) (Figure 12). The molecular weight of this ion, 408, is also consistent with that of the underivatized dihydroxy hydroperoxy eicosatetraenoic acid present as a potassium salt as described above. Additionally, a characteristic ion of an n-6 olefin was observed with the same retention time at m/z 338 (M + H - 71, loss of C_5H_{11}) providing further support for the presence of this metabolite (Oliw et al., 1993). As can be seen in the UV/TIC chromatograms of the control and treated samples, the peak observed in the TIC at 18.7 min of the treated sample is not present, or at least to

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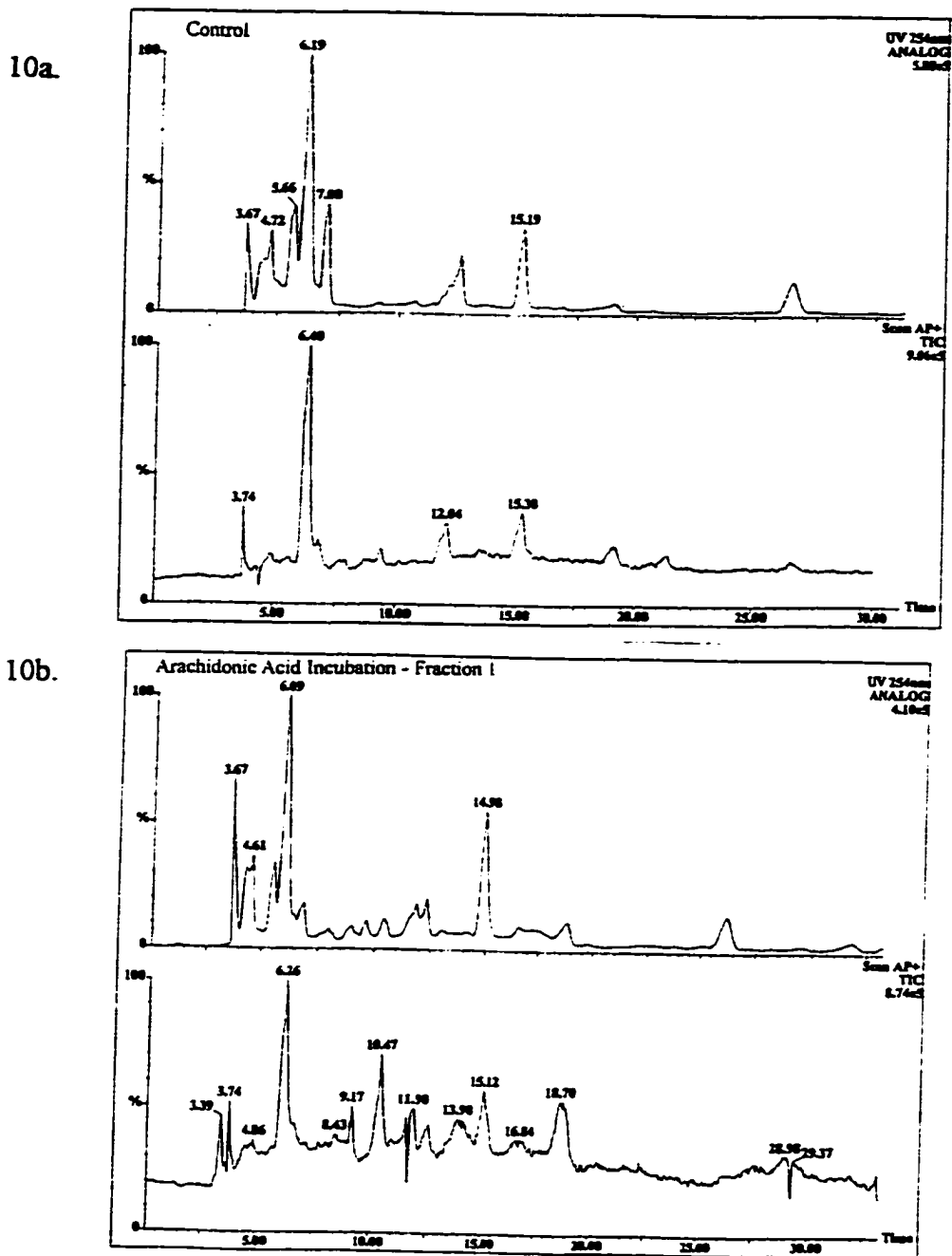
Figure 10. LC/MS data for ADAM derivatizations, UV chromatogram (top), TIC (bottom). 10a) control, 10b) arachidonic acid incubation (200 μM), Fraction 1.

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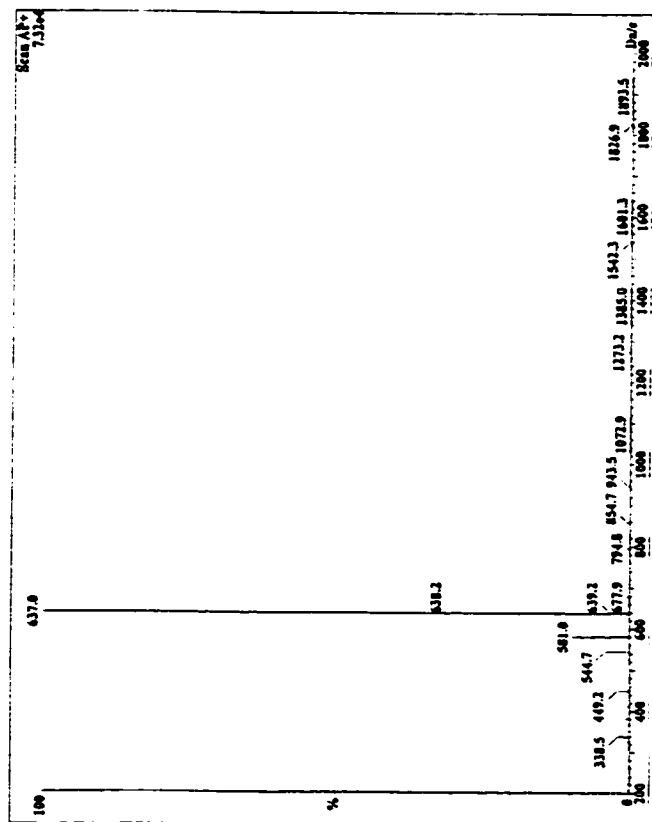
Figure 11. APcI positive-ion mass spectra of oxygenated trienoic fatty acid intermediate, potentially dihydroxy-hydroperoxy eicosatrienoic acid, derivatized with ADAM, and present as a potassium salt (m/z 637).

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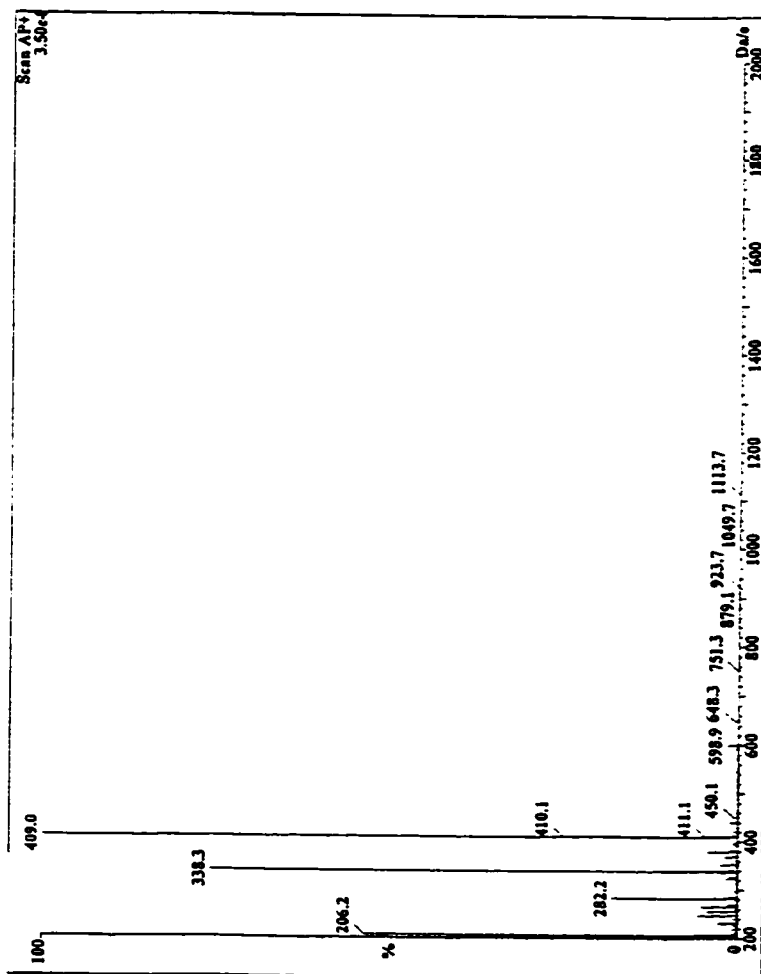
Figure 12. APcI positive-ion mass spectra of oxygenated trienoic fatty acid intermediate, potentially dihydroxy-hydroperoxy eicosatrienoic acid, as a potassium salt (m/z 409). Characteristic [M+1-71] peak at m/z 338.

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a much lesser extent in the control sample (Figure 10). Since a variety of options as to the structure of this metabolite exist, a confident assignment could not be made at this time.

3.4 Conjugated Diene Formation in *Anadyomene stellata* Chloroplasts

3.4.1 Preparation of a Semi-Purified Enzyme

Three series of fractions eluting off the Macro-Prep High Q column (elution vols. 475 - 511 mls (F1), 699 - 745 mls (F2), and 910 – 963 mls (F3)) were concentrated separately with a 10.000 Da molecular weight cutoff membrane (YM10) in an Amicon Ultrafiltration cell. The series were chosen from peaks in A_{280} and A_{234} data and are seen on the elution profile illustrated in Figure 13. All three series had corresponding peaks in both protein concentration and LO activity. The concentrate from each peak was ran separately on the Sephacryl gel filtration column. Active fractions from each run eluted within the same volume, 33.3 – 38.6 mls (Figure 14). However, F3 was found to be most active when analyzed for LO activity with hydroperoxy product formation equal to 7.44 nmol over the three minute incubation period. In the other two fractions, 6.64 nmols (F1) and 4.88 (F2) nmols of hydroperoxy product was formed over
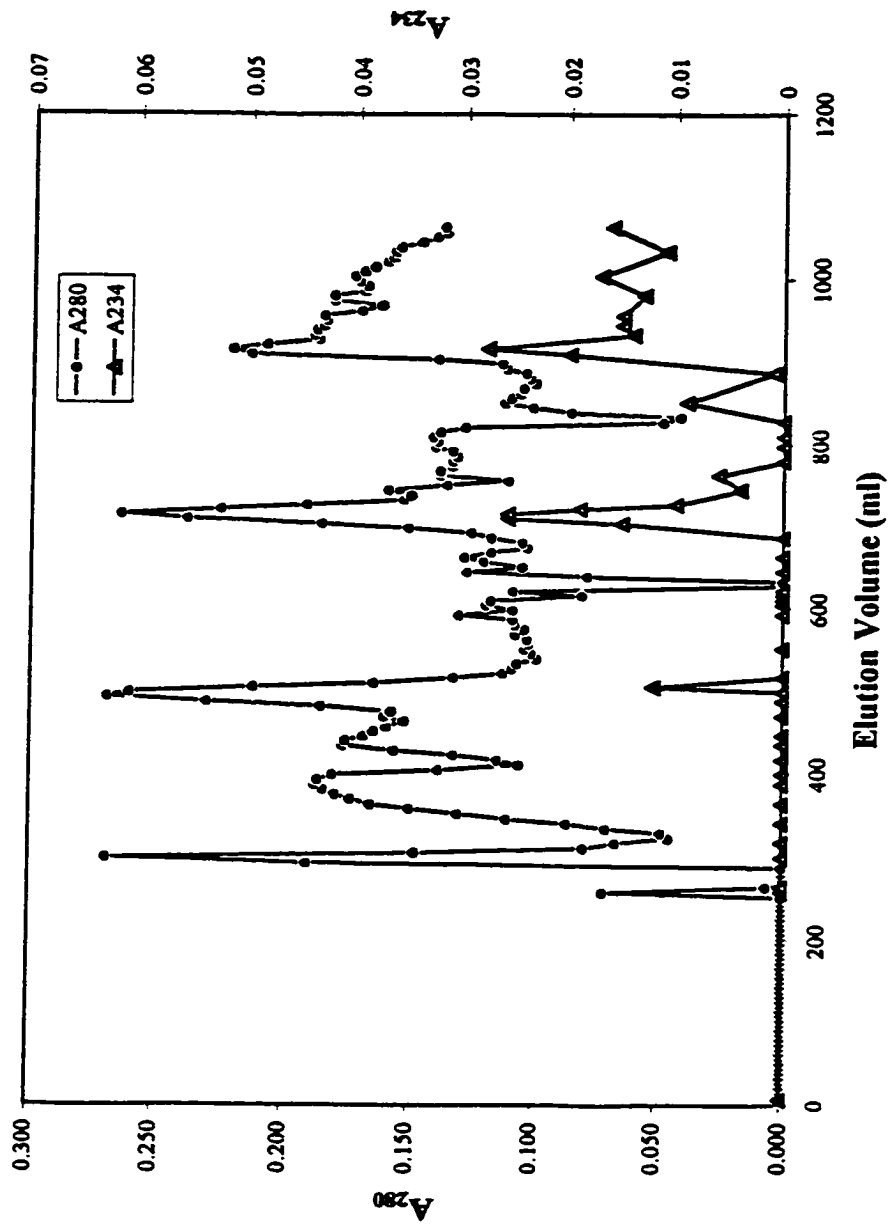
Figure 13. Anion exchange column elution profile. Protein concentration monitored at A_{280}, and conjugated diene formation analyzed at A_{234}.

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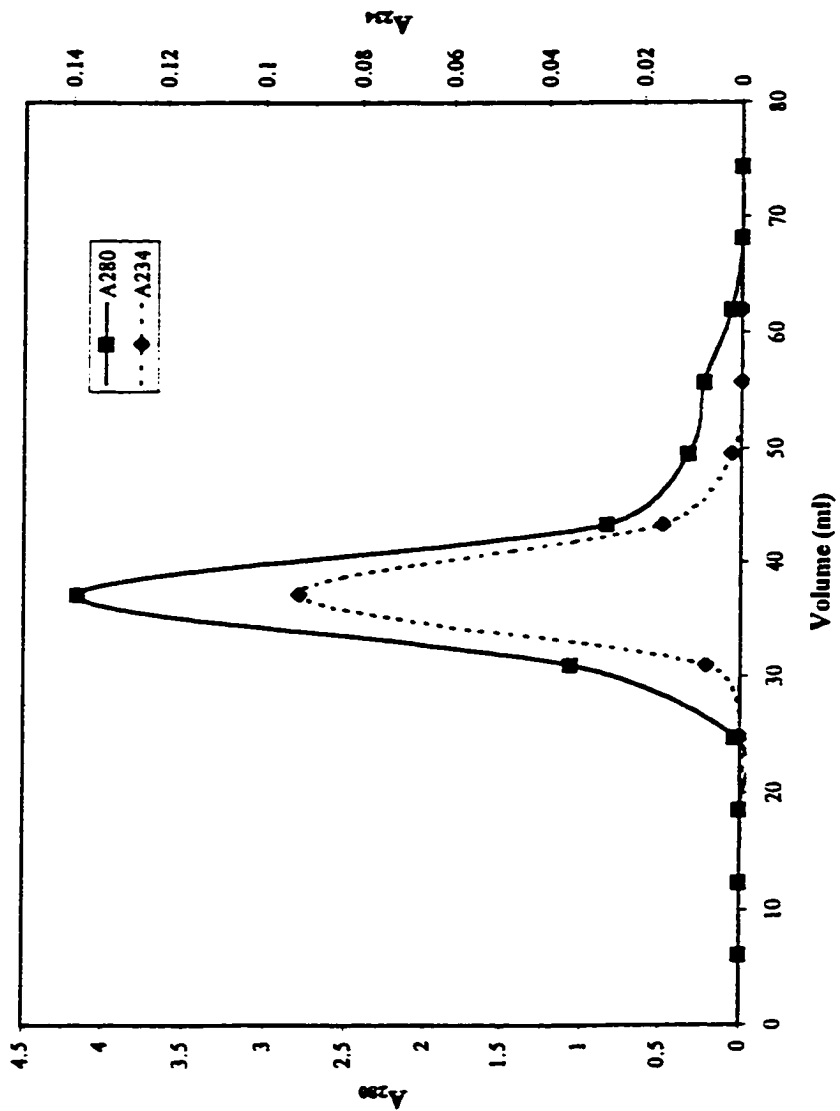
Figure 14. Gel filtration column elution profile. Protein concentration monitored at A_{280}, and conjugated diene formation analyzed at A_{234}.

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the three minute incubation period. By estimating a molecular weight of 182,000 g/mol based on that determined for LO isolated from *Chlorella pyrenoidosa* (Vick and Zimmerman. 1989) and an extinction coefficient of 5600 $M^{-1}cm^{-1}$ as known for tryptophan at 280 nm (Freifelder. 1982). a total protein concentration of the active preparation was calculated to be 135.2 mg/ml. This allowed for an estimation of the specific activity of the preparation to be 0.092 nmol min^{-1} mg^{-1}. This fraction. F3, was used for all biosynthesis experiments.

To gain additional evidence that the increase in A_{234} values observed were resultant of enzymatic activity. aliquots of the enzyme were boiled for 15 minutes prior to incubation with the substrate and 0.05 M potassium phosphate buffer (0.2 mM $CaCl_2$. pH 7.0). In regards to conjugated diene formation. the specific activity of the boiled preparation (0.016 nmol min^{-1} mg^{-1}) was 48% less than the un-boiled preparation (0.031 nmol min^{-1} mig^{-1}). Complete obliteration of enzymatic activity was demonstrated by boiling the enzyme preparation in the presence of 5% B-mercaptoethanol.

3.4.2 Dependency of Diene Formation on Calcium Concentrations

Calcium dependency of the crude enzyme preparation was analyzed by a series of experiments using 0.05 M potassium phosphate buffers with varying concentrations of $CaCl_2$. including 0. 0.2 , 1.0, and 5.0 mM $CaCl_2$. Additionally,

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controls with enzyme preparation in buffer (0.2 mM $CaCl_2$) and substrate in buffer (0.2 mM $CaCl_2$) were also analyzed. Incubations were prepared with constant enzyme concentrations as described for the LO activity assay. As seen in Table 4, a 0.05 M potassium phosphate buffer with 0.2 mM $CaCl_2$ resulted in the greatest rate of hydroperoxy product formation with 0.845 nmol of hydroperoxy products formed per minute. However, calculation of standard error of the mean between data sets demonstrated that no significant differences existed between the $CaCl_2$ concentrations tested (0, 0.2, 1.0, and 5.0 mM $CaCl_2$, n=5 for each data set). From this data, it appears that the enzyme preparation was calcium independent.

Table 4. Conjugated Diene Product Formation As
A Function of $CaCl_2$ Concentration

	A_{234} (Avg.)	SEM	nmol/min
Linoleic acid alone	0.002	0.002	0.030
Enzyme prep. alone	0.007	0.002	0.140
0 mM $CaCl_2$	0.033	0.006	0.660
0.2 mM $CaCl_2$	0.042	0.004	0.845
1 mM $CaCl_2$	0.036	0.004	0.715
5 mM $CaCl_2$	0.030	0.008	0.595

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3.4.3 Thin Layer Chromatography of Products

Following incubations of the enzyme preparation with linoleic acid and arachidonic acid, acidification, and chromatographic separation over C18 Amprep minicolumn, samples were analyzed by TLC. Several compounds were visualized in the samples incubated with substrate that are likely to be resultant of enzymatic activity since they were not identified in the controls of enzyme alone and substrate alone (Figure 15). The product of most interest to us was identified in the sample incubated with linoleic acid and had an R_f value of 0.150 which is equivalent to the R_f value of the standard 9-hydroxyoctadecaenoic acid (9-HODE). R_f =0.155. This spot was visible by UV and turned a grey/blue color following treatment with the lipid- and hydroxy fatty acid- specific spray, phosphomolybdic acid. Together with the R_f value comparison, UV absorbing properties, and visualization with phosphomolybdic acid, the compound appears to have the same chemical characteristics as 9-HODE. The solvent system used allowed reasonable separation between 9-HODE (R_f = 0.155) and 13-HODE (R_f = 0.212), thereby suggesting that the unknown compound is most likely 9-HODE. Furthermore, no compounds with similar migratory characteristics were observed in the control lanes. The R_f value of linoleic acid alone was observed to be 0.478 in this system.

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Lane	Contents
1	9-HODE standard
2	13-HODE standard
3	1/10 dilution of sample: enzyme incubation with linoleic acid
4	Sample: enzyme incubation with linoleic acid
5	Linoleic acid standard
6	Control: substrate
7	Control: enzyme
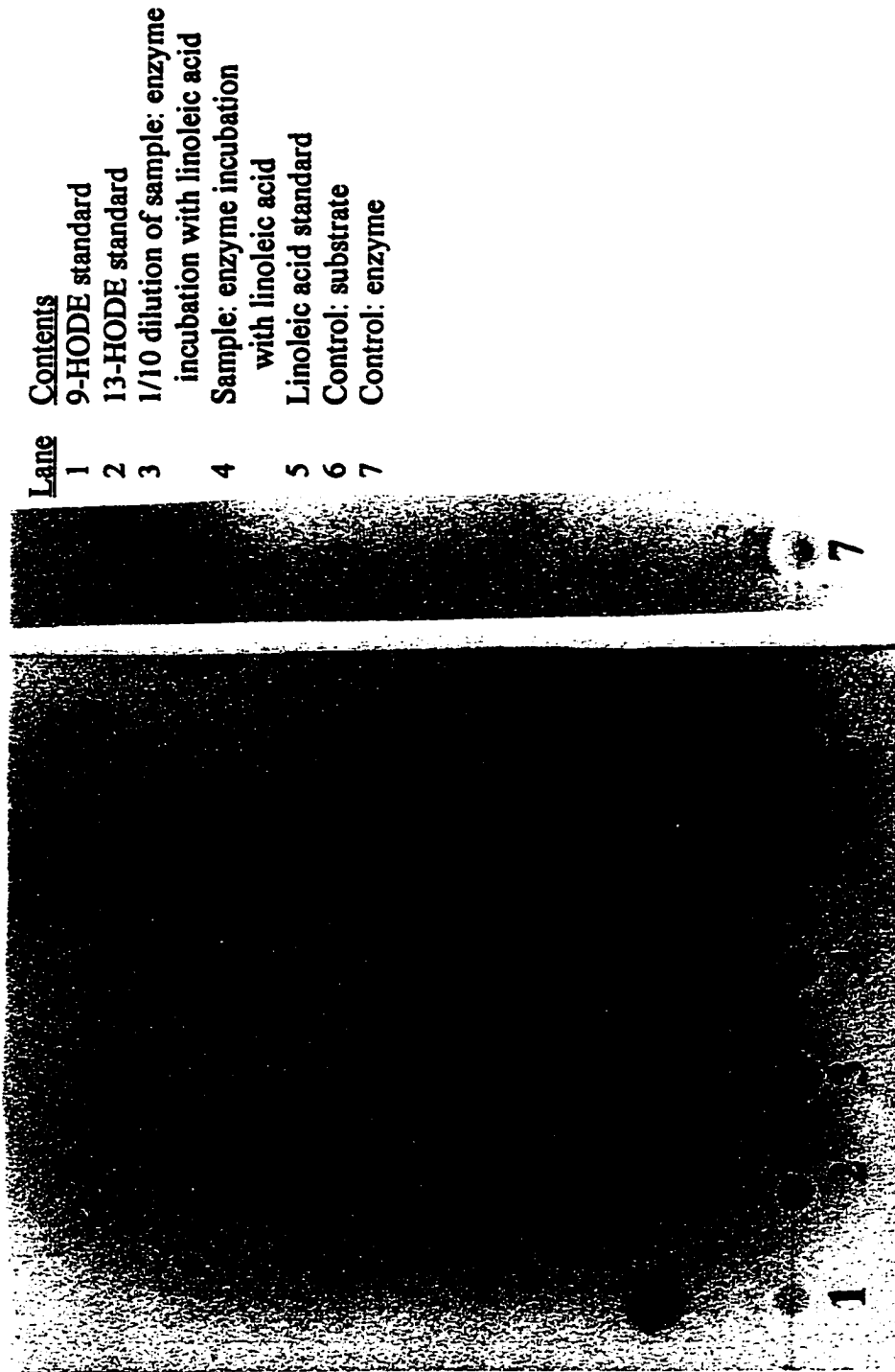
Figure 15. Straight phase TLC plate developed with phosphomolybdic acid spray.

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3.5 Effects of Arachidonic Acid on Oxygen Flux in Whole Algae Thalli

Since the fatty acid oxidative processes described thus far are O_2 consuming, we carried out the following physiological experiments to explore potential relationships. To determine if AA in the local sea water environment had an effect on oxygen flux in *A. stellata*, oxygen concentrations in a controlled environment containing algae thalli were monitored in the presence and absence of the fatty acid. An effect elicited by the AA vehicle (ethanol) on the algae was ruled out with a set of preliminary experiments demonstrating no significant change in the mean rates of oxygen production for thalli with and without the addition of ethanol. 6.99 ± 1.87 μmol O_2 g^{-1}WWh^{-1} and 6.41 ± 2.41 μmol O_2 g^{-1}WWh^{-1}, respectively.

Initial analysis of individual control values for the experiments conducted under photosynthetic conditions showed that variations in the pre-treatment O_2 values were independent of the treatment values when differences were analyzed. In several cases, treatment effects were exaggerated by using pre-treatment values to examine the magnitude of effect. Unpaired statistical analysis was therefore utilized on the assumption that addition of AA may have no direct effect on photosynthetic rates. Pooled pre-treatment values from all photosynthetic experiments averaged about 10 μM g^{-1} WW hr^{-1} (Table 5). Under

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conditions of darkness. oxygen flux changed direction shifting from evolution to uptake (Table 5) with the thalli averaging -6.41 μM $g^{-1}WWhr^{-1}$. The mean differences between pre-treatment groups in the photosynthetic and dark periods were statistically significant ($P < 0.05$) clearly delineating the periods of oxygen production occurring during photosynthesis from net oxygen uptake during the dark period.

In the photosynthetic (light) period. exposure of the thalli to various concentrations of AA (3.13. 6.25. 12.5. 25. 50. and 100 μM final concentrations) reversed the direction of oxygen flux in a manner qualitatively and quantitatively similar to that seen in the control dark period ($P < 0.01$. Table 5). Although the magnitude of response to AA treatment was not dependent on concentration. it was sufficient to mitigate over 50% of the oxygen production. Individual responses to AA administration were all within normal variation and the means were not statistically different from each other. It was evident from these results that AA oxidation had saturated and the threshold for activation was less than 3.13 μM. This may be the result of AA partitioning into a non-aqueous phase in the alga or precipitating out of its aqueous environment due to physical properties of the sea water. thus resulting in an apparent saturation.

Table 5. Effects of Arachidonic Acid on O_2 Flux in *A. stellata* thalli

AA Concentration (µM)	n	O_2 Flux (µM O_2 g^{-1} WWh^{-1}) (mean ± SE)
Light Period		
Control	63	10.17 ± 1.95
3.13	7	$-9.40 \pm 3.36^{\dagger}$
6.25	10	$-4.14 \pm 2.25^{\dagger}$
12.50	11	$-4.28 \pm 2.53^{\dagger}$
25.00	14	$-6.05 \pm 3.03^{\dagger}$
50.00	13	$-2.86 \pm 3.24^{\dagger}$
100.00	8	$-6.73 \pm 6.53^{\dagger}$
Dark Period		
Control	13	$-6.41 \pm 0.62^{\ddagger}$
100.00	13	$-11.69 \pm 1.85^{*}$

†Significant difference between means of treatment groups relative to control in the light (Student's t-test: $P<0.01$)

‡Significant difference between means of control group relative to treatment groups in the dark. $P<0.05$

*Mean O_2 uptake values for 100 µM treated groups in both light and dark not statistically different, $P>0.05$

Addition of 100 µM AA to the thalli in the dark resulted in a marked increase in oxygen uptake (P < 0.05. Table 5). These data indicate that under conditions of darkness. AA administration was able to stimulate oxidative

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pathways in addition to those already occurring. It should be noted that the pre-treatment group for the dark period had a smaller standard error relative to the pre-treatment group of the photosynthetic period indicating more uniform values were obtained in the dark period (c.v. = 0.182 and 0.097, respectively). AA exposure appears to alter oxygen flux to the same extent during both photoperiods, albeit perhaps indirectly. Overall, these experiments suggest that the enhanced oxygen uptake occurring in both photoperiods following exposure to AA may not be directly coupled to oxygen fluxes caused by the photosynthetic / respiratory mechanisms of the alga.

These data raised the question of whether the observed changes in oxygen fluxes after additions of AA resulted from a process which required the uptake and metabolism of this PUFA or whether it was due to other effects such as the activation or inhibition of a cell signaling mechanism. This question was pursued in a series of experiments in which intact algal thalli were incubated for 1.5h in the presence of 100 μM AA. The resultant metabolites were extracted and scanned by UV for the presence of specific oxidation products with the absorbance spectrum indicative of a conjugated tetraene functionality ($\lambda = 294$, 306, and 322 nm). Only trace levels of these compounds were present in control samples incubated in the absence of AA. These data indicate that at least a fraction of the AA added passed from the seawater into the algal cells during the incubation period, and activated a PUFA oxidative process.

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3.6 Fatty Acid Analyses of Isolated Chloroplasts from *Anadyomene stellata*

3.6.1 *Anadyomene stellata* Collected in the Florida Keys

The fatty acid profile of *A. stellata* chloroplasts was analyzed to determine the endogenous substrates available for the fatty acid oxidative pathways under study. FAMEs were prepared and analyzed GC/MS (Figure 16). The resultant profile is illustrated in Figure 17. Approximately 35% of all fatty acids in the chloroplasts are palmitic acid (16:0). The next highest contributors are isomers of 18:1 and 20:4, presumably oleic acid (n-9) and arachidonic acid (n-6), which comprise about 14% and 12% of the total fatty acids, respectively. The PUFA 18:2 was also found to contribute a significant amount (10%) to the total. Saturated fatty acids comprise 43% of total fatty acids.
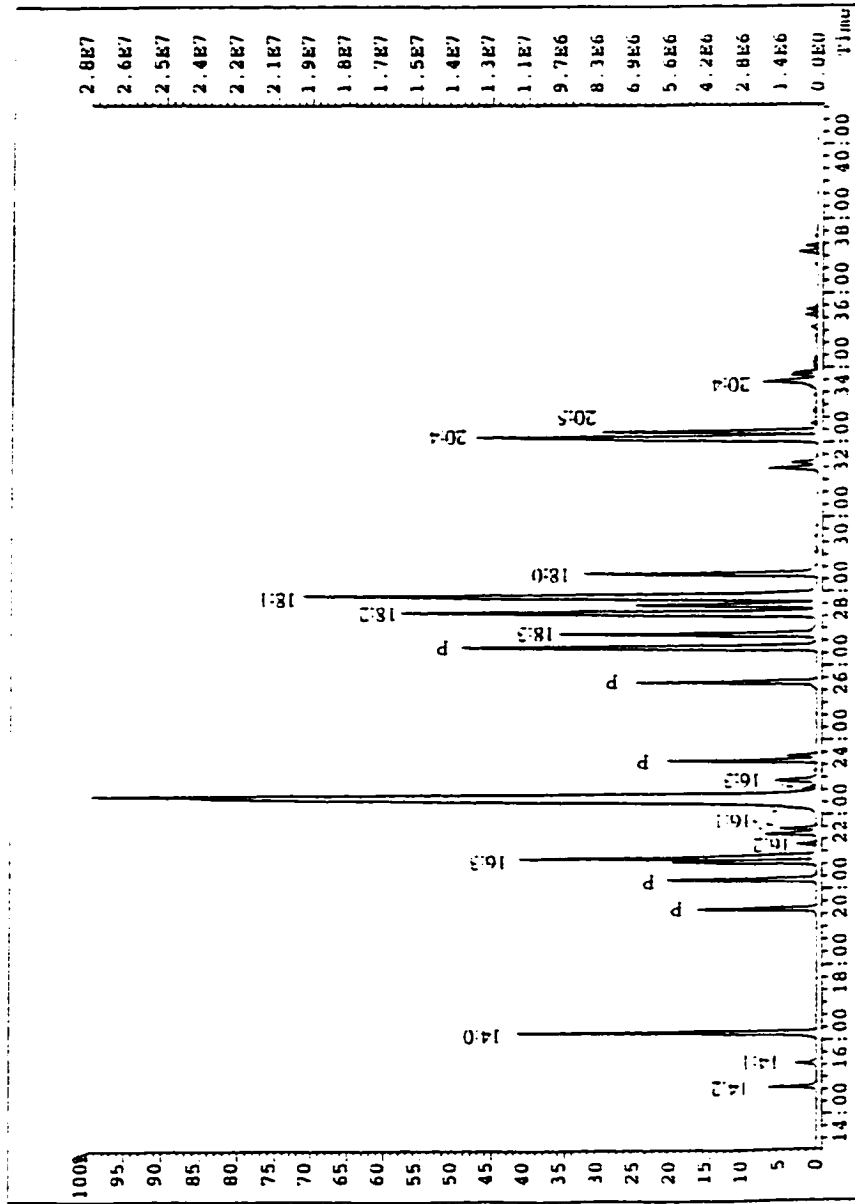
83

Figure 16. GC/MS chromatogram of fatty acid methyl esters in isolated chloroplasts from A. stellata. (Florida Keys collection)
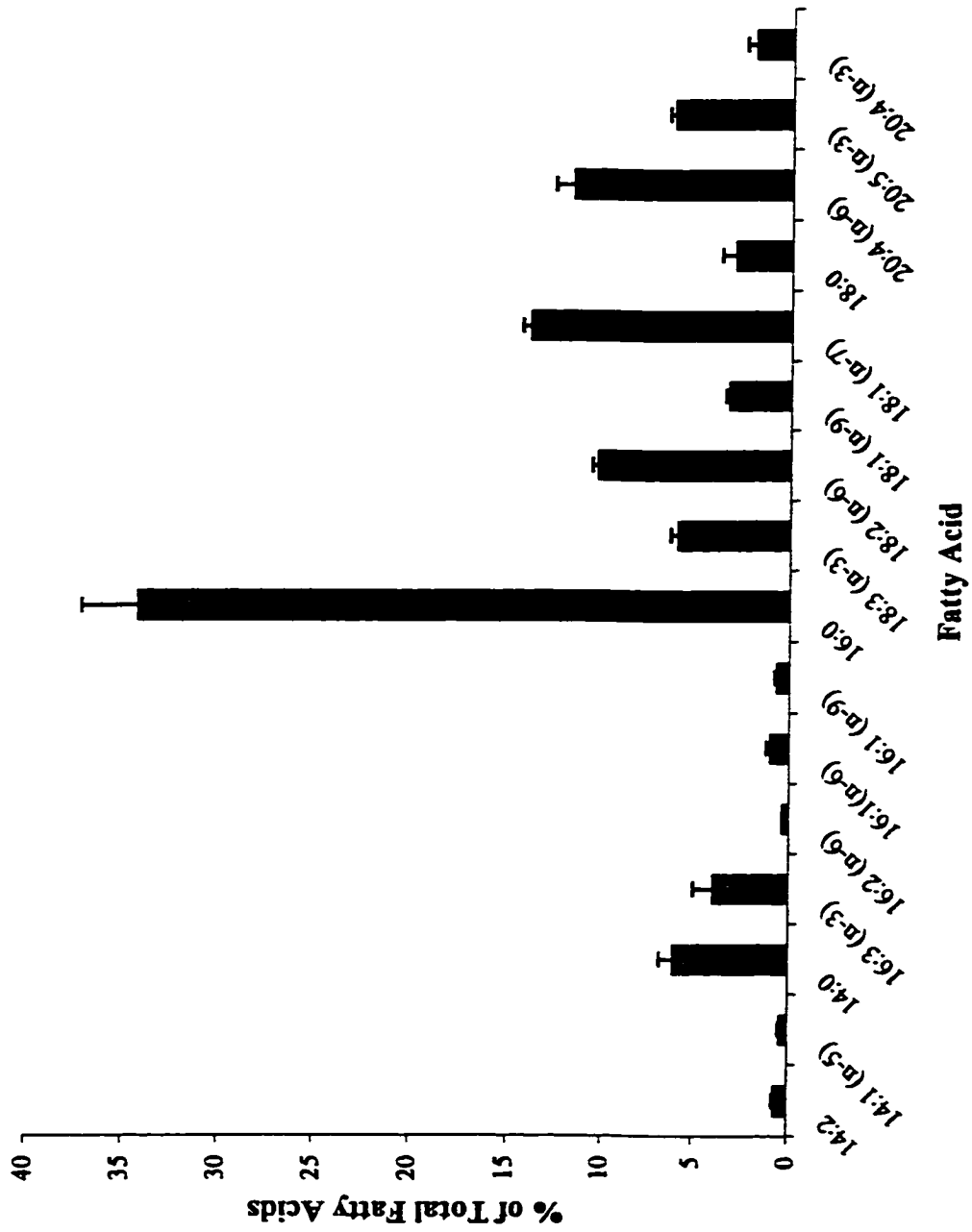
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Figure 17. Fatty acid profile of chloroplasts isolated from *A. stellata* (Florida Keys collection).

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3.6.2 Comparison of Fatty Acid Compositions of *Anadyomene stellata* Collected at Three Mediterranean Sites

The major fatty acids of *A. stellata* chloroplasts isolated from algae collected from the three Mediterranean locations were found to be 16:0, 18:1 (n-9, n-7), 18:2 and 20:4 (n-6, n-3). The saturated fatty acid, palmitic acid, was present in the highest amounts in all samples consisting of approximately 25 to 27% of total fatty acids. Similar to data obtained from the Florida Bay algae, Mediterranean samples also contained an unusually high level of C20 PUFAs for a Chorophyte. On average, C20 PUFAs comprised 17% of total FAs in these samples. Surprisingly, averaged data collected from all three Mediterranean sites illustrated unexpected levels of 14:1 and 14:2 fatty acids, 0.25% and 0.88%, respectively. Although these values are quite small, both fatty acids are not common constituents of Chlorophytes and are, therefore, of interest in *A. stellata*. Saturated fatty acid content was 33.7%, 33.7%, and 32.8% for the samples collected at Daskalio, Ftelios, and Vagias Beach, respectively. The complete fatty acid profile is illustrated in Figure 18.

An analysis of variance was employed to determine if differences between the populations exist for the contribution of each fatty acid (Table 6). Including isomers, 19 fatty acids were identified in all samples: 4 saturated, 6 monounsaturated, and 9 polyunsaturated fatty acids. Twelve were found to have significant differences between either all locations or one other site. Although

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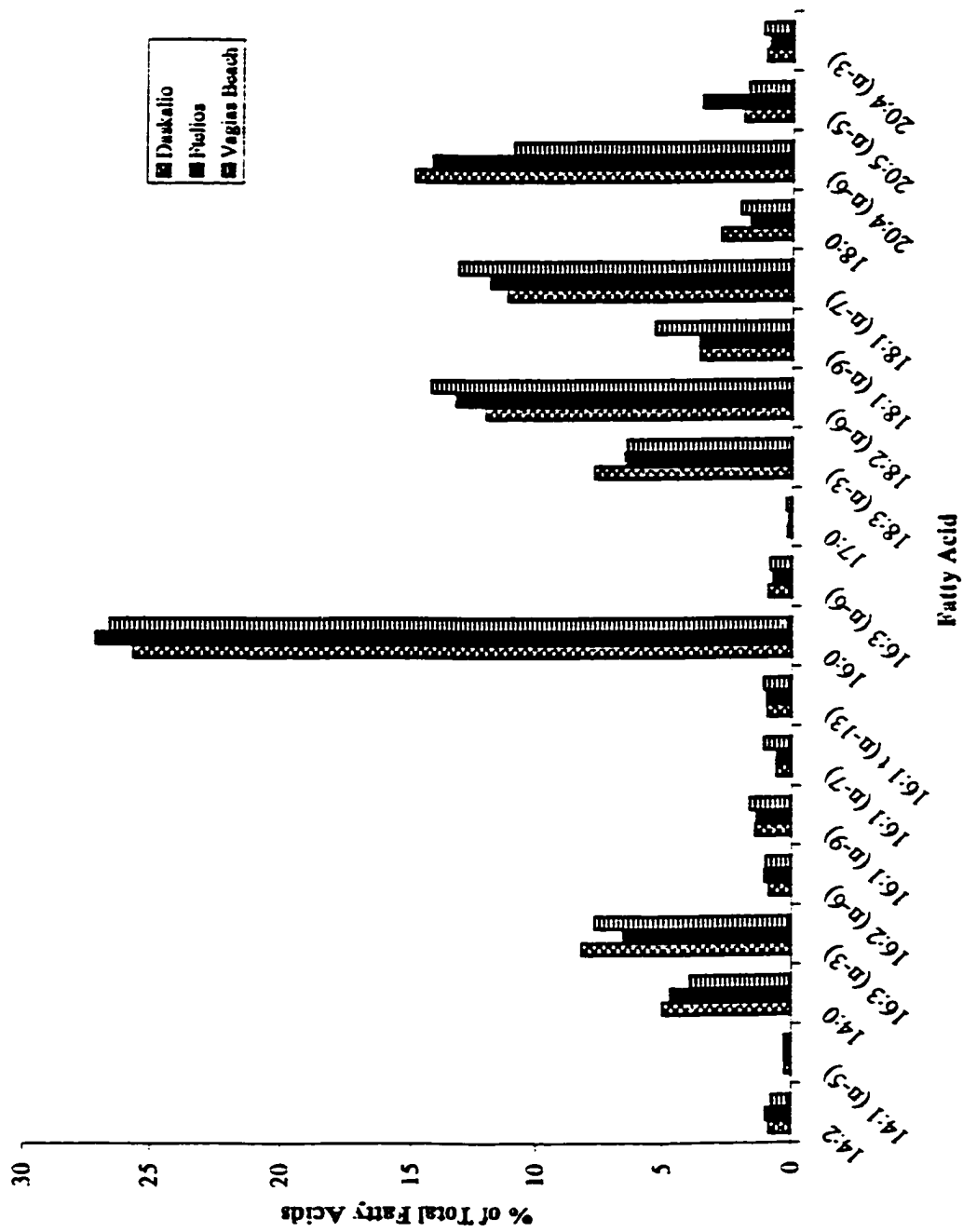
Figure 18. Fatty acid profile of chloroplasts isolated from *A. stellata* (Mediterranean Sea collection).

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Table 1. Chloroplast fatty acid composition from *Anadyomene stellata*

Fatty Acid	Average % of Total Fatty Acids		
	Daskalio	Ftelios	Vagias Beach
14:2 *	0.87	1.01[t]	0.77
14:1 (n-5)	0.23	0.27	0.26
14:0	5.00[t]	4.70[t]	3.94
16:3 (n-3)	8.17[t]	6.52	7.66[t]
16:3 (n-6)	0.92[‡]	0.73	0.84
16:2 (n-6)	0.86	1.05	1.00
16:1 (n-9)	1.40	1.35	1.62
16:1 (n-7)	0.59	0.59	1.09[t]
16:1 *trans* (n-13)	0.94	0.96	1.08
16:0	25.78	27.29	26.70
17:0	0.17	0.13	0.21
18:3 (n-3)	7.70[‡] [0.69][§]	6.46 [0.55][§]	6.37 [0.48][§]
18:2 (n-6)	12.02[t]	13.28[t]	14.27[t]
18:1 (n-9)	3.59	3.60	5.30[t]
18:1 (n-7)	11.15	11.85	13.16[t]
18:0	2.75[t]	1.61	1.99
20:4 (n-6)	14.97[t]	14.24[t]	10.91
20:5 (n-3)	1.88	3.48[t]	1.68
20:4 (n-3)	1.01	0.89	1.14

*Location of double bonds is unknown.

[t]Values significantly greater than values without [t] based on ANOVA analysis. $P < 0.05$.

[‡]Values that are significantly greater than lowest value of the group, but no significant difference from the mid-ranged value (ANOVA. $P < 0.05$)

[§]Ratio of 18:3 to 18:1

statistically significant. these differences were relatively minor. and were in the range of 2% or less with the exception of 20:4 (n-6) which was approximately 4% less at the Vagias Beach site than the other two sites.

3.6.3 Comparison Between Florida and Mediterranean Collections of *Anadyomene stellata*

Comparison of the fatty acid profiles from both Florida Bay and Mediterranean waters revealed some interesting observations (Figure 19). First. levels of palmitic acid appear much higher in the Florida algae (34% of total fatty acids) than the Mediterranean samples (26-27% of total fatty acids). Also. 20:5 (presumably eicosapentaenoic acid) is found in greater amounts in the algae collected in Florida (6% of total fatty acids) than in the Mediterranean samples (2-3% of total fatty acids). The presence of 16:3 (n-6) and 17:0 were not detected in significant levels in the Florida algae. but were identified in the Mediterranean samples (0.8% and 0.2%. respectively).
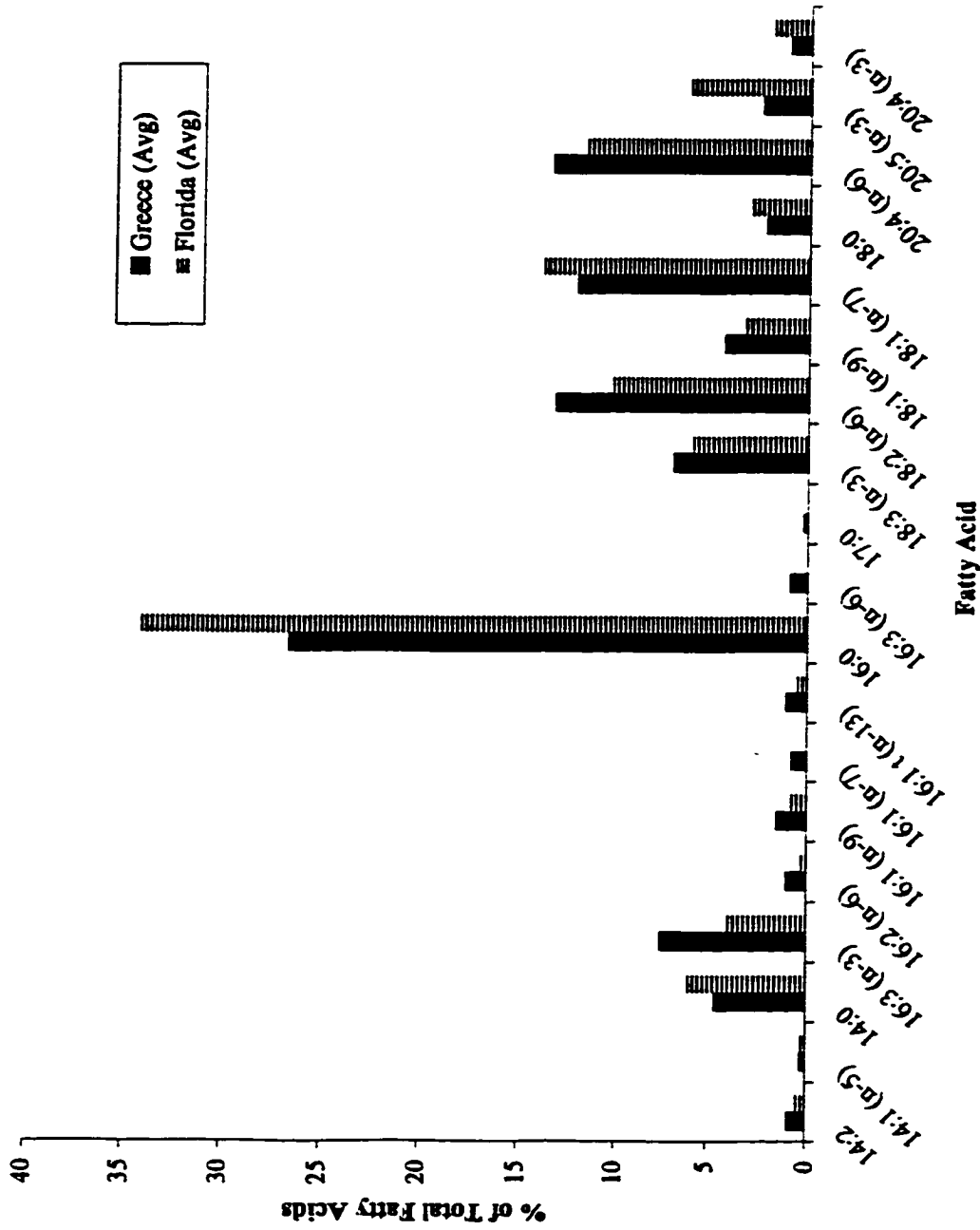
Figure 19. Comparison of chloroplast fatty acid compositions from *A. stellata* collected in the Florida Keys and the Mediterranean Sea.

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4.0 DISCUSSION

Oxylipins are widely distributed in plants, animals, and a variety of other organisms including macrophytic and unicellular algae. Many recent studies have uncovered a diverse array of chemical structures within this family of compounds, along with equally unique biosynthetic pathways (reviewed in Gerwick and Bernart, 1993). However, the exact physiological roles of oxylipins in algae are much less clear than in plants and animals. Intra and inter-organismal signaling events, such as chemical defense, reproduction, and growth regulation, have been implicated based on parallel pathways described in plants (Vick and Zimmerman, 1989; Gerwick and Bernart, 1993).

4.1 Conjugated Tetraene PUFAs in *Anadyomene stellata*

A series of five conjugated tetraene containing PUFAs were identified in whole algal homogenates prepared from *A. stellata*. These included 16:5, 18:4, 20:5, 20:6, and 22:7 PUFAs. GC/MS and NMR techniques enabled us to identify one of these compounds (22:7) as a novel PUFA not described elsewhere in the literature, and another one (20:5) as the recently described bosseopentaenoic acid (Burgess *et al.*, 1991). We proposed the name,

stellaheptaenoic acid, for the former compound. The discovery of conjugated

tetraene containing PUFAs in *A. stellata* suggests a unique oxidative pathway is

present.

Additional studies pointed to the chloroplasts of *A. stellata* as a main

location for the biosynthesis of these unique conjugated tetraenes. This data is

consistent with recent work illustrating the presence of fatty acid oxidative

pathways in spinach chloroplast membranes (Blee and Joyard, 1996).

Furthermore, plastid enzymes have been recognized to play a major role in

signaling fundamental physiological processes in plants via the production of

lipid mediators (Joyard *et al.*, 1991; Bell *et al.*, 1995; Voros *et al.*, 1998). Based

on our data and that of others, isolated chloroplast preparations were utilized to

further study the biosynthetic capabilities and enzymatic characteristics of

conjugated tetraene production.

As outlined in the results, incubation with six different PUFA substrates

resulted in production of a variety of combinations of the same conjugated

tetraenes identified in the whole algae homogenate work. Although all substrates

were capable of initiating the formation of at least one species of conjugated

tetraenes, only incubations with the 22 carbon substrates were found to result in

the production of all five species. We have proposed a tentative explanation for

this observation suggesting that the various exogenous fatty acids supplied, or

metabolites produced from them, are each eliciting the release of specific profiles

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of fatty acids from complex lipid species which then potentially feed into this tetraene biosynthetic pathway. A possibility for the source of these fatty acids is the galactolipids which are found in high amounts in chloroplast membranes. Additional work is necessary to uncover this complex regulation of the flow of fatty acids into this pathway. as well as the physiological roles of the products.

Our studies on the enzymatic characteristics of this fatty acid oxidative pathway in isolated chloroplasts preparations provided information regarding the behavior of the system. Suggesting that the oxidation occurring was enzymatically driven. the activity was significantly diminished following boiling of the chloroplasts prior to the incubations. Furthermore, we were able to observe classical Michaelis-Menten-type kinetics following incubation with various concentrations of AA. beginning with a pseudo-first order linear response phase followed by saturation equilibrium kinetics. Estimates of apparent K_m and V_{max} values were empirically determined. Incubations with all of the chosen PUFA substrates. palmitoleic (16:1). 6,9,12,15-octadecatetraenoic (18:4). arachidonic (20:4). eicosapentaenoic (20:5), 7,10,13,16-docosatetraenoic (22:4). and 4,7,10,13,16,19-docosahexaenoic (22:6) acids, illustrated the ability of the enzyme system to produces a variety of chromophores with absorption spectra typical of conjugated tetraenes. Kinetic properties for each substrate were graphically determined. This data demonstrated preference of the enzyme

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preparation to AA. as seen by the lowest K_m^{app} value (55 μM). However, 22:6 was observed to react with the fastest velocity (V_{max}^{app} = 8.380 μM min^{-1} mg^{-1}).

Analyses of the conjugated tetraene structures (Figure 7) produced and the kinetic parameters of the various substrates suggest that the enzyme system introduces the conjugated tetraene with a range of specificity relative to the carboxy terminus. In all products, the conjugated tetraene functionality begins at either the 5th, 6th or 7th carbon from the carboxy terminus. It is conceivable that a critical range of distance between the carboxy terminus to the first point of unsaturation is required for proper fit of the substrate into the catalytic site. Palmitoleic acid does not offer a point of unsaturation until the ninth carbon of the molecule, which may result in a saturated chain that is too long for efficient catalysis. The catalytic efficiencies (V_{max}/K_m) observed for each substrate support this critical range of distances concept. The 22:4 and 18:4 substrates had the lowest V_{max}/K_m values and the longest saturated chain portions following the carboxylic acid group with stretches of 5 and 4 saturated carbons, respectively. These distances may be inhibiting optimal binding of the substrate to the catalytic site. However, 22:6, 20:4, and 20:5 had high efficiency values and shorter saturated carbon chain lengths following the carboxylic acid group, with stretches of 2, 3, and 3 saturated carbons, respectively. Furthermore, comparison of K_m^{app} values suggest that the enzyme preparation does not require a specific

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arrangement in regards to the olefinic group proximal to the methyl terminus since no preference towards n-3 or n-6 fatty acids was observed.

In recent studies of the unrelated red marine algae. *Lithothamnion corallioides* (Corallinales). Hamberg reported conjugated tetraene biosynthesis in cell free preparations (1992. 1993). This work postulated that conjugated tetraene formation from γ-linolenic acid was catalyzed by a fatty acid oxidase. The enzyme appeared to be oxygen-dependent. as suggested by the production of equivalent levels of hydrogen peroxide and the ability to support the oxidation under anaerobic conditions with a number of artificial electron acceptors such as *p*-benzoquinone. Figure 20 illustrates the biosynthetic mechanism proposed in these studies. A similar pathway is conceivable in the formation of at least some of the conjugated tetraenes observed in *A. stellata*. It is important to note that 6.9.12.15-octadecatetraenoic acid was utilized in our studies. not 6.9.12-octadecatrienoic acid as in Hamberg's work. However. both have the same unsaturated region between carbons 6 and 13 in the olefinic chain and result in the presence of the conjugated tetraene 6.8.10.12-octadecatetraenoic acid. If similar fatty acid oxidase systems are present in both of these divergent algal phyla. then this biosynthetic pathway must be of utmost biochemical and physiological significance to have survived evolutionary pressures.

Other mechanisms of conjugated tetraene formation are feasible. as well. For example, Burgess *et al.* proposed that a completely different mechanism was
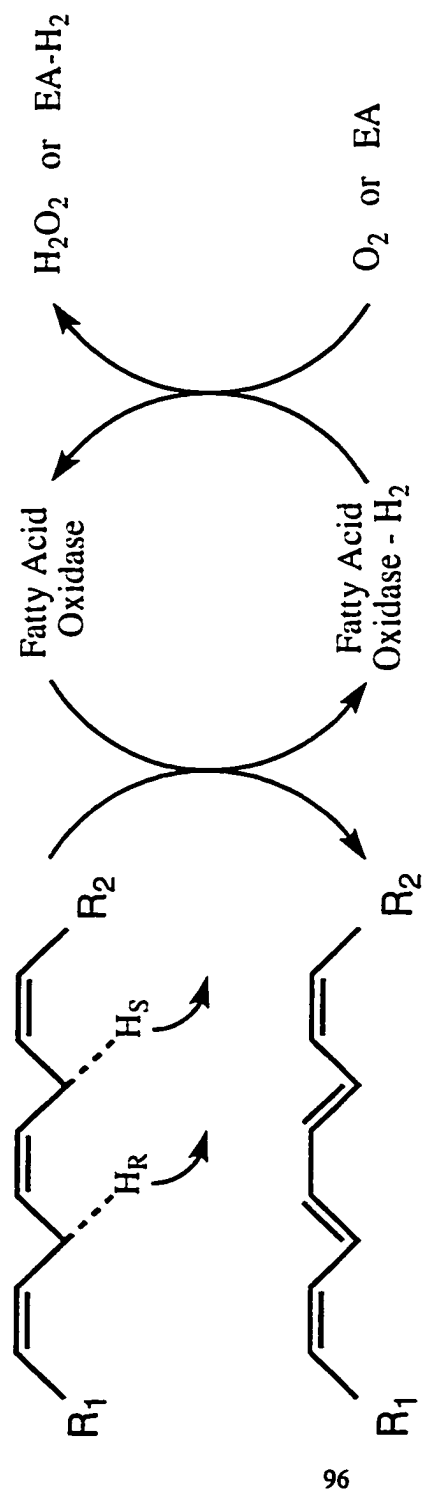
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Figure 20. *From* Hamberg, Mats. Metabolism of 6,9,12-Octadecatrienoic Acid in the Red Alga *Lithothamnon coralloides*: Mechanism of Formation of a Conjugated Tetraene Fatty Acid. Biochemical and Biophysical Research Communications. **188:**1220-1227, 1992. "EA" - Artificial Electron Acceptor.

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involved in the formation of the conjugated tetraene. bosseopentaenoic acid.

This compound was originally isolated and identified from the red algae

Bossiella orbigniana following incubation of the algal preparation with AA

(Burgess *et al.*. 1991). and has also been identified in our preparations of *A.*

stellata. The formation of the conjugated tetraene functionality was proposed to

involve a lipoxygenase or a cytochrome P450-type enzyme resulting in the

production of a more polar intermediate. such as 12-HETE. Enzymatic

dehydration of this intermediate would then result in the formation of the

conjugated tetraene. Although further experimentation focused on the

elucidation of the enzymatic mechanism resulting in conjugated tetraene

formation in *A. stellata* is necessary. pathways such as those proposed for *L.*

corallioides and *B. orbigniana* are quite feasible.

4.2 Hydroxy and Hydroperoxy Polyenoic Fatty Acids from *Anadyomene stellata*

Enzymes involved in the biosynthetic pathways of important lipid

mediators have been identified in plant chloroplast preparations. These include

lipoxygenases and enzymes of the jasmonic acid (octadecanoid) pathway, such as

hydroperoxide lyase, hydroperoxide dehydratase (allene oxide synthase), and

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alcohol dehydrogenase (Blee and Joyard, 1996; Vick and Zimmerman, 1987; Song *et al.*, 1993). Plant lipoxygenases are believed to play a key role in the production of oxygenated fatty acids involved in vital signaling systems regulating developmental and defense responses to the environment (Bell *et al.*, 1995). LO activity and oxylipin production has been observed in many species of algae (Gerwick and Bernart, 1993; Kuo *et al.*, 1997; Kuo *et al.*, 1996; Beneytout *et al.*, 1989). However, there are no reports of studies concerning the compartmentalization of algal LOs in the literature.

Interest in the identification of hydroxy polyenoic fattty acids in *A. stellata* chloroplasts originated when scanning UV spectrophotometric analysis of these preparations consistently illustrated the presence of a single UV absorbing peak with a λmax of 238 nm. This absorbance maximum is characteristic of a conjugated diene such as that produced following lipoxygenase activity (Smith and Lands, 1972). The identification of potential LO products then became a focus of this work. However, due to the hydrophobic nature of most of the intermediates and their instability, isolation and correlation to their physiological roles has proved to be a difficult challenge.

The work reported here on lysed chloroplast preparations failed to detect diene formation. This was most likely the result of the presence of enzymes competing for the putative LO products, the high level of background interference in studies involving a crude preparation, and / or the inherent

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instability of intermediate oxylipin products. However, the use of partially purified enzyme preparations from the chloroplast lysate allowed conjugated diene formation to be detected as an increase in UV absorbace at 234 nm over a three minute incubation period following the addition of 353 μM linoleic acid (Surrey. 1964: Chen and Whitaker. 1986). Conjugated diene formation, suspected to be catalyzed by LO activity. proceeded at a rate of 2.48 nmol/min, and the specific activity of the enzyme preparation was calculated to be 0.092 nmol min^{-1} mg^{-1}. The oxidation occurring appears to be enzymatically driven since boiling the enzyme preparation prior to incubation partially inactivates it. Furthermore. upon denaturing disulfide bonds between cysteins with B-mercaptoethanol and boiling. catalytic activity was completely obliterated.

The significant increases in conjugated diene concentrations following incubation of the enzyme preparation with linoleic acid strongly suggest LO catalyzed hydroperoxy fatty acid formation. A compilation of indirect evidence further supports hydroperoxy formation via LO activity in the isolated chloroplasts of $A. stellata$. Based on R_f values from TLC data, the hydroxy fatty acid. 9-HODE. appears to be present in preparations of enzyme incubated with linoleic acid. A compound with the same migratory characteristics was not observed in the controls. thereby suggesting its biosynthesis.

A series of experiments designed to evaluate the effects of varying calcium concentrations on catalytic activity were performed on the partially

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purified enzyme preparation from *A. stellata* chloroplasts. Surprisingly, the putative LO activity did not seem to be dependent on calcium concentrations, as no significant differences were seen between the various concentrations including the absence of the divalent cation. These data are in opposition of that observed with partially purified LO preparations from the macrophytic green algae *Ulva lactuca*. LO activity in the *U. lactuca* preparations were found be calcium dependent as marked stimulation in activity was seen with 0.2 mM Ca^{2+} in the buffer (Kuo *et al.*, 1997). However, LO preparations from the unicellular green alga *Chlorella pyrenoidosa* were found to be active without calcium addition to the buffer (Bisakowski *et al.*, 1995). It is interesting to point out that both calcium dependent (5-LO) and calcium independent (12- and 15-LO's) enzymes are present in mammalian cells (Rouzer and Samuelsson, 1985; Lagarde *et al.*, 1984; Narumiya *et al.*, 1981). The lipoxygenase class of enzymes appear to have different catalytic properties that potentially correspond to cellular location and physiological roles.

The capability of various algae to produce fatty acid hydroperoxides via LO activity and the variety of oxylipin structures isolated from these organisms has led to a question of considerable evolutionary, biochemical, and ecological interest. Are hydroperoxide metabolic pathways, such as jasmonate production, existent in algae? Little work has been done to address this issue. However, recent studies in the unicellular green alga *Chlorella pyrenoidosa* demonstrate

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enzymatic activities characteristic of plant hydroperoxide metabolic pathways leading to jasmonate production (Vick and Zimmerman. 1989). These data strongly suggest that fatty acid hydroperoxide metabolism via similar pathways observed in plants is highly conserved (Vick and Zimmerman. 1989). The presence of such metabolic schemes in the macrophytic green algae. *A. stellata.* is quite possible. In addition. enzymes of the jasmonic acid pathway have been identified in the envelope membranes of spinach chloroplasts (Blee and Joyard. 1996). Structurally. the chloroplast membranes of Chlorophytes are similar to those of higher plants. as both are surrounded by a two-membrane envelope (Staehelin. 1986). These membranes could potentially house such enzymes in Chlorophytes. as well.

Observations of hydroperoxide formation and identification of potential intermediates of hydroperoxide metabolism in *A. stellata* support the possibile existence of hydroperoxide metabolic pathways in *A. stellata* chloroplasts. LC/MS (ApcI⁻) data obtained from our biosynthesis experiments involving the incubation of isolated chloroplasts with AA and labeled with ADAM revealed the presence of putative oxylipin compounds likely originating from the metabolism of lipoxygenase derived products. The structures of these compounds and a proposed biosynthetic mechanism for their formation are illustrated in Figure 21. This proposed pathway begins with double LO activity on AA leading to the formation of a dihydroperoxy fatty acid. Such enzymatic

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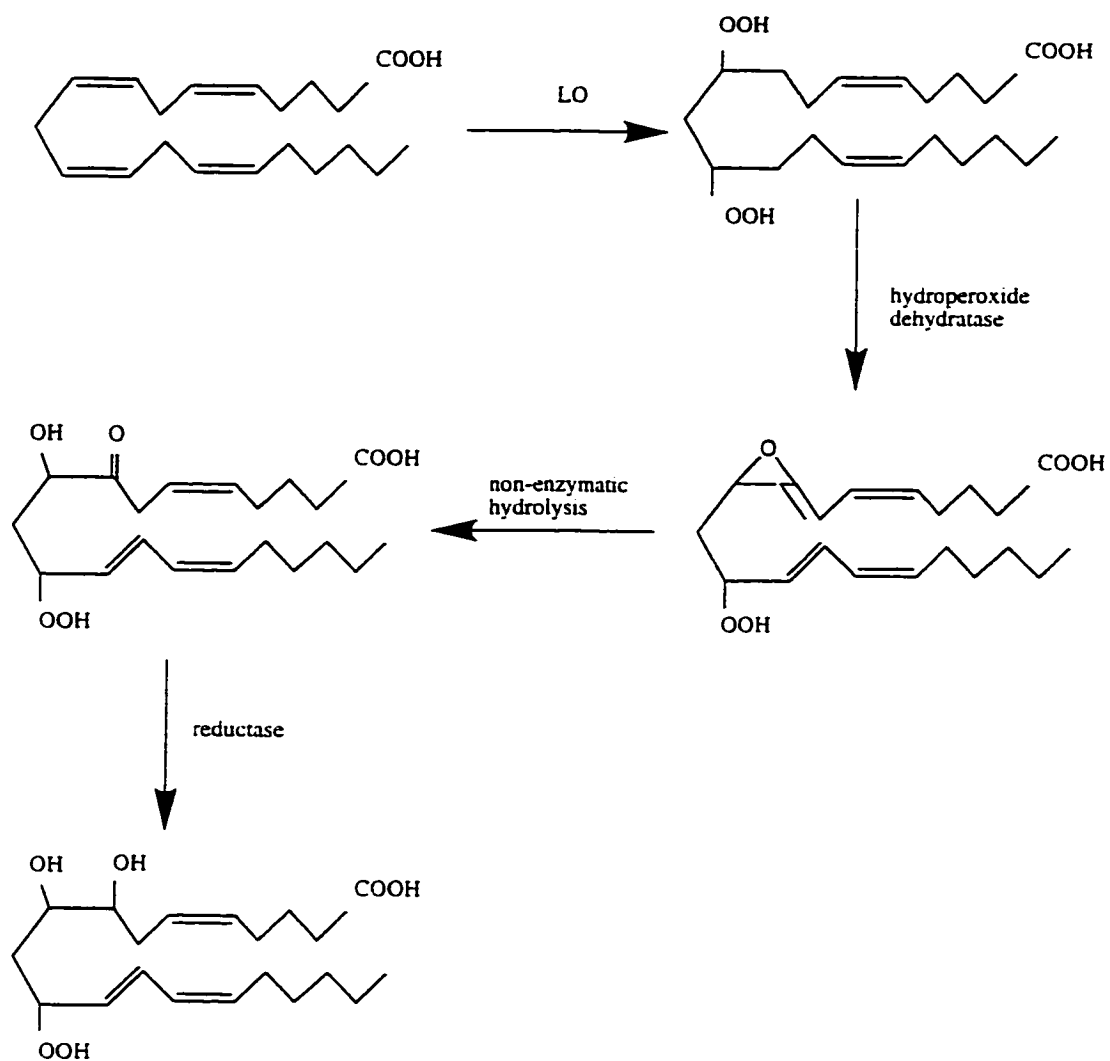
Figure 21. Potential biosynthetic pathway up to the intermediate, dihydroxy-hydroperoxy-eicosatrienoic acid (mw 370). The first three steps are common to octadecanoid biosynthesis.

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activity has been observed in the conversion of arachidonic acid to 8.15-dihydroperoxyeicosatetraenoic acid via soybean lipoxygenase (Bild *et al.*, 1977). The next step proposed involves hydroperoxide dehydratase (allene oxide synthase) activity on a dihydroperoxide resulting in the formation of an allene oxide (Song and Brash, 1991). Hydrolysis of this unstable allene oxide to an α-ketol followed by reductase activity could form the proposed structure. The presence of all such enzymes has been confirmed in the unicellular green algae. *Chlorella pyrenoidosa* (Vick and Zimmerman, 1989). However work of this type has not yet been conducted in *A. stellata* or other closely related macrophytic Chlorophytes.

The findings presented here support the existence of at least two PUFA metabolic pathways in chloroplast preparations from *A. stellata*. These data include the formation of PUFAs containing the conjugated tetraene functionality. LO activity resulting in putative hydroxy fatty acids, and the detection of oxylipins likely produced via hydroperoxide metabolism in *A. stellata* chloroplasts. Studies of conjugated tetraene production in the red algae, *L. corallioides*, also suggest that at least two different enzyme systems are present acting upon the fatty acid substrate and resulting in the formation of the conjugated tetraene and hydroxy fatty acids (Hamberg, 1992). However, characterization of these pathways has proven difficult for researchers due to a

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variety of reasons. These include the inherent instability of the intermediates. the possibility that expression of these pathways may not be constitutive. and the short half-lives of the products. This latter explanation is seen for oxylipin production in plants which appears to be initiated by extracellular signals and results in immediate release without storage capabilities (Blee and Joyard. 1996). It is important to note that although Vick and Zimmerman demonstrated *C. pyrenoidosa* extracts possessed hydroperoxide metabolism similar to that of the jasmonic acid pathway. they were unable to isolate jasmonic acid or other jasmonates under normal laboratory conditions (1989).

4.3 Effects of Exogenous Arachidonic Acid on Oxygen Flux in Whole Algal Thalli

To consider the potential for PUFAs to act as signaling molecules in the environment. whole algal thalli were exposed to AA while oxygen flux within the controlled environment was determined. The results from these studies demonstrated that AA administered directly to the local environment can pass through the cell membranes of the algae and elicit a significant effect by either mitigating oxygen production during light periods or enhancing oxygen uptake

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during periods of darkness. Oxygen flux appeared to be effected to the same extent during both photoperiods.

To our knowledge. a thorough investigation of the physiological relationship and the stoichiometry between PUFA oxidation. oxygen productivity. and metabolite production has not been explored. Few hypotheses have been put forth regarding such a relationship. Jiang and Gerwick (1990) have suggested that reactive oxygen produced during photosynthesis in the macrophyte *Gracilariopsis lemaneiformis* (Gracilariales. Rhodophyta) may be utilized for PUFA oxidation. and thus. the two processes could be coupled. This is not likely a complete explanation for our observations in *A. stellata* since significant PUFA oxidation was detected in the absence of photosynthesis. However. exposure to saturating conditions of substrate may be stimulating pathways otherwise turned off. Hence. a role of PUFA oxidation within the chloroplasts of contributing to sustaining an electron transport cascade cannot be ruled out.

On a quantitative basis. probably one molecule of AA could consume up to four molecules of oxygen in the first few minutes for the production of dihydroperoxy fatty acids. Under conditions of complete AA absorption and presumed metabolism. 3 μM AA could result in an uptake of 12 μM oxygen molecules. This is within the same order of magnitude observed and the range of AA concentrations used in these studies. Therefore. it is reasonable to assert that

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a physiologically significant effect on oxygen flux is associated with PUFA oxidation.

These observations in *A. stellata* thalli support and extend recently reported investigations of PUFA metabolism in cell-free homogenates. Burgess *et al.* (1991) described the O_2 dependent synthesis of a 20:5 metabolite (bosseopentaenoic acid) following AA addition to the calcified red alga *Bossiella orbigniana*. Conversion of 6,9,12-octadecatrienoic acid to 6,8,10,12-octadecatetraenoic acid in the crustose coralline red alga *Lithothamnion corallioides* was also reported to be an oxygen-dependent process (Hamberg, 1992). Similarly, a requirement for molecular oxygen has been observed in the biosynthesis of the naturally occurring conjugated tetraene parinaric acid, found in seed oils of the terrestrial flowering plant *Impatiens balsamina* (Noda *et al.*, 1980). As discussed previously, AA addition to isolated chloroplasts from *A. stellata* produced a wide variety of unique fatty acid oxidative metabolites. The data reported here suggest at least two O_2-dependent PUFA metabolic pathways are involved in the production of these compounds, both of which could successfully utilize AA as a substrate. The oxygen uptake observed in our experiments with whole algal thalli could be resultant of its use in O_2 dependent fatty acid metabolic pathways of this sort.

It is interesting to consider that the oxidative fatty acid metabolites formed could act as signaling molecules that enable the organism to respond to

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environmental stresses. An example of such a role can be seen in the algae *Cladisiphon okamuranus*. Oxylipin production in this algae has been suggested as a means of chemical defense by inhibiting other algal competitors (Kakisawa *et al.*, 1988). Antifungal or antibacterial activities have also been noted for a variety of oxylipins (Gerwick and Bernart, 1993). Although much work is needed to clearly understand the physiological significance of oxylipins, their involvement in a variety of pathways enabling the algae to successfully respond to its environment is conceivable.

4.4 Fatty Acid Analysis of Isolated Chloroplasts from *Anadyomene stellata* Collected in the Florida Keys

Analysis of fatty acid methyl esters (FAMEs) prepared from *A. stellata* collected in the Florida Bay resulted in a typical Chorophyte fatty acid profile, except for one major difference. Surprisingly, the 20 carbon PUFAs, 20:4 and 20:5, comprised about 20% of the total fatty acids with AA alone contributing 12%. Chlorophytes typically contain high amounts of 16 and 18 carbon PUFAs while rhodophytes produce a significantly higher amount of 20 carbon PUFAs (Khotimchenko and Vaskovsky, 1990). Although many examples of C_{20} PUFAs are seen in Chlorophytes, the levels are generally below 10% of total fatty acids

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for whole cell preparations. and more commonly below 5% (Pohl and Zurheide. 1979: Dembitsky *et al.* 1991). This unique finding points to *A. stellata* as an important organism for studies of fatty acid metabolic pathways. Although these samples were not analyzed for the presence of PUFAs containing the conjugated tetraene functionality. the only PUFA found that could potentially be a conjugted tetraene as identified in our previous work is 20:5. However, structural analysis studies were not completed on these samples.

It is important to point out that our data represent chloroplast fatty acid content. not cellular content. However. fatty acid synthesis originates in the chloroplasts resulting 16:0 and 18:1 production (Stumpf *et al.* 1982). Further elongation and desaturation occurs in the endoplasmic reticulum after which PUFAs can be exported back into the chloroplast. Therefore. fatty acid synthesis is an integrated pathway involving the chloroplast and other cellular constituents.

4.5 Comparison of Chloroplast Fatty Acid Content of Three Mediterranean Populations of *Anadyomene stellata*

The fatty acid profiles of *A. stellata* chloroplasts isolated from algal collections made in three Mediterranean locations, Daskalio. Ftelios, and Vagias Beach revealed high amounts of 16:0. 18:1 (n-9, n-7), 18:2, and 20:4 (n-6. n-3).

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The saturated fatty acid 16:0 was present in the highest amounts in all samples consisting of approximately 25 to 27% of total fatty acids. The high levels of 16:0 and the 18:1 isomers found are not surprising since these fatty acids are the endpoints of fatty acid synthesis and primary desaturation reactions in the chloroplasts (Stumpf *et al.*. 1982). The percent of total saturated fatty acids was 33.7%. 33.7%. and 32.8% for the samples collected at Daskalio. Ftelios. and Vagias Beach. respectively. These values are quite comparable to data collected from a variety of Chlorophytes. Rhodophytes. and Phaeophytes which yielded an average of 30% saturated fatty acids (Fleurence *et al.*. 1994). As was the case with the Florida samples. the overall fatty acid content is quite similar to that of other Chlorophytes. with the exception of the high levels of 20 carbon fatty acids (approximately 17% of total fatty acids) (Khotimchenko. 1993). Such high levels of these PUFAs are considered to be more characteristic of Rhodophytes. as mentioned earlier (Khotimchenko and Vaskocsky. 1990).

The significant levels of 14:2 and 14:1 fatty acids found in the *A. stellata* chloroplast preparations were an unexpected observation in this study. The average levels of both of these fatty acids from data collected at all three sites was calculated to be 0.88% of total fatty acids for 14:2 and 0.25% for 14:1. Interestingly. no reports of the presence of 14:2 were found in the literature for a marine macrophytic Chlorophyte. and only a few reports of 14:1 were found

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(Jameison and Ried, 1972: Vaskovsky *et al.*, 1996: Fleurence *et al.*, 1994; Khotimchenko, 1993: Dembitsky *et al.*, 1991: Pohl and Zurheide, 1979).

The apparent lack of 16:4 is particularly important due to its recently proposed taxonomic significance for macrophytic green algae (Leurence *et al.*, 1994: Khotimchenko, 1993). This PUFA is believed to be a common chloroplast glycolipid constituent in a variety of macrophytic green algae (Johns *et al.*, 1979). Observations made by Khotimchenko between the two classes Chlorophyceae and Siphonophyceae, suggest that specific differences in the composition of the 16:4 can be used to group algae into the appropriate classes (1993). They reported that the algae in the class Chlorophyceae were able to synthesize 16:4 (n-3) and 16:3 (n-3), while algae in the class Siphonophyceae were found to either synthesize 16:3 (n-3) or 16:4 (n-3), but not both. Our data are in accordance with these findings since *A. stellata* is grouped in the class Siphonophyceae.

Data from a variety of plants and algae including soybean and *ricinus communis* cell cultures, and the red alga *Porphyridium cruentum*, demonstrate that increased light levels, decreased water temperatures, and overall optimal growth conditions all result in higher levels of PUFA unsaturation and faster growth rates (Cohen *et al.*, 1988: Gemmrich, 1982; Pohl and Zurheide, 1979; and Wilson *et al.*, 1978). A comparison of the ratio of 18:3 / 18:1 from algae grown under different conditions can offer useful information regarding the

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environmental effects on the alga's ability to produce the polyunsaturate. The ratios of 18:3 to 18:1. as bracketed in Table 1. decrease from 0.69 at Daskalio to 0.48 at Vagias Beach. In addition. Vagias Beach was found to have a higher water temperature and greater salinity than the other two sites. The *A. stellata* population at Vagias Beach was also observed to be scarcest and in the worst condition in comparison to Daskalio and Ftelios. Both quantitative and qualitative observations made at the collection sites. suggest that Vagias Beach had the least optimal growth conditions.

As with the Florida Bay samples. apparently no conjugated tetraene containing fatty acids were present in the Mediterranean chloroplast preparations. These samples also were not analyzed for the presence of conjugated tetraene containing PUFAs. The PUFA 20:5 was observed. but the location of the double bonds was not determined. However. the previously identified conjugated tetraenes in *A. stellata*. 16:5. 18:4. 20:6. and 22:7 were not found (Mikhailova *et al.*. 1995). These data may offer important clues regarding the physiological roles of these unique PUFAs in *A. stellata*. If such compounds serve the purpose of signaling molecules. it is possible that the stimulus leading to their production was environmental and present at a much lesser extent or not at all in comparison to previous studies. Additionally. the concentration of conjugated tetraenes within the chloroplasts may have been quite small at the time of collection and the half-life relatively short rendering them undetectable

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by the methods employed in this study. Variations in seasonal conditions could also play a role in conjugated tetraene production.

4.6 Comparative Analysis Between Chloroplast Fatty Acid Content from *Anadyomene stellata* Populations in the Florida Keys and the Mediterranean Sea

Comparison in the fatty acid content of the Florida samples and the Mediterranean samples revealed a few marked differences. the most obvious of those being the higher levels of palmitic acid in the Florida samples. This observation may be explained when considering the need to maintain membrane fluidity at low temperatures which is accomplished by an increase in fatty acid unsaturation (Marr. 1962). Studies conducted by Cohen *et al.* (1988). illustrated a direct effect of supraoptimal growth temperatures (30 °C) on fatty acid content of the red alge. *Porphyridium cruentum*. The higher growth temperature resulted in an increase in both saturated fatty acid content (16:0 and 18:0) and 20:4 levels. followed by a reduction in concentrations of 20:5. In agreement with this study. our data comparing both the Florida samples to the Mediterranean samples illustrates a significant increase in palmitic acid levels in the Florida samples which were typically collected in waters that were much warmer waters than the

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Mediterranean samples. 27 °C verses 17 °C. However, higher levels of 20:5 found in the Florida samples contradict the findings of Cohen's group for *P. cruentum* (Cohen *et al.*, 1988). Average water salinity was relatively constant between the Florida (36.2 g/L) and the Mediterranean (36.7 g/L) samples. Therefore, this environmental parameter is not thought to markedly contribute to the differences observed. One factor that could have led to these differences is average light levels.

Another important deviance between the two groups of algae collected from both bodies of water concerns the appearance of small but detectable levels of 17:0 and 16:3 in the Mediterranean samples, and their absence in the Florida samples. This could be explained in a few ways. First, it is possible that the GC/MS system used to analyze the Mediterranean samples was more sensitive than that used to analyze the Florida samples. Additionally, these data could suggest the presence of impurities contributed by organisms living in close relationship to the algae and not removed by the isolation techniques employed. One last possibility is that of contamination from extraplastidal sources.

In conclusion, this work has demonstrated the presence of at least two different fatty acid oxidation reactions occurring in the chloroplast of the macrophytic green algae. *A. stellata*. The first of these reactions involves potential fatty acid oxidase activity resulting in the formation of five metabolites

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containing conjugated tetraene systems following the addition of fatty acid substrates. The structure of one of these metabolites, stellaheptaenoic acid (22:7), has not been observed in nature prior to our work. The second pathway observed is suggestive of LO activity resulting in the oxygenation of linoleic acid to produce hydroperoxy and hydroxy fatty acid metabolites. A plausible scheme for the biosynthesis of these compounds was presented which suggests the involvement of enzymes of the octadecanoid pathway. Chloroplast fatty acid composition was profiled to identify endogenous fatty acid precursors for these oxidation reactions, and analyzed between the various collection sites. Lastly, we detected a pronounced effect on oxygen flux of whole algal thalli exposed to AA that is of the same order of magnitude as oxygen consumed during the oxygenase reactions described.

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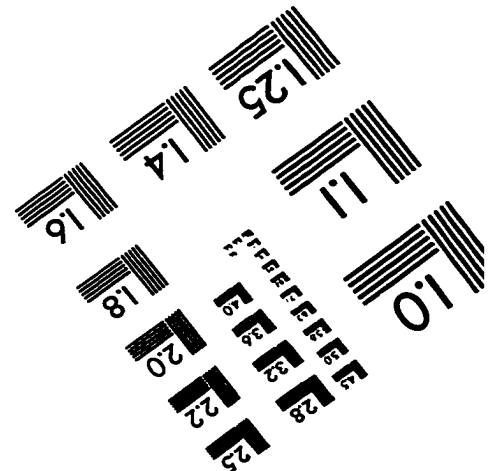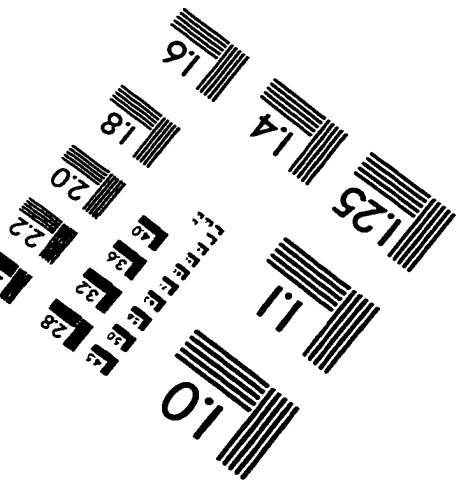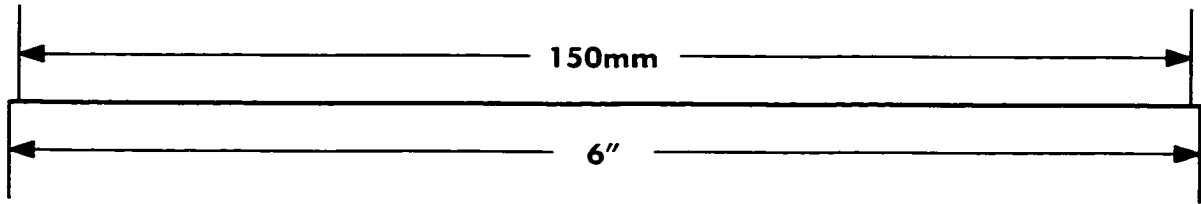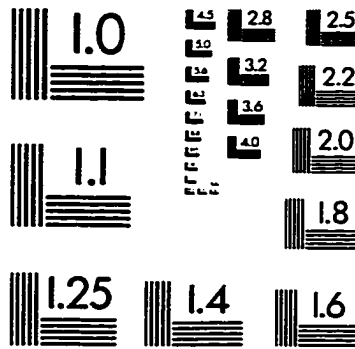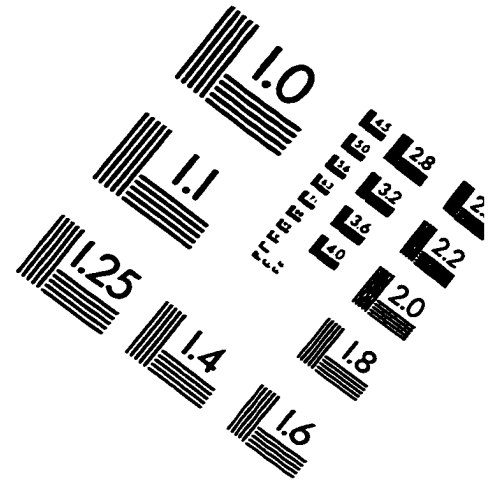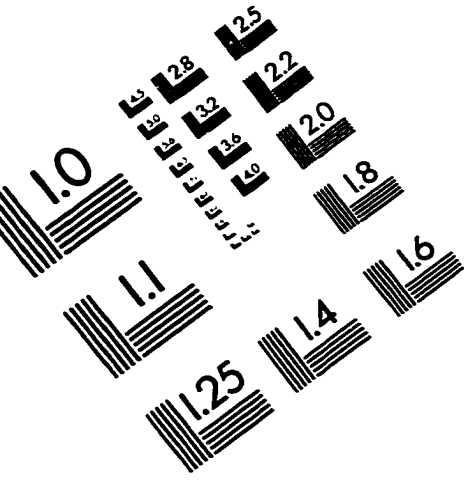
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IMAGE EVALUATION
TEST TARGET (QA-3)