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Biogenesis of Lipid-Derived Volatile Aroma Compounds in the Emerald Shiner (*Notropis atherinoides*)

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The formation of carbonyls and alcohols that characterize the fresh fish aroma of emerald shiners (*Notropis artherinoides*) was almost completely inhibited when shiners were sacrificed and immediately exposed to acetylsalicylic acid or stannous [tin(II)] chloride (inhibitors of fatty acid cyclooxygenase and lipoxygenase, respectively). These observations are interpreted as a demonstration of the involvement of enzymic conversions of ω-6 and ω-3 fatty acids to volatile aroma compounds in fresh fish. Postulated reaction mechanisms for the enzymic conversions of prostaglandins and polyunsaturated fatty acids to volatile aroma compounds are presented.

Earlier workers generally have attributed much of the characteristics aroma of fresh fish to nonenzymic autoxidation products of highly unsaturated fatty acids in fish (Yu et al., 1961; Stansby, 1962; Mejboom and Stroink, 1972; Badings, 1973; McGill et al., 1974, 1977; Crawford et al., 1975; Ke et al., 1975; Crawford and Kretsch, 1976; Ikeda, 1979). Fish-like aromas that resemble those of oxidized fish oil also develop in oxidizing butter, soybean, and linseed oils (Forss et al., 1960; Badings, 1970; Seals and Hammond, 1970), and these occurrences have usually been interpreted as support for the view that nonenzymic processes are responsible for the aroma of fishes. However, conflicting descriptions of fishy odors, and the inability to chemically account for the different fresh aromas of various species, even when considering volatile amines (Moncrieff, 1944; Tokunaga, 1970; Yamada, 1967) and sulfur compounds (Ackman et al., 1972; Tokunaga et al., 1977; Shiomi et al., 1982), suggest that previously unrecognized reaction pathways are involved in the formation of volatile aroma compounds that characterize fresh seafoods.

Obata et al. (1950) have reported that gauze used to wipe nearly odorless, fresh herring did not exhibit the aroma of fresh fish when it was dried rapidly. However, when a portion of the gauze was allowed to remain wet, an odor described as freshwater fish was observed. These observations suggest an enzymic involvement in the development of fresh fish aromas that was inhibited in the dried

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gauze. Additionally, it is well recognized that a marked suppression of fresh fish aromas occurs in fish held 3–5 days on ice when they exhibit neutral odors before the onset of putrid spoilage odor development (Howgate, 1977). Although microbial activity may account for some changes in aromas, the predictable decrease in odor strength suggests that specific enzymic processes play roles in the alteration of compounds responsible for fresh fish aromas.

Fish and other aquatic organisms contain abundant polyunsaturated ω-3 fatty acids (Ackman, 1974; Kinsella et al., 1977; Viswanathan Nair and Gopakumar, 1978) that have been shown recently to function as precursors in prostaglandin and monohydroxy fatty acid biosyntheses (Marcus, 1978; Budowski, 1981). Positionally specific hydroperoxidation of precursor fatty acids by novel lipoxygenases (Hamberg and Samuelsson, 1974a; Nugteren, 1975; Marcus, 1978; Samuelsson et al., 1978; Nelson et al., 1982) followed by reduction of the hydroperoxide to a corresponding alcohol (and chain cyclization for the case of prostaglandins) occurs in these pathways. Similarities between lipoxygenases in plants and those found in the arachidonic acid cascade of animals suggest that compounds produced through these enzymic processes may be involved in the development of the distinctive aromas of fresh fish.

Other studies have demonstrated the selective occurrence of volatile carbonyls and alcohols in several species of fresh and saltwater fish (Josephson et al., 1984), and this paper presents evidence for the selective biogenesis of these aroma compounds from the polyunsaturated lipids in the emerald shiner (*Notropis atherinoides*). Experiments were devised to demonstrate the involvement of certain enzymes in the biogenesis of volatile aroma compounds in fish.

MATERIALS AND METHODS

Live emerald shiners (*N. atherinoides*) were purchased from a local bait shop; each weighed about 1.25 g. Lots of 10 shiners each were sacrificed through immediate homogenization (6 °C; high-speed Waring Blendor; 5–10 s) in either 200 mL of a solution containing 360 mg of acetylsalicylic acid (0.01 F; F = formal; Skoog and West, 1965; acetylsalicylic acid is sparingly soluble in water), 0.01 M stannous [tin(II)] chloride, or distilled water. Each solution, which contained an internal standard (3-methyl-1-butanol; 98 ppb), was then saturated with sodium chloride and analyzed for volatile compounds by using the Tenax GC collection system and GC–MS analysis described previously (Josephson et al., 1984).

RESULTS AND DISCUSSION

Initial studies to demonstrate the involvement of enzymes in the generation of fresh fish aroma compounds involved submersion of live fathead minnows (*Pimephales promelas*) or emerald shiners (*N. atherinoides*) in solutions of ethanol or mercuric chloride to denature surface and subsurface enzymes. These treatments virtually eliminated fresh fish aromas compared to those of untreated fish, which developed typical aromas within a few minutes. Extraction of volatile aroma compounds by ethanol did not appear responsible for the absence of fish-like aroma character in ethanol-treated fish. However, because of the general enzyme denaturing properties of both ethanol and mercuric chloride, more specific inihibition of enzyme activity was needed to define the overall mechanism of fresh fish aroma biogenesis. This was accomplished by utilizing experiments that specifically demonstrated the inhibition of fatty acid cyclooxygenase with acetylsalicylic acid (Hamberg and Samuelson, 1974a,b; Freas and Grollman, 1980) and lipoxygenase with stannous [tin(II)] chloride (German and Kinsella, 1983).

Table I. Volatile Aroma Compounds Identified in Emerald Shiners That Were Blended in Distilled Water (Control), 0.01 F Acetylsalicylic Acid (Aspirin), or 0.01 M Stannous Chloride [Sn(II)Cl] prior to Purge Collecting onto Tenax GC

peak no.	compound	samples[a] control	aspirin	Sn(II)Cl	I_E[b]
1	ethanol	93[c]	147	64.8	2.66
1a	2-pentanone	–[d]	7.4	5.1	3.26
2	pentanal	20	–	–	3.31
3	1-penten-3-one	12.2	<2.1 (coelute)	–	3.67
4	2,3-pentanedione	49.8	–	–	4.13
5	hexanal	418.3	13.7	11.3	4.49
6	undecane	40	30.7	20.6	4.81
7	1,3,5-octatriene	57.6	–	–	4.87
8	1,3,5-octatriene	2.2	–	–	4.97
9	unknown	5.2	–	2.3	5.03
10	2-pentenal	25.4	–	–	5.07
11	1,3-dimethylbenzene	8.2	4.7	5.3	5.17
12	1-penten-3-ol	49	–	–	5.23
13	2-methyl-2-pentenal	10.9	14.6	13.9	5.30
14	myrcene	7.8	2.1	–	5.33
15	heptanal	16.3	4.4	7.4	5.50
16	3-methyl-1-butanol (internal standard)	98	98	98	5.58
17	limonene	9.8	2.8	4.6	5.60
18	(*E*)-2-hexenal	6.3	–	–	5.81
19	1-pentanol	10.5	–	–	6.03
20	trimethylbenzene	7.4	2.7	4.3	6.12
21	3-octanone	5.4	–	–	6.18
22	trimethylbenzene	18.1	8.3	–	6.40
23	1-octen-3-one	20.1	–	–	6.67
24	unknown	12.3	–	–	6.74
25	2,3-octanedione	97.1	–	–	6.89
26	1-hexanol	18.4	–	–	7.14
27	unknown	2.4	–	–	7.42
28	nonanal	10.3	8.8	14.1	7.62
29	acetic acid	80	32.6	81.6	7.95
30	1-octen-3-ol	110	–	4.8	8.15
31	(*E,Z*)-2,4-heptadienal	26.4	–	–	8.24
32	1,5-octadien-3-ol	31.7	–	2.2	8.48
33	(*E,E*)-2,4-heptadienal	36	–	–	8.52
34	pentadecane	34.7	25	22.9	8.75
35	3,5-octadien-2-one	4.8	–	–	8.80
36	benzaldehyde	3.3	3.8	20.7	8.91
37	(*E*)-2-nonenal	4.1	–	1.1	9.00
38	3,5-octadien-2-one	3.4	–	–	9.30
39	(*E,Z*)-2,6-nonadienal	3.2	–	–	9.43
40	2-octen-1-ol	12.7	–	–	9.72
41	2,5-octadien-1-ol	10.9	–	–	10.34
42	heptadecane	22.2	17.3	19.7	10.67
43	naphthalene	16.5	13.9	14.7	11.14
44	(*E,E*)-2,4-decadienal	3.5	–	–	11.62
44a	hexanoic acid	–	–	7.7	11.91
45	2-methylnaphthalene (?)	5.7	3.6	5.1	12.51

[a] Corresponding chromatograms shown in Figure 1. [b] Retention indices: Van den Dool and Kratz (1963). [c] Compound concentration (μg/kg) based on 10 shiners (ca. 12–13 g) blended in 200 mL with internal standard (98 μg/kg). [d] Not detected.

Homogenization of live shiners, conditioned to 6 °C, in 0.01 F acetylsalicylic acid (Figure 1A) or 0.01 M stannous chloride (Figure 1B), markedly suppressed the formation of volatile aroma compounds compared to fish that were similarly prepared in distilled water (Figure 1C). Identifications and estimated concentrations of compounds resulting from these experimental treatments of emerald shiners are presented in Table I. With the exception of 1-octen-3-ol and 1,5-octadien-3-ol in the sample containing stannous chloride, characterizing volatile aroma compounds were not instrumentally detected in any of the

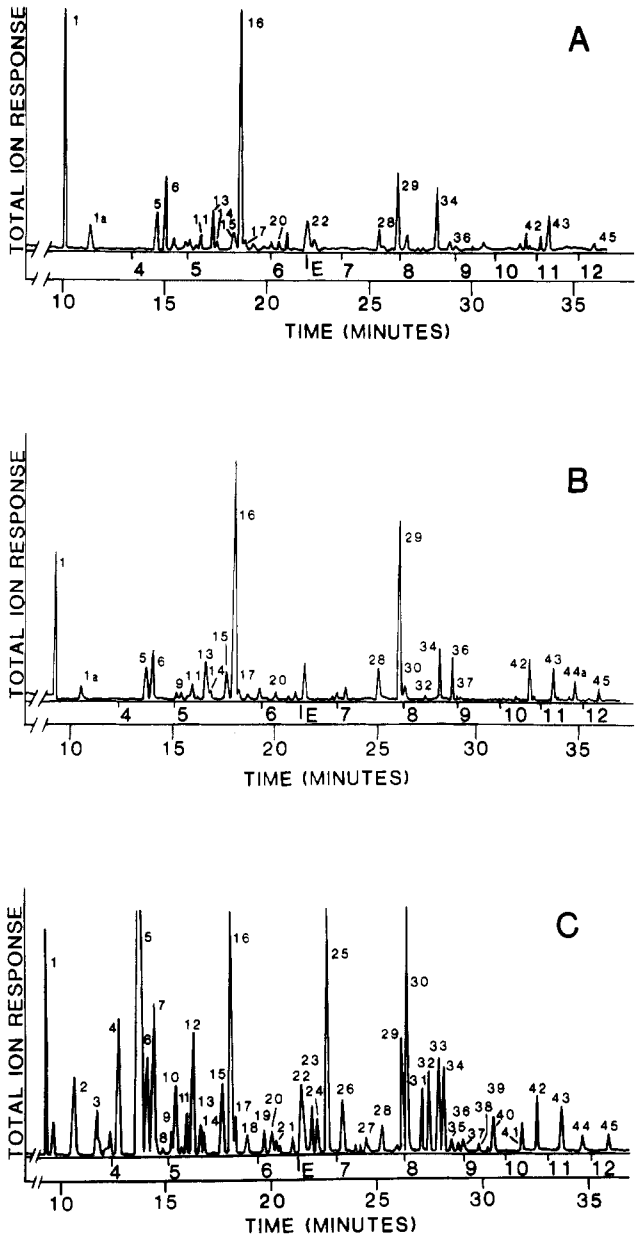

Figure 1. Capillary Carbowax 20M gas chromatograms of volatiles from 10 emerald shiners (ca. 12–13 g), homogenized in 200 mL of (A) 0.01 F acetylsalicylic acid (aspirin), (B) 0.01 M stannous chloride [Sn(II)Cl], or (C) distilled water. Peak identifications are reported in Table I.

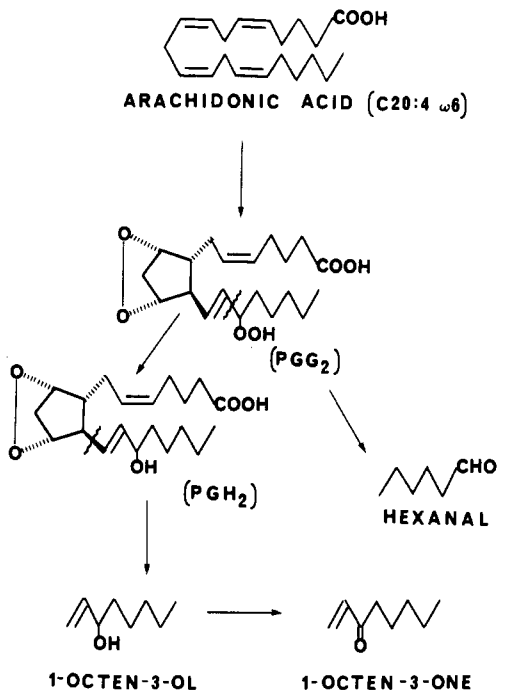

Figure 2. Postulated reaction mechanism in fresh fish for the formation of 1-octen-3-ol, 1-octen-3-one, and hexanal from prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2).

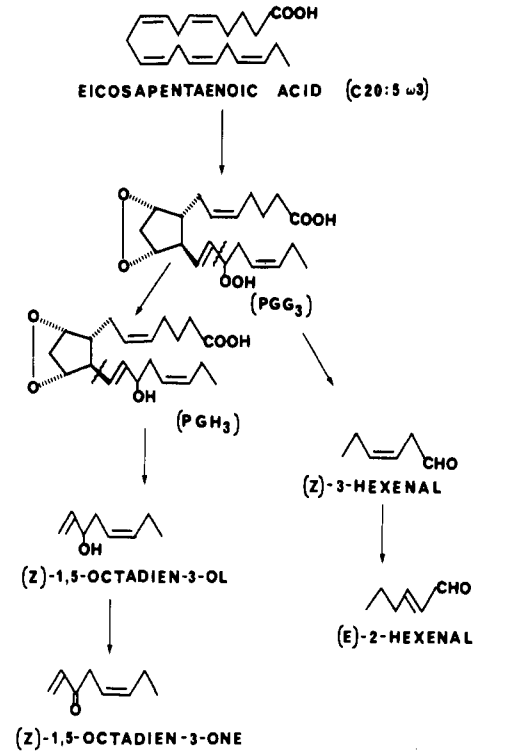

Figure 3. Postulated reaction mechanism in fresh fish for the formation of (*Z*)-1,5-octadien-3-ol, (*Z*)-1,5-octadien-3-one, and (*E*)-2-hexenal from prostaglandin G_3 (PGG_3) and prostaglandin H_3 (PGH_3).

treated samples. Even though present, the concentration of these two compounds was suppresed greatly in the stannous chloride treated sample compared to the control sample. The low pH associated with a 0.01 F acetylsalicylic acid solution (pH 2.9) could have influenced the activity of enzymes, but similar experiments with solutions of distilled water adjusted to pH 2.9 with 0.1 N HCl yielded nearly the full intensity of the characteristic odor of the emerald shiners.

Earlier experiments with live rainbow trout (*Salmo gairdneri*) and yellow perch (*Perca flavenscens*), which were held in solutions of acetylsalicylic acid (aspirin; 0.008 F) before sacrificing, showed only modest suppression of fresh fish odors (Josephson et al., 1983b). Lack of efficient penetration and equilibration of aspirin into the slime layer and skin cells, and/or exchange across the gills into the blood and lymph systems, of these fish appeared responsible for the limited suppression of fresh fish odor under the conditions of these experiments. However, the experiments collectively have demonstrated that the biogenesis of volatile aroma compounds in fresh fish is associated with the formation of prostaglandins and possibly other biologically active lipoxygenase-formed hydroxy compounds, such as 5-hydroxy-6,8,11,14-eicosatetraenoic acid, which is an intermediate in leucotriene synthesis (Nelson et al., 1982).

Postulated mechanisms for the formation of 1-octen-3-ol and 1-octen-3-one via cleavage of prostaglandin H_2 (PGH_2)

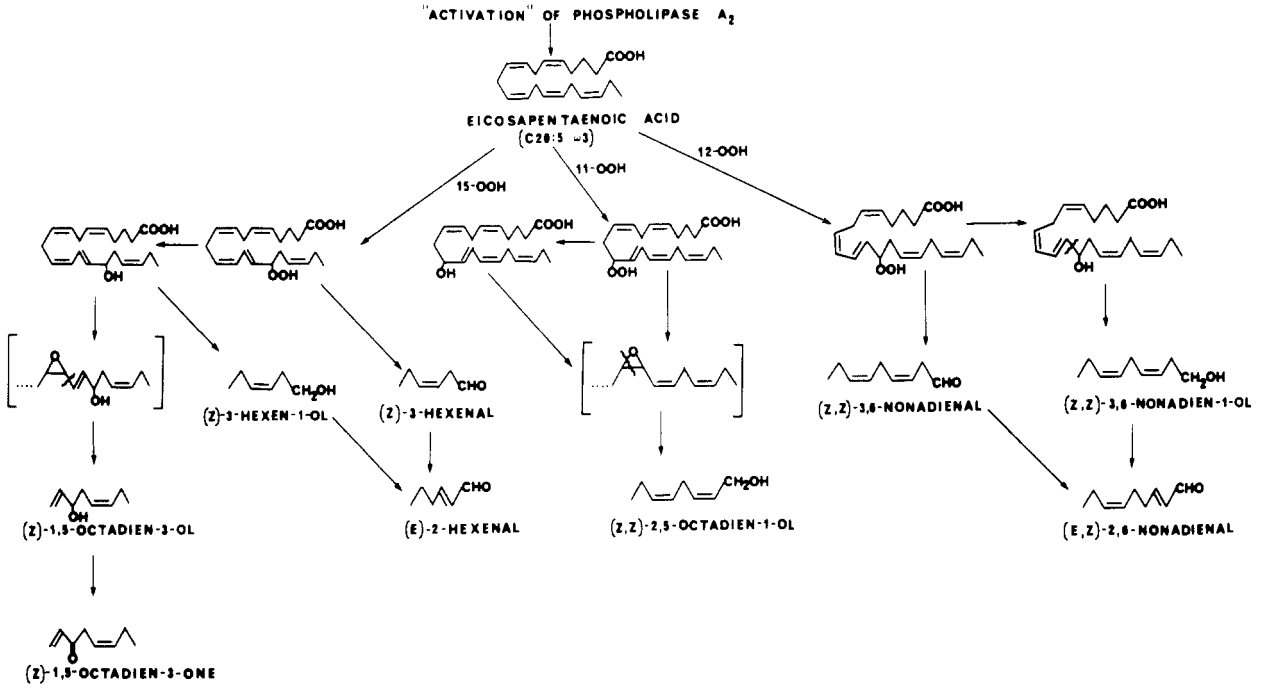

Figure 4. Postulated reaction mechanism for the formation of characterizing volatile aroma compounds in fresh fish from eicosapentaenoic acid (C20:5) via lipoxygenase and lyase action.

and the formation of 1,5-octadien-3-ol and 1,5-octadien-3-one via cleavage of prostaglandin H_3 (PGH_3) are shown in Figures 2 and 3, respectively. These eight-carbon compounds occurred in all fish species surveyed and are responsible for the fresh, heavy, plant-like aroma exhibited by fresh fish. Hexanal can likewise be biosynthesized from prostaglandin G_2 (PGG_2; Figure 2) and (*E*)-2-hexenal can be formed from prostaglandin G_3 (PGG_3; Figure 3).

Greater than normal stress (i.e., stuggling in nets, removing from water, or temperature shocking) induces many changes in the physiological systems of fish, including the defenses mediated by components of the lymph system (Ellis, 1981). The skin and gills usually provide the first contact with environmental stressors that result in local injury, and this leads to the accumulation of fluid and blood cells (Flethcher, 1981). Smith and Ramos (1976) noted that dying fish often show red blotches on skin surfaces that originate from hemorrhages and free hemoglobin. Fatty acid cyclooxygenase and lipoxygenase activity have been reported in human blood platelets (Hamberg and Samuelsson, 1974a; Nugteren, 1975; Ho et al., 1977) and rabbit peritoneal neutrophils (Borgeat et al., 1976), and this suggests the involvement of similar enzymes in the circulatory systems in fish in the biogenesis of characteristic volatile aroma compounds.

The highest concentrations of volatiles from enzymic conversions appear to be associated with the slime and surfaces of fish. Prostaglandins act as pharmacological mediators (Fletcher, 1981) and have been found in especially high concentrations in the skin and gills of numerous fish (Christ and Van Dorp, 1972; Nomura and Ogata, 1976). Prostaglandins have been identified additionally in the skin of frogs (Hall et al., 1977; Nomura and Ogata, 1976), in mollusks (Freas and Grollman, 1980), and in numerous invertebrates (Nomura and Ogata, 1976).

The involvement of lipoxygenases in the biogenesis of volatile aroma compounds in fish probably parallels similar reactivities in fruits and vegetables (Wardale and Galliard, 1975; Grosch and Laskawy, 1975; Hatanaka et al., 1975; Phillips et al., 1979; Tressl et al., 1981). The 5-hydroxy (Borgeat et al., 1976; Samuelsson et al., 1978), 11-hydroxy (Nelson et al., 1982), 12-hydroxy (Hamberg and Samuelsson, 1974a; Hammarstrom et al., 1975; Nugteren, 1975; Ho et al., 1977), and 15-hydroxy (Nelson et al., 1982) derivatives of arachidonic acid have been identified in mammalian systems and are derived directly from arachidonic acid by novel lipoxygenases. Relatives of the 12-hydroxy compound derived from eicosapentaenoic (C20:5) and docosahexaenoic acid (C22:6) have been reported (Nugteren, 1975), and the existence of analogous ω-3 hydroxy fatty acids in fish where ω-3 fatty acids dominate the percentages of fatty acids found in lipids (Ackman, 1974; Kinsella et al., 1977; Viswanathan Nair and Gopakumar, 1978; Budowski, 1981) is strongly suggested. A plausible pathway for the biogenesis of diunsaturated aroma compounds in fish from eicosapentaenoic acid (C20:5 ω-3) following initial lipoxygenation is shown in Figure 4. Mechanistic pathways can also be constructed for the enzymic conversion of arachidonic acid (C20:4 ω-6) to the monounsaturated aroma compounds. Cleavage of the 15-hydroxy fatty acid of eicosapentaenoic acid would result in the production of 1,5-octadien-3-ol. Similar enzymic conversions of the 15-hydroxy fatty acid of arachidonic acid would result in the production of 1-octen-3-ol. Oxidation of alcohols by alcohol oxidoreductases would account for the formation of the corresponding vinyl ketones. In this scheme, the direct formation of the eight-carbon alcohols occurs first, and they have been found at higher concentrations than the corresponding vinyl ketones in fresh fish (Josephson et al., 1983a, 1984; Table I).

(*E*)-2-Hexenal is predicted from the 15-hydroperoxy fatty acid derivative of eicospentaenoic acid after conversion of the initial product [(*Z*)-3-hexenal] by a *cis*-3,*trans*-2-isomerease and/or from the oxidation of (*Z*)-3-hexen-1-ol derived from the 15-hydroxy fatty acid derivative of eicosapentaenoic acid. The saturated aldehyde, hexanal, would likewise be formed from the 15-hydroperoxy fatty acid derivative of arachidonic acid. The 12-hydroxy fatty acid of eicosapentaenoic acid could serve as a precursor to (*Z*,*Z*)-3,6-nonadien-1-ol, which can be isomerized and oxidized to (*E*,*Z*)-2,6-nonadienal. Additionally, the 12-hydroperoxy fatty acid derivative of eico-

sapentaenoic acid could serve as precursor to (*Z,Z*)-3,6-nonadienal, which could then be isomerized to (*E,Z*)-2,6-nonadienal. The 12-hydroperoxy fatty acid of arachidonic acid would be predicted to serve as the precursor to (*E*)-2-nonenal. The 11-hydroperoxy and/or 11-hydroxy fatty acid of eicosapentaenoic acid would serve as precursor to (*Z,Z*)-2,5-octadien-1-ol while the 11-hydroxy fatty acid of arachidonic acid would serve as the precursor to 2-octen-1-ol and 2-octenal.

The biochemical pathways responsible for the formation of volatile aroma compounds in fish may serve as a means for producing physiochemically active compounds. The occurrence of 12-oxo-*trans*-10-dodecenoic acid (traumatin) in plant tissues has been shown to function as a wound hormone (Zimmerman and Coudron, 1979) and is formed from the enzymic cleavage of 13-hydroperoxylinoleic acid and 13-hydroperoxylinolenic acid by a hydroperoxide lyase. In addition to the formation of 12-oxo-*trans*-10-dodecenoic acid, hexanal and 2-hexenal are formed, respectively, and these six-carbon volatile compounds characterize many freshly cut plant tissues (Eskin, 1979; Tressl et al., 1981; Grosch, 1982). Thus, the enzymic oxidation and cleavage of 20-carbon polyunsaturated fatty acids in fish may provide a means to induce cellular repair and would result in the formation of the commonly occurring eight-carbon volatile aroma compounds as well as a 12-oxoenoic acid. Volatile compounds in fish slime may also provide signals that induce feeding, schooling, or fright responses.

Experiments in the current investigation have demonstrated the involvement of enzyme-mediated pathways in the development of species-specific fresh fish aromas. The biogenesis of aroma compounds in various species is probably influenced by the peculiar physiological status of individual fish. For example, flavor differences that occur between prespawning (saltwater) and spawning (freshwater) salmon (*Oncorhyncus sp.*) are likely to be at least partially caused by related enzymic activity. It now appears that certain reactions occur that regulate the enhanced output of biologically active compounds that are generated in response to greater than normal stress, and these regulatory processes result in the biogenesis of characteristic fresh fish aromas. The enzymic formation of hydroperoxides in fresh fish can also accelerate the rate of formation of compounds, causing oxidized flavors in fish during subsequent frozen storage through a reactive hydroperoxide-seeding action (Josephson et al., 1983b, 1985).

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Registry No. Acetylsalicylic acid, 50-78-2; fatty acid cyclooxygenase, 39391-18-9; lipoxygenase, 9029-60-1; stannous chloride, 7772-99-8; ethanol, 64-17-5; 2-pentanone, 107-87-9; pentanal, 110-62-3; 1-penten-3-one, 1629-58-9; 2,3-pentanedione, 600-14-6; hexanal, 66-25-1; undecane, 1120-21-4; 1,3,5-octatriene, 26555-19-1; 2-pentenal, 764-39-6; 1,3-dimethylbenzene, 108-38-3; 1-penten-3-ol, 616-25-1; 2-methyl-2-pentenal, 623-36-9; myrcene, 123-35-3; heptanal, 29381-66-6; limonene, 138-86-3; (*E*)-2-hexenal, 6728-26-3; 1-pentanol, 71-41-0; trimethylbenzene, 25551-13-7; 3-octanone, 106-68-3; 1-octen-3-one, 4312-99-6; 2,3-octanedione, 585-25-1; 1-hexanol, 111-27-3; nonanal, 124-19-6; acetic acid, 64-19-7; 1-octen-3-ol, 3391-86-4; (*E,Z*)-2,4-heptadienal, 4313-02-4; 1,5-octadien-3-ol, 83861-74-9; (*E,E*)-2,4-heptadienal, 4313-03-5; pentadecane, 629-62-9; 3,5-octadien-2-one, 38284-27-4; benzaldehyde, 100-52-7; (*E*)-2-nonenal, 18829-56-6; (*E,Z*)-2,6-nonadienal, 557-48-2; 2-octen-1-ol, 22104-78-5; 2,5-octadien-1-ol, 83861-75-0; heptadecane, 629-78-7; naphthalene, 91-20-3; (*E,E*)-2,4-decadienal, 25152-84-5; hexanoic acid, 142-62-1; 2-methylnaphthalene, 91-57-6.

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Peroxidase and Chilling Injury in Banana Fruit

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Storage of unripe banana fruit (*Musa sapientum*) at 10 °C lead to development of chilling injury. The chill-injured fruit showed low peroxidase activity as compared to fruit stored at normal ripening temperature (healthy). Polyacrylamide gel electrophoretic analysis showed that the isozyme pattern for peroxidase was different for chill-injured and healthy fruit. Studies with partially purified chill-injured and healthy enzyme suggest that peroxidase may be one of the enzymes affected during low-temperature storage.

Temperature is one of the most important environmental condition that affects the ripening process. When fruits are stored below a certain critical temperature, the process of normal ripening does not occur. Instead, the fruits develop "chilling injury", a physiological abnormality that manifests with symptoms such as lack of flavor, lack of taste, and delayed ripening (Murata, 1969).

Haard (1973) observed that during ripening of banana there was an increase in the particulate (60000*g* pellet) peroxidase. This fraction was identified as "intracellular bound peroxidase". The soluble (60000*g* supernatant) and wall-bound peroxidases (solubilized from 60000*g* pellet by 0.1–0.2 M $CaCl_2$) were found to be invariant during normal ripening. During chilling injury the soluble and wall-bound peroxidases increased after 10–15 days of storage, whereas intracellular bound peroxidase exhibited negligible change (Haard and Timbie, 1973). The increase in soluble and wall-bound peroxidases after prolonged storage was viewed as a "hardening" effect.

In the present investigation we observed that during normal ripening of banana fruit the soluble (20000*g* supernant) peroxidase activity increased considerably as the fruit ripened. Since peroxidases are also implicated in cold adaptation of hardy plant tissues (McGown et al., 1969), we investigated the effect of low-temperature storage of banana fruit on the soluble peroxidase activity.

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MATERIALS AND METHODS

The unripe banana fruit were given ethylene treatment (10 ppm for 1 h) and subsequently stored at room temperature (28 ± 1 °C) and at 10 °C. The relative humidity was between 85 and 90% at both the temperatures. The fruits were sampled on different days, and their respiratory rate was measured as described earlier (Mattoo and Modi, 1975).

Enzyme Extraction and Assay. Peeled bananas were frozen at −15 °C and were ground in a mortar and pestle to form a pulp. PVP (1 g/2 g of pulp) was then added followed by twice the volume of 0.05 M PO_4 buffer, pH 7.2, to make a 50% homogenate. The homogenate was centrifugated at 20000*g* for 20 min at 4 °C. The resultant supernantant was taken as the soluble fraction and used for enzyme assay.

The peroxidase was assayed by Worthington's method ("The Worthington Manual", 1963). One unit of peroxidase activity is the amount of enzyme that catalyzes the conversion of 1 μmol of peroxide/min under the given assay conditions. Indole-3-acetic acid (IAA) oxidase was assayed by method described by Meudt and Gaines (1967). One unit of IAA oxidase activity is the amount of enzyme that degrades 1 μmol of IAA/min under the given assay conditions.

Protein in the cell-free extracts was estimated by analysis of 10% trichloroacetic acid (TCA) precipitates of

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