AN ABSTRACT OF THE THESIS OF

Donald R. Marino for the degree of Master of Science in Food Science and Technology presented on October 31, 1988

Title: Effects of Cyclopropenoid Fatty Acids on Liver Plasma Membranes of Rainbow Trout (Salmo gairdneri)

Abstract approved: _____
Daniel P. Selivonchick

Cyclopropenoid fatty acids (CPFA), which are a group of fatty acids produced by plants of the order Malvales, are known to induce adverse physiological effects when administered to a variety of animal species. A structurally strained cyclopropene ring is present in all CPFA and is believed responsible for the toxic action of these fatty acids. Dietary consumption of CPFA by mammals, poultry and fish has resulted in toxic responses including hepatic damage, impaired reproductive capabilities and sizeable alterations in lipid metabolism. Furthermore, CPFA have been identified as mildly carcinogenic and strongly cocarcinogenic towards rainbow trout (Salmo gairdneri). The mechanism by which CPFA enhance carcinogenesis is currently not understood. The research in this thesis has therefore been directed toward obtaining a better understanding as to how CPFA induce toxic responses in rainbow trout.

Hepatic plasma membranes were isolated from both control trout and trout which had consumed dietary CPFA. The plasma membranes were then compared via the use of electron microscopy, chromatographic analysis of phospholipid and fatty acid content, two dimensional polyacrylamide gel electrophoresis of proteins, and Western blot analysis of concanavalin A sensitive glycoproteins. Electron micrographs revealed that control plasma membranes appeared more homogeneous than CPFA membranes and were characterized by more membrane sheets and less vesicularization. The analysis of enzyme activities revealed that CPFA caused a decrease in whole liver glucose-6-phosphatase activity and that control plasma membranes expressed slightly higher glucose-6-phosphatase and 5'-nucleotidase activities as compared to CPFA membranes. Although dietary CPFA appeared to have no effect on the phospholipid content of the plasma membranes, significant alterations in the fatty acid profiles of ethanolamine and choline phospholipids were observed. CPFA caused a decrease in palmitic, palmitoleic and oleic acids while the level of stearic and docosahexaenoic acids subsequently increased. Differences between the protein content of control and CPFA plasma membranes were made clear through the analysis of electrophoretic and Western blotting data. Membranes isolated from fish fed CPFA contained several proteins of high molecular weight (above 66,000 daltons) and other proteins of high isoelectric point that were not present in control plasma membranes. Additionally, two families of glycoproteins which had previously been identified as microsomal in origin were

detected only in CPFA plasma membranes. A discussion concerning the possible causes and biological ramifications of the observed subcellular alterations caused by CPFA insult is also presented in this thesis.

# Effects of Cyclopropenoid Fatty Acids on Liver Plasma Membranes of Rainbow Trout (*Salmo gairdneri*)

by

Donald R. Marino

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed October 31, 1988

Commencement June 1989

APPROVED:

Professor of Food Science and Technology in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented October 31, 1988

Typed by Tracy Mitzel for Donald R. Marino

ACKNOWLEDGMENTS

I wish to thank my major professor, Dan Selivonchick, for his friendship and guidance both in and out of the laboratory. Knowing Dr. Selivonchick has truly been one of the high points for me during my stay in Corvallis. I would also like to thank Jan Williams, Mark Einerson and Gary Perdew for their help in the lab, Ted Will for his care of the fish, and Tracy Mitzel for typing this thesis.

This research was sponsored by Grant 1R01 CA30087-04A1 from the United State Public Health Service.

## DEDICATION

This thesis is dedicated to my parents, Russell and Argentina, for their constant love, support and patience which has made this research possible.

TABLE OF CONTENTS

## LIST OF FIGURES

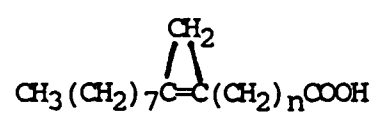
## LIST OF TABLES

# EFFECTS OF CYCLOPROPENOID FATTY ACIDS ON LIVER PLASMA MEMBRANES OF RAINBOW TROUT (SALMO GAIRDNERI)

## I. LITERATURE REVIEW

### Occurrence and Structure of CPFA

Cyclopropenoid fatty acids (CPFA) are a group of fatty acids that are produced by plants in the order Malvales. The major CPFA of this plant order have been isolated and identified as 7-(2-octyl 1 cyclopropenyl) heptanoic acid and 8-(2-octyl 1 cyclopropenyl) octanoic acid. These compounds have been given the common names of malvalic acid (1) and sterculic acid (2), respectively. The chemical structures of these two acids are given below.



$CH_3(CH_2)_7C{=}C(CH_2)_nCOOH$

n=6 for malvalic acid

n=7 for sterculic acid

The sterically strained cyclopropene ring of these two acids is responsible for their high degree of reactivity (3,4). The peculiar structure of CPFA is also responsible for a variety of adverse effects that have been observed in several biological situations. CPFA readily undergo such reactions as oxidation, reduction, addition and polymerization (4-6). The traditional method of detecting CPFA is by the Halphen test in which sulfur

compounds react with the cyclopropene ring, thereby producing a pink or red colored compound (1,7). Additional methods used for the detection of CPFA include titration with hydrogen bromide (8), infrared absorption (9), gas-liquid chromatography (10), nuclear magnetic resonance spectroscopy (11) and high pressure liquid chromatography (12). The laboratory synthesis of CPFA (13,14), especially the synthesis of [9,10-methylene-$^{14}$C]-sterculic acid (15), has proven to be a valuable tool for research purposes.

CPFA have been detected in a number of plant oils, some of which are commonly used for human and animal consumption. The highest concentration of CPFA is found in the oil of Sterculia foetida which contains up to 70% CPFA (16). Sterculia foetida oil is not considered an edible oil but it has served as a useful source of CPFA for research purposes. The most common CPFA containing oils that are used for human and animal consumption are derived from Gossypium hirsutum (cottonseed oil) and Eriodendron anfractuosum (kapok oil). Cottonseed oil has been reported to contain between 1.5 and 2.4% CPFA (6) while kapok oil has been reported to contain between 12 and 14% CPFA (17). Because of the high reactivity of the cyclopropene ring, most CPFA are destroyed by modern processing methods (18) although small levels may survive processing (17,19). However, under conditions where modern processing methods are not widely used, significant amounts of CPFA are consumed by both animals and humans (20). The following discussion concerns the effects

that CPFA can exert on biological systems. More detailed discussions on the occurrence, chemistry and biological implications of CPFA can be obtained from several review articles (4-6,18).

## Gross Physiological Effects of CPFA

### Effects of CPFA on poultry:

CPFA have been shown to induce adverse physiological changes in a variety of biological systems. The earliest observed effect arose in hens that were fed meal that contained cottonseed oil. Early in the twentieth century, an agricultural problem arose when many chickens produced eggs that developed pink egg whites and large, watery, salmon-colored yolks during periods of storage. In 1928, Sherwood (21) reported that cottonseed oil in chicken feed was responsible for the egg damage. This problem came to be know as pink white discoloration of eggs and many investigators since Sherwood have confirmed CPFA as the causative agent (22-24). Currently, it is commonly accepted that pink white discoloration signifies the presence of an iron-conalbumin complex (25). CPFA cause an increased permeability of the egg yolk vitelline membrane, which consequently allows iron to leach out of the yolk and react with conalbumin in the white, thus producing the pink iron-conalbumin complex (26). Shenstone and Vickery (24) report that CPFA also cause an uptake of water by the egg yolk, diffusion of amino acids from the yolk to the egg white, and a conversion of pH

values between the yolk and the white during periods of storage. These circumstances may be responsible for some of the undesirable attributes of the discolored eggs, such as the presence of the large, salmon-colored yolks.

In addition to pink white discoloration of eggs, CPFA have been shown to produce other adverse physiological effects on poultry. White Leghorn hens fed CPFA have exhibited a delay in sexual maturity (27), a reduction in egg production and hatchability (28,29) and an increased chick embryo mortality rate (30). Increased embryo mortality was also exhibited by Japanese quail ingesting CPFA (31).

Effects of CPFA on mammals:

Adverse physiological effects have also been observed in rats exposed to dietary CPFA. Schneider et al. (32) have reported that moderate levels of CPFA in the diet resulted in decreased growth rates of weanling rats while the same animals consuming 5% Sterculia foetida oil exhibited a high mortality rate. Increased prenatal and postnatal mortality rates were observed in rats fed CPFA (33), and dietary CPFA have also been reported to impair reproduction in rats (34).

Rats exposed to dietary CPFA have also exhibited damage on both the cellular and subcellular levels. Nixon et al. (35) have demonstrated a decrease in microsomal codeine demethylase activity, an inhibition of mitochondrial swelling by reduced glutathione and an increase of erythrocyte hemolysis in 0.3M glycerol in rats fed 2% S. foetida oil. These investigators

also noted areas of hepatic necrosis and kidney tubule degeneration due to CPFA exposure. Scarpelli (36,37) has reported that CPFA have a mitogenic effect in rat pancreas and liver, characterized by increases in total liver DNA, mitotic index and [$^3$H]-thymidine incorporation into DNA.

Effects of CPFA on rainbow trout:

Rainbow trout are especially susceptible to deleterious effects induced by CPFA. Rainbow trout readily absorb, transport and incorporate dietary CPFA into tissue lipids (38,39). Young trout consuming 100 or 200 ppm dietary CPFA have exhibited depressed growth rates during the initial 90 days of exposure; thereafter they resumed normal growth (40). CPFA have been shown to alter trout liver histology as characterized by enlarged, very firm and pale-colored livers (40,41). Struthers et al. (42) have found that 200 ppm dietary CPFA caused altered liver histology in trout characterized by increased liver weight, increased liver lipids, necrosis of hepatocytes, unusual glycogen deposition, appearance of fibers in the cytoplasm of many cells and fibrotic bile ducts and blood vessels. Similar histological damage has been reported by other investigators (40,43-45) and Malevski et al. (45) have shown that this damage can be reversed by removing CPFA from the trout's diet.

CPFA have been shown to alter trout liver cytosolic and microsomal protein levels (46-50) and the activity of numerous hepatic enzymes. Scarpelli et al. (44) have noted a decrease in the activity of hepatic glucose-6-phosphatase, while Taylor et

al. (51) have reported decreased activities of hepatic glucose-6-phosphate dehydrogenase, NADP-linked isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in trout. Malevski et al. (41) have shown that dietary CPFA decreased total hepatic protein content and suppressed the enzyme activities of glutamate dehydrogenase and alanine amino-transferase.

Several investigators have studied the effects of CPFA on the hepatic drug metabolizing enzymes of rainbow trout (47-49). Because of the importance of these enzymes in the processes of chemical detoxification and the formation of ultimate carcinogens, these studies are discussed in this thesis under the section entitled "The Role of CPFA in Carcinogenesis."

## Effects of CPFA on Lipid Metabolism

Perhaps the most studied effect of CPFA is their ability to alter lipid metabolism in a variety of biological situations. Fish (40,49,52), mammals (34,35,53-58) and poultry (26,27,31,59-62) that were fed CPFA have been shown to develop altered lipid profiles characterized by higher proportions of saturated fatty acids, especially higher levels of stearic acid, as compared to animals on a control diet. Even the unicellular green alga Chlorella vulgaris has exhibited an inhibited ability to desaturate stearic acid to oleic acid in the presence of sterculic acid (63). It is generally accepted today that CPFA produce this effect via an inhibition of the fatty acyl

desaturase enzyme complex. A general summary of the known effects of CPFA on lipid metabolism and a discussion concerning the current understanding of the mechanism of desaturase inhibition are presented below.

Rainbow trout lipid metabolism:

Rainbow trout lipid metabolism has been shown to be effected by CPFA in a number of ways. Trout readily absorb, transport and incorporate dietary CPFA into tissue lipids (38,39) and a resultant increase in total liver fat content has been reported (41,42). Einerson et al. (64) have monitored the incorporation of sterculic acid into trout hepatic microsomal phospholipids. At 72 hours after intraperitoneal injection of [9,10-methylene-$^{14}$C] sterculic acid, radioactivity was detected primarily in choline (57%) and ethanolamine (22%) phospholipids. These investigators also report that the sterculic acid was preferentially esterified to the one position on the glycerol backbone of phospholipids. In 1963, Reiser et al. (52) noted that the marine fish Fundulus grandis developed increased levels of tissue stearic acid due to dietary cottonseed oil. This study marked the first time that CPFA were found to inhibit fatty acid desaturation in fish. Subsequently, Roehm et al. (40) demonstrated this phenomena in rainbow trout when they found CPFA to cause increased palmitate:palmitoleate and stearate:oleate ratios in trout hepatic triglycerides and phospholipids. These investigators also reported that young trout fed methyl sterculate for 87 days contained substantially

lowered docosahexaenoic acid in hepatic triglycerides as compared to control fed trout. This latter result suggests a CPFA mediated inhibition on the biosynthesis of long chain polyunsaturated fatty acids. In a similar investigation, Morrissey (49) reported that 50 ppm, and to a greater extent 300 ppm, dietary CPFA caused increased stearate:oleate ratios in both the choline and ethanolamine microsomal phospholipids of rainbow trout.

In a recent study concerning trout lipid metabolism, Perdew et al. (50) have shown that dietary CPFA reduce both the hepatic content and synthesis of acetyl CoA-carboxylase. In this study, polyacrylamide gel electrophoreis exhibited a CPFA mediated 80% decrease in the mass of a high molecular weight microsomal protein. A Western blotting procedure revealed that the protein was sensitive to both avidin-peroxidase staining and antibodies raised against goose acetyl-CoA carboxylase. A double labeling experiment using both [$^{14}$C]- and [$^{3}$H]-leucine demonstrated the reduced synthesis of the protein believed to be acetyl-CoA carboxylase, as well as a reduced synthesis of a 68,000-74,000 dalton cytosolic protein of unknown identity. Struthers et al. (65) studied a different aspect of lipid metabolism via the use of radioactive isotopes. Trout were fed 200 or 300 ppm CPFA for 14 days and subsequently were monitored for the incorporation of both [$^{32}$P]$O_4$ and [$^{14}$C]-oleic acid into hepatic lipids. CPFA feeding lowered the incorporation of [$^{32}$P]$O_4$ into hepatic phospholipids whether the phosphate was administered by

intraperitoneal injection or in vitro perfusion of the liver. Compared to controls, trout exposed to dietary CPFA also incorporated reduced amounts of [$^{14}$C]oleic acid into both microsomal and mitochondrial hepatic lipids.

Mammalian lipid metabolism:

Mammals have also been observed to exhibit a higher saturated lipid profile due to dietary CPFA. Hogs fed cottonseed oil developed hard fat, characterized by a higher melting temperature and a higher saturated fatty acid content (53). Goats consuming approximately 1 gram of CPFA per day and cows consuming approximately 3 grams of CPFA per day both produced hard butter fat that was characterized by a substantial increase in stearate:oleate ratios as compared to controls (54). Mice fed CPFA have exhibited higher palmitate:palmitoleate and stearate:oleate ratios in tissue lipids (55). In an investigation by Raju and Reiser (56), lactating mice were exposed to dietary CPFA and, as a result, nursing pups expressed altered hepatic microsomal enzyme activities. Pups nursed for 30 days by CPFA-fed females exhibited lowered stearoyl-CoA desaturase activity and elevated sn-glycerophosphate-acyltransferase (GPAT) activity as compared to pups nursed by control-fed females. Even after CPFA-nursed pups were weaned for 30 days on a control diet, the desaturase and GPAT activities remained altered as compared to control animals. Rats have also expressed a higher degree of lipid saturation due to CPFA insult. Elevated stearate:oleate ratios have been

observed in rat adipose tissue (35) and liver triglycerides (57,58). Dietary CPFA are readily incorporated into the tissue of male rats (66) and into the tissue and milk of female rats (34). A resultant increase in the stearate:oleate ratio has been observed in the females' tissues, milk, fetuses and pups (34). Sterculic acid has also been shown to inhibit fatty acyl desaturase *in vitro* in rat liver extracts (58,67). Jeffcoat and Pollard (67) also demonstrated the specificity of sterculic acid for delta-9 desaturase inhibition by showing the inability of sterculate to inhibit the *in vitro* delta-6 desaturation of linoleic acid.

*Lipid metabolism in poultry*:

An increase in saturated fatty acids of tissues from poultry fed CPFA has also been noted in a number of previous experiments. White Leghorn hens consuming CPFA have exhibited a lowered iodine absorption value in omental fat (27), and increased stearate:oleate ratios in blood plasma lipids (59,60), egg yolk lipids (60,61,68), and ovarian and liver lipids (59). *In vitro* inhibition of fatty acyl desaturase has also been observed in hen liver extracts (61,62,68-70), and several investigators have used this *in vitro* technique to demonstrate the importance of the position of the cyclopropene ring on the fatty acid in desaturase inhibition (69,70). Quail dosed orally with 30 mg/day of *S. foetida* oil showed an increased stearate:oleate ratio in laid eggs and a decreased rate of desaturation of stearic acid by liver homogenates (31).

The mechanism of desaturase inhibition:

Although the ability of CPFA to increase the saturation of tissue lipids is a widely recognized phenomenon occurring in numerous biological situations, the mechanism by which CPFA exert this effect is not fully understood. One of the most useful approaches to understanding this problem has been to monitor the effects of CPFA on the *in vitro* desaturation of fatty acids by hepatic extracts. This approach led to an early hypothesis (58,62) that suggested the formation of a covalent bond between the cyclopropene ring and sulfhydryl sidechains at the active site of the desaturase enzyme. This mechanism seemed reasonable because of its similarity to the Halphen reaction, but data collected by Jeffcoat and Pollard (67) suggests otherwise. These investigators first demonstrated a decreased activity of rat microsomal delta-9 desaturase caused by [$^3$H]-sterculic acid, and then washed the microsomes with organic solvent. After the solvent wash, microsomal protein exhibited only background radioactivity. Since a simple chloroform-methanol wash is not sufficient to break covalent bonds, they concluded that no covalent attachment had occurred between the enzyme and the sterculic acid. Also in this study, polyacrylamide gel electrophoresis did not yield a [$^3$H]-labeled protein band, further suggesting the lack of a covalent interaction. The small amount of sterculate needed to produce desaturase inhibition suggested to these investigators the existence of a very tight noncovalent bond between CPFA and the

desaturase enzyme. These investigators also noted the inability of sterculic acid to inhibit desaturation unless NADH, NADPH, ATP, Co-enzyme A and $MgCl_2$ were added as co-factors, indicating that the actual inhibitor of delta-9 desaturase is sterculoyol-CoA and not sterculic acid.

A separate hypothesis which may explain the inhibition of lipid desaturation is that CPFA exposure can cause a decrease in the content of hepatic enzymes involved in lipid metabolism. For example, although it has been reported that dietary CPFA have no effect on the activity of acetyl-CoA carboxylase in trout liver extracts (41), these extracts were prepared from livers that had reduced protein content. As previously noted, Perdew et al. (50) have recently reported a decrease in both the mass and synthesis in trout hepatic acetyl-CoA carboxylase due to CPFA insult. These data, along with the findings of Jeffcoat and Pollard (67) suggest a multi-faceted mechanistic effect of CPFA on lipid metabolism.

Effects of CPFA on cholesterol metabolism:

Dietary CPFA have also been shown to alter cholesterol metabolism in a few animal species. White Leghorn cockerels have exhibited a higher incidence of aortic atheromatosis (71), higher plasma cholesterol and a higher incidence of aortic atherosclerosis (72). New Zealand rabbits consuming 0.27% dietary CPFA have also exhibited altered cholesterol metabolism which has been characterized by higher plasma cholesterol, higher liver cholesterol and an increased incidence of aortic

atherosclerosis (73). This same study also noted altered liver histology marked by the loss of the orderly arrangement of parenchymal cells and the appearance of enlarged cells with abnormal nuclei. It should be noted that these rabbits exhibited a wide range of susceptibility to CPFA; some animals showed a marked difference in cholesterol metabolism after 3 weeks of feeding while others showed only a slight change after 5 weeks of exposure. Matlock (74) has reported that Swiss-Webster mice exposed to dietary CPFA have also exhibited altered cholesterol metabolism as characterized by hypercholesteremia and increased levels of free cholesterol. CPFA-fed mice exhibited slower blood clearance and fecal elimination of exogenous [$^{3}$H]-cholesterol which may have been due to altered fatty acid profiles of cholesterol esters. A CPFA mediated increased ratio of saturated:monounsaturated fatty acids was observed in mouse blood serum cholesterol esters, high density lipoproteins, triglycerides and C-2 fatty acyl residues of serum phosphatidyl choline (which serve as the source of fatty acids in the formation of cholesterol esters). Matlock theorized that the altered cholesterol ester profile inhibited cholesterol esterase activity, resulting in the slower clearance of cholesterol as bile acids.

The metabolic fate of CPFA:

In order to best understand the biological mode of action by which CPFA exert toxic responses, a discussion concerning the metabolic fate of these fatty acids is essential. CPFA are

readily absorbed, transported and incorporated into the tissue lipids of fish (38,39,64), mammals (34,66,75), and poultry (76,77). In addition, CPFA can be incorporated into the milk and fetuses of mammals (34), and into the egg yolk lipids of poultry (31,68,76,78). Rainbow trout exposed to dietary methyl sterculate have exhibited an accumulation of sterculic acid into both muscle lipid and depot lipid (38). These investigators report that the accumulation of sterculic acid was predominantly present as triglycerides and that withdrawal of dietary methyl sterculate resulted in the clearance of Halphen-positive compounds from tissue lipids. CPFA have also been shown to accumulate into trout hepatic phospholipids (39) and microsomal phospholipids (64). Eisele et al. (39) utilized [9,10-methylene-$^{14}$C]-sterculic acid in a thorough study of CPFA metabolism by rainbow trout. Trout exposed to dietary CPFA absorbed and incorporated more of a single intragastric dose of [$^{14}$C]-methyl sterculate than trout raised on a control diet. Over a 168 hour time period, most of the label was found in the liver, blood, bile and excreta of the fish. A lack of radioactive carbon dioxide production suggests that trout cannot metabolize the cyclopropene ring via $\beta$-oxidation. Similarly, chickens have been shown to incorporate CPFA into organs and depot lipids and to excrete the CPFA in the feces (77). As in the case of trout, chickens do not seem to expire $^{14}CO_2$ in response to methylene labelled sterculic acid exposure. Rats have also exhibited a similar deposition profile of dietary CPFA

(66,75) and an apparent inability to degrade the cycloprene ring via β-oxidation (75,79). These data are in agreement with reports that rats are unable to metabolically degrade the cyclopropane ring of cyclopropane fatty acids via β-oxidation (80,81). Eisele et al. (79) report that the major sterculate metabolites found in rat urine are short chain cyclopropane dicarboxylic acids. The formation of these metabolites requires that sterculic acid undergo α-, β- and ω-oxidation, plus reduction of the cyclopropene ring to a cyclopropane ring. These investigators postulate that the metabolic products of sterculic acid may be responsible for renal damage as noted by Nixon et al. (35), and further speculate that the dicarboxylic acids may act as inhibitors of the Krebs cycle.

## The Role of CPFA in Carcinogenesis

### Occurrence of CPFA mediated carcinogenesis:

During the past two decades, special interest has been directed toward the ability of CPFA to enhance carcinogenesis in several biological situations. The most recognized role that CPFA play in carcinogenesis is that of a cocarcinogen. In 1968 Sinnhuber et al. (43,82) first discovered hepatomas in rainbow trout fed diets containing cottonseed meal and aflatoxin, then subsequently showed that CPFA exhibit a potent cocarcinogenic effect with aflatoxin $B_1$ ($AFB_1$) in trout (83). Trout that were fed a diet containing 220 ppm CPFA plus 4 ppb $AFB_1$ exhibited an increase in both incidence and growth rate of hepatomas as

compared to trout fed 4 ppb $AFB_1$ without added CPFA. Many investigators have since confirmed the cocarcinogenicity of CPFA when administered to rainbow trout in conjunction with aflatoxins. Consequently, CPFA have been identified as cocarcinogenic with $AFB_1$ (84,85) and the related metabolites aflatoxin $M_1$ (86), aflatoxin $Q_1$ (87,88) and aflatoxicol (89). In these studies the above species of aflatoxin each caused a greater hepatic tumor incidence over positive controls when 50-220 ppm CPFA were included in the trout's diet. More recently, CPFA have been identified as promoters of $AFB_1$ induced carcinogenesis as reported by Hendricks (90). In this study, 21-day-old trout embryos were exposed to as little as 0.05 ppm $AFB_1$ for 60 minutes. After hatching, trout fed a 50 ppm CPFA diet for 9 months developed significantly more tumors than control fed trout.

In addition to the synergistic effect of CPFA and aflatoxin in carcinogenesis, CPFA have also been identified as carcinogenic in rainbow trout. Early investigators who studied the cocarcinogenic action of CPFA with aflatoxins have noted an increased incidence of hepatic tumors in trout fed diets containing CPFA but no dietary aflatoxin (84,88,89). In 1976, Sinnhuber et al. (91) reported an increased incidence of liver cancer in fish fed 45, 135 and 405 ppm methyl sterculate for 8 months. A similar study in 1980 noted increased hepatocellular carcinoma in rainbow trout fed less than 300 ppm CPFA for 12 months (92).

Studies designed to investigate possible roles for CPFA in mammalian carcinogenesis have been less conclusive than studies involving trout. In 1969 Lee et al. (93) found a slight increase in total liver and renal tubule tumors in rats fed aflatoxin and CPFA over rats fed aflatoxin alone. Similarly, Nixon et al. (94) found a slight increase in hepatic tumors in rats fed CPFA plus aflatoxin but this data failed to statistically signify CPFA as a cocarcinogen in the rat. This same study also showed a lack of cocarcinogenic action of CPFA with diethylnitrosamine. The possibility of CPFA acting as a carcinogen in mice was tested by Tinsley et al. (55) who found that C3H mice ingesting 10% cottonseed oil developed more spontaneous mammary tumors at a quicker rate than mice fed diets containing 10% other oils of comparable fatty acid composition but without CPFA. Since CPFA was not administered in conjunction with any known carcinogen, the results suggest that CPFA act as a promoter rather than as a cocarcinogen in this situation. In a similar investigation by Carroll et al. (95) Sprague-Dawley rats exhibited a trend towards increased tumorigenesis due to dietary cottonseed oil as compared to corn or soybean oil. These rats were exposed to dietary cottonseed oil before and after, but not during a three day period when they were dosed once with the carcinogen 7,12-dimethylbenz($\alpha$) anthracene. Once again, the protocol of this experiment suggests CPFA may be acting as a promoter in this situation. It should be noted that research directed towards uncovering a role

of CPFA in mammalian carcinogenesis has failed to produce statistically significant data implicating CPFA.

Mechanism of CPFA related carcinogenesis:

The mechanism by which CPFA exert a role in carcinogenesis is not clearly understood. A number of studies directed toward understanding the action of CPFA in carcinogenicity has concentrated on the combined effects of CPFA and $AFB_1$ in rainbow trout. $AFB_1$ is metabolized by the mixed function oxidase (MFO) enzyme system to the putative ultimate carcinogen: the 2,3-oxide derivative of $AFB_1$ (96). Consequently, research concerning the effects of CPFA on the MFO system is of special interest.

In a study designed to elucidate the effects of CPFA on the MFO system of rainbow trout, Eisele et al. (47) found that 300 ppm dietary sterculic acid caused a decrease in cytochrome P-450 (P-450), cytochrome b5 and total microsomal protein content. These investigators also showed that CPFA caused both an increase of benzo (a) pyrene aryl hydrocarbon hydroxylase activity and a decrease in the activity of NADPH-cytochrome c reductase. In a follow-up study, Eisele et al. (48) have reported that higher doses of dietary CPFA (450 ppm and 600 ppm) inhibited all aspects of the trout MFO system that were examined. These higher doses of CPFA resulted in all the depressive effects on the MFO system that were observed at 300 ppm dietary CPFA. In addition, however, fish exposed to these higher doses now exhibited a decrease in the activity of benzo

(a) pyrene aryl hydrocarbon hydroxylase instead of the increased activity this enzyme displayed at the lower CPFA dosage. These investigators also reported that CPFA decreased the enzyme activities of ethoxycoumarin-o-deethylase, p-nitroanisole-o-demethylase, epoxide hydrase and glutathione transferase. In a similar study, the CPFA mediated inhibition of trout MFO enzymes was demonstrated by Voss et al. (97), who also used the MFO inducer Aroclor 1254 (a polychlorinated biphenyl or PCB) in a further experiment. Trout fed PCB and CPFA had lowered microsomal MFO activity as compared to trout exposed to the PCB alone.

Recently in this laboratory, Morrissey (49) investigated the effects of CPFA on the hepatic microsomal content of two isozymes of cytochrome P-450. Antisera raised against trout P-450 and P-448 were used to quantitate P-450 and P-448 microsomal content by the method of peroxidase-antiperoxidase (PAP) immunochemical staining of proteins on nitrocellulose sheets. Compared to control fish, trout exposed to 50 ppm dietary CPFA had a reduce P-448 content while trout fed 300 ppm CPFA exhibited an even greater reduction in P-448. Neither level of dietary CPFA had a discernable effect on the content of microsomal P-450. In a study relevant to Morrissey's findings, Williams and Buhler (98) used in vitro reconstituted enzyme systems to monitor $AFB_1$ metabolism by trout P-450 and P-448. Under these experimental conditions, the P-448 enzyme system exhibited very low activity of $AFB_1$ metabolism and failed

completely to produce detectable aflatoxin-DNA adducts. On the other hand, the reconstituted P-450 system was very efficient in producing both $AFB_1$-2,3-dihydroxy-2,3-dihydrodiol and DNA adducts. These investigators also studied the effect of exposing trout to $\beta$-naphthoflavone (BNF), which is a known inducer of P-448. Microsomes isolated from BNF-exposed trout tended to convert more $AFB_1$ to $AFM_1$ and subsequently produced fewer DNA adducts, as compared to control microsomes. These data seem to indicate that the relative balance between P-448 and P-450 content is important in determining the metabolic fate of $AFB_1$. These results, along with the PAP findings of Morrissey (49), suggest that CPFA exposure may cause a shift in $AFB_1$ metabolism, ultimately resulting in less $AFM_1$ production and more aflatoxin-DNA adduct formation. These findings are consistent with another experiment conducted by Loveland et al. (99), who report that CPFA inhibit the conversion of $AFB_1$ to $AFM_1$ under in vitro experimental conditions.

Despite the above in vitro evidence that exposure to CPFA may alter $AFB_1$ metabolism in such a way as to increase genomic damage, the majority of available data seem to indicate otherwise. The suppressive effect of CPFA on the trout MFO system suggests that CPFA do not exert cocarcinogenecity with $AFB_1$ by enzyme induction resulting in more ultimate carcinogen production. Indeed, the S20 liver fraction isolated from CPFA fed trout does not enhance the mutagenic activity of $AFB_1$ as determined by the Ames' assay (48). Bailey et al. (100) also

found that dietary CPFA repressed aflatoxin binding to DNA which is believed to be the initiation step of $AFB_1$ carcinogenesis. Bailey et al. (100) reported a CPFA mediated shift of $AFB_1$ metabolism in hepatocytes isolated from rainbow trout. Although the reaction rate of $AFB_1$ was unaffected, CPFA caused an increase in the formation of aflatoxicol and inhibited the formation of DNA adducts, $AFM_1$ and water soluble aflatoxin conjugates.

The majority of available data suggests that although the inhibitive effects of CPFA on trout hepatic enzymes may increase the half-life of parent aflatoxin compounds, CPFA actually decreased the genomic damage caused by aflatoxin when administered in conjunction with aflatoxin. These data suggest that the enhancement of carcinogenesis by CPFA is due to post initiation promotion of aflatoxin toxicity. Hendricks (90) has demonstrated that CPFA can act as strict promoters of $AFB_1$ carcinogenesis and studies by Tinsley et al. (55) and Carroll et al. (95) strongly suggest a promotional role for CPFA. The promotional theory is also consistent with data from many of the studies conducted on CPFA's role in $AFB_1$ carcinogenesis since most "cocarcinogenic" investigations can be explained in a promotional manner.

# II. EFFECTS OF CYCLOPROPENOID FATTY ACIDS ON LIVER PLASMA MEMBRANES OF RAINBOW TROUT (*SALMO GAIRDNERI*)

Donald R. Marino and Daniel P. Selivonchick

Oregon State University
Department of Food Science and Technology
Corvallis, OR 97331-6602

## ABSTRACT

Hepatic plasma membranes were isolated from rainbow trout which had been exposed to different levels of dietary cyclopropenoid fatty acids (CPFA). Electron microscopy revealed that control plasma membranes existed mainly in the form of membrane sheets. In comparison, plasma membranes isolated from CPFA-fed trout contained membrane vesicles, fewer membrane sheets and more small membrane fragments. Control and CPFA plasma membranes both contained an enriched 5'-nucleotidase activity and low enzyme activities of succinate dehydrogenase and glucose-6-phosphatase, thus indicating that the isolation procedure was valid. Additionally, whole liver glucose-6-phosphatase activity was inhibited by dietary CPFA. Chromatographic analysis revealed little change in the phospholipid composition of plasma membranes due to CPFA. However, dietary CPFA significantly altered the fatty acid profiles of choline and ethanolamine phospholipids. In both phospholipid pools, CPFA caused a decrease in palmitic, palmitoleic and oleic acids and an increase in stearic and docosahexaenoic acids. Two-dimensional gel electrophoresis of proteins revealed substantial differences between the protein content of control and CPFA plasma membranes, particularly in the high molecular weight fraction. Western blotting followed

by concanavalin A-peroxidase staining revealed the presence of three families of glycoproteins only in CPFA plasma membranes. Two of these families have been previously identified as being associated with microsomes and sensitive to CPFA exposure. The third glycoprotein family appears to be characteristic only of CPFA plasma membranes.

## INTRODUCTION

Cyclopropenoid fatty acids (CPFA) are a group of structurally peculiar fatty acids that are produced by plants of the order Malvales. The presence of a sterically strained cyclopropene ring in the CPFA molecule is responsible for a number of adverse physiological reactions in a diverse range of biological situations. Exposure to dietary CPFA has resulted in impaired reproduction in both poultry (28-31) and mammals (32-34), hepatic damage to mammals (35-37) and fish (40-45), and altered lipid metabolism in mammals (34,35,53-58), poultry (26,27,31,59-62) and fish (40,49,50,52,65). Furthermore, CPFA have been identified as playing a role in the enhancement of carcinogenesis in fish, especially when CPFA are administered in cunjunction with various carcinogenic forms of aflatoxin (43,82-90). The toxic nature of CPFA is of special research interest because these fatty acids are found in certain edible plant oils, most notably the oil obtained from Gossypium hirsutum (cottonseed oil). Although most CPFA are destroyed by the modern processing methods used for edible oils (18), situations do exist where CPFA are consumed by both animals and humans (17,19,20).

The mechanism by which CPFA enhance hepatocarcinogenesis is presently not well understood, although these toxins are known to alter hepatic protein content (46-50), induce mitosis in the

liver and pancreas (36,37), and inhibit numerous hepatic enzymes, including microsomal mixed function oxidase enzymes (47-49). In addition, CPFA are know to alter the composition (46,49,64) and to inhibit the natural function (26,35) of biological membranes. This membrane damage, along with the suggestion of some researchers that alteration of plasma membranes may play an important role in carcinogenesis (101), direct attention to the need for further research regarding the relationship between CPFA mediated membrane damage and carcinogenesis.

The following research is directed towards achieving a better understanding as to the effects of dietary CPFA on the hepatic plasma membranes of rainbow trout. Plasma membranes isolated from CPFA fed fish will be compared to plasma membranes from control fish via the techniques of electron microscopy, analysis of enzyme activities, thin layer chromatographic analysis of phospholipids, gas-liquid chromatographic analysis of phospholipid fatty acids, two dimensional gel electrophoresis of proteins, and the Western blotting analysis of concanavalin A sensitive glycoproteins. The purpose of this study is to determine what effect, if any, that CPFA exert on hepatic plasma membranes in the hope that such information may help in the further understanding of CPFA toxicity.

# MATERIALS AND METHODS

## Care of Fish

All experimental animals used in this study were Mount Shasta strain rainbow trout (Salmo gairdneri) that were spawned and raised at the Oregon State University Toxicology Laboratory. All fish were housed in 90 cm fiberglass tanks which received 12°C well water at a flow rate of 7.6 liters per minute. Control fish were fed a basal diet as described by Sinnhuber et al. (102) while test fish were fed the basal diet plus either 50 or 300 ppm methyl sterculate.

## Chemicals and Equipment

All electrophoretic equipment and chemicals used in these studies were purchased from Bio-Rad Laboratories (Richmond, CA) unless otherwise noted. A Buchler Model 3-1500 constant power supply (Buchler Instruments Inc., Fort Lee, NJ) was used for isoelectric focusing and gradient gel electrophoresis. The power supply used for Western blotting was a model EC-420 (E-C Apparatus Corporation, St. Petersburg, FL). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. All spectrophotometric values were determined with the use of a Varian model DMS 80 UV-visible spectrophotometer (Varian Associates, Palo Alto, CA). All solutions were made with distilled, deionized water and all chemicals were of reagent grade quality.

Preparation of Plasma Membranes

Hepatocyte plasma membranes were prepared essentially by the method of Lutz (103). Fish to provide at least 10 grams of whole liver were sacrificed by a cranial blow and the livers were immediately excised and washed in a cold buffer consisting of 10% sucrose (w/v) in 5 mM Tris-HCl (pH 7.4). All further procedures were performed on ice in order to keep the temperature at about 4°C. The livers were next trimmed of connective tissue, diluted with one volume of the above buffer and then finely minced with a razor blade. The minced liver suspension then was further diluted with three additional volumes of the above buffer and subsequently homogenized with two careful strokes of a Kontes Dounce homogenizer with a loosely fitting (type A) pestle. The resulting homogenate was diluted to 160 ml with the above buffer and a small aliquot (labelled as whole liver homogenate) was set aside for use in the marker enzyme assays. The remainder of the liver homogenate was centrifuged for six minutes at 120 x g (1000 rpm in a Sorvall RC-2B centrifuge with a Sorvall SS-34 rotor). The resulting supernatant and a thin layer of erythrocytes that laid on top of the pellet were discarded. The pellet was resuspended in 40 ml of the above buffer and then homogenized again with two careful strokes of the loose Dounce. This homogenate was filtered through a 1 mm nylon mesh (Tetko, Inc., Elmsford, NY),

diluted to 160 ml and then centrifuged under the same conditions as above. Once again, the resulting supernatant and any remaining erythrocytes were discarded. The pellet was brought to 35 ml with the same buffer and this suspension was homogenized with 14 vigorous strokes of the loose Dounce. This homogenate was brought to 40 ml with the same 10% sucrose buffer, divided into two equal portions and each was subjected to high speed, discontinuous gradient centrifugation. Two centrifuge tubes were prepared in which 8 ml of 33% sucrose (w/v) in 5 mM Tris-HCl (pH 7.4) had been carefully layered on top of 5 ml of 38% sucrose (w/v) in 5 mM Tris-HCl (pH 7.4). For each tube, one portion of the liver homogenate was carefully layered on top of the 33% sucrose. The samples were then centrifuged at 105,000 x g for 150 minutes (24,000 rpm in a Sorvall OTD-65 centrifuge using a Sorvall 627 rotor). This centrifugation resulted in a cloudy region at the 10%-33% interface which was collected using a bent Pasteur pipette. The collected material was diluted to 40 ml in the 10% sucrose/Tris solution and centrifuged for 50 minutes at 27,000 x g (15,000 rpm in the SS-34 rotor). The supernatant was discarded and the small white pellet containing plasma membranes was suspended in 1 ml of 1mM $NaHCO_3$. This 1 ml suspension was layered on top of a continuous linear sucrose gradient that ranged in concentration from 1% to 24% sucrose (w/v) in 1 mM $NaHCO_3$. The sucrose gradient had been carefully layered on top of a cushion that consisted of 50% (w/v) sucrose in 1 mM $NaHCO_3$. The sample

was then centrifuged at 105,000 x g in the 627 rotor for 30 minutes. The cloudy interface between the gradient and the cushion was collected with a bent Pasteur pipette and diluted to 40 ml with 1 mM $NaHCO_3$. This suspension was centrifuged for 50 minutes at 27,000 x g using the SS-34 rotor. The supernatant was discarded and the small white pellet was suspended in a minimal amount of 1 mM $NaHCO_3$. Protein content of this final product was checked by the method of Lowry et al. (104), enzyme assays were performed and the remainder of the product was stored at -80°C for future analysis.

Electron Micrographs of Plasma Membranes

Plasma membranes were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 1 hr, rinsed in buffer, postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr and then dehydrated in a graded series of ethanol (15 min each). The membranes were stained in 1% uranyl acetate in 100% ethanol for 90 min before infiltration and embedding in Spurr's low viscosity medium (105). Silver-gold sections were mounted on uncoated 200 mesh copper grids, stained with lead citrate (106) and examined at a magnification of 9593x.

Marker Enzyme Assays

In order to determine the degree of purification of plasma membranes achieved by the isolation procedure, assays were performed on enzymes that are known to be associated with certain subcellular components. Levels of succinic acid

dehydrogenase, glucose-6-phosphatase and 5'-nucleotidase were determined in order to check for the presence of mitochondria, endoplasmic recticulum and plasma membranes, respectively.

Succinic acid dehydrogenase activity was determined essentially by the method of Khouw and McCurdy (107). Solutions used were 50 mM KCN, 4 mM 2,6-dichlorophenolindophenol (DCPIP), 2.5 mM phenazine methosulfate, and 250 mM sodium succinate (all of which were prepared in 100 mM Tris-HCl at pH 7.4). Assays were performed at room temperature in the Varian DMS 80 double beam spectrophotometer. The reference cuvette (which contained no substrate) was filled with 1.0 ml of 100 mM Tris-HCl, 0.2 ml KCN, 0.2 ml phenazine methosulfate, 0.2 ml sample (containing approximately 0.5 mg of protein) and 1.2 ml water. The sample cuvette was filled with the same amount of Tris-HCl, KCN, phenazine methosulfate and protein sample but 0.2 ml of sodium succinate and only 1.0 ml of water were added. The reaction was initiated by the addition of 0.2 ml DCPIP to both cuvettes and the progress of the reaction was monitored by recording the decrease in absorbance at 600 nm for 5 minutes.

Glucose-6-phosphatase activity was determined by the method of Swanson (108). Solutions used for this assay were 0.1 M sodium maleate buffer (pH 6.5 with 0.1 N NaOH), 21.4 mM glucose-6-phosphate (G-6-P) prepared in the maleate buffer, and 25% (w/v) trichloroacetic acid (TCA). First of all, 1.4 ml of the G-6-P solution was incubated at 25$^{o}$C for 5 minutes. At this time, 0.1 ml of the sample (containing approximately 0.1 mg of

protein) was added and the hydrolysis reaction was allowed to proceed for 20 minutes at 25°C. The reaction was stopped by adding 1.0 ml of the TCA, the resulting protein precipitate was centrifuged to a pellet and 2 ml of the supernatant were subjected to phosphate analysis. A zero-time control reaction was also performed in which 1.4 ml G-6-P was added to 1.0 ml TCA. This mixture was incubated for 5 minutes at 25°C, 0.1 ml sample was added, and the mixture was then incubated, centrifuged and 2 ml of the supernatant were analyzed as above. The amount of phosphate liberated by the experimental sample was determined by subtracting the zero-time phosphate value from the experimental phosphate value.

The method used to determine 5'-nucleotidase activity was that of Michell and Hawthorne (109). The substrate solution used for this assay was 5 mM adenosine-5'-monophosphate (AMP) in a buffer consisting of 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium-potassium tartrate and 50 mM Tris-HCl (pH 7.4). First, 1.9 ml of the AMP solution was incubated at 25°C for 5 minutes after which time 0.1 ml sample (containing approximately 0.1 mg of protein) was added. After an additional 20 minute incubation, the reaction was stopped by adding 1.0 ml of 25% TCA. The protein precipitate was pelletized and 2.0 ml of the supernatant were assayed for free phosphate. As in the glucose-6-phosphatase assay, a zero-time control was prepared by adding the TCA to the substrate prior to the addition of the sample. Once again, the amount of phosphate liberated by the enzymatic

reaction was determined by subtracting the zero-time value from the experimental value.

The amount of free phosphate liberated by the glucose-6-phosphatase and 5'-nucleotidase assays was determined by the method of Fiske and Subbarow (110). A 0.5 mM standard phosphate solution was prepared by dissolving 68 mg $KH_2PO_4$ in 10 ml concentrated $H_2SO_4$ and diluting to 1 liter with water. A 2 mM ammonium molybdate solution was prepared by first dissolving 2.5 g $(NH_4)_6Mo_7 \cdot 4H_2O$ in 500 ml of water and diluting 14 ml of concentrated $H_2SO_4$ in 200 ml of water. These two solutions were combined, diluted to 1 liter and designated as the ammonium molybdate reagent. The 1-amino-2-naphthol-4-sulfonic acid (ANSA) reagent was prepared by dissolving 5.7 g of $NaHSO_3$ and 0.2 g of $Na_2SO_3$ in 50 ml of water and then adding 0.1 g of ANSA and allowing it to dissolve. This solution was diluted to 1 liter with water, filtered and then stored in the dark.

To run the assay, 2 ml of supernatant from the appropriate marker assays (or 2 ml of the standard phosphate solution) were added to 5 ml of the ammonium molybdate reagent and allowed to incubate at room temperature for 10 minutes. Next, 1 ml of the ANSA reagent was added and the reaction mixture was allowed to incubate for 20 minutes at room temperature during which time a blue color developed. Optical density at 700 nm was measured immediately after this incubation and the free phosphate content of each sample was determined by comparison with the optical density developed by the standard phosphate solution. The

statistical significance of the enzyme data, as well as other statistical analyses performed in this thesis, was calculated with the use of a Z table.

Phospholipid Analysis

In order to analyze phospholipid content, total lipid was first extracted from purified plasma membranes by the method of Bligh and Dyer (111). Plasma membrane samples were mixed well with 2.0 ml water, 5.0 ml methanol and 2.5 ml $CHCl_3$. This mixture was stored under nitrogen at 4°C overnight, after which time an additional 2.5 ml $CHCl_3$ and 2.5 ml water were added. After thorough mixing, the chloroform phase was separated from the aqueous phase with a clinical centrifuge. The chloroform phase was collected and evaporated to dryness under nitrogen. The remaining lipid sample was dissolved in 100 microliters of benzene and then subjected to two dimensional thin layer chromatography (TLC). TLC analysis was performed as described by Rouser et al. (112). TLC plates were coated with 0.5 mm silica gel H, washed with $CHCl_3/CH_3OH/NH_4OH$ (65:35:5, by volume) and then activated at 120°C for one hour. Lipid samples were spotted on a corner of the plate and the first dimension was run in $CHCl_3/CH_3OH/NH_4OH$ (65:35:5, by volume) until the solvent front was approximately 2 cm from the top of the plate. After drying the plate under nitrogen, the second dimension was run in

a solvent system that consisted of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by volume). The plates were then dried under nitrogen and lipid spots were visualized with iodine. Appropriate spots were scraped from the plates and the phospholipid content was determined for each spot by the method of Bartlett (113). The scraped phospholipid spots were placed in test tubes and any remaining organic solvent was evaporated under nitrogen. The samples were then mixed with 1.0 ml of 70% perchloric acid and digested at 190°F for one hour. After cooling, 4.5 ml of .44% (w/v) ammonium molybdate in 0.5N sulfuric acid was added. Next, 0.2 ml of a solution containing 0.2% (w/v) 1-amino-2-naphthol-4-sulfuric acid, 12% (w/v) $NaHSO_3$ and 2.4% (w/v) $Na_2SO_3$ was added. The resulting solution was mixed well and then heated in a boiling water bath for 10 minutes. The final blue colored solution was diluted to 10 ml with water and centrifuged to precipitate the silica gel. Optical density readings at 830 nm were determined (Varian DMS 80 spectrophotometer) and the phosphorous content of each sample was determined by comparison against a known standard $KH_2PO_4$ solution. Background phosphate levels were determined by scraping the TLC plates in areas which failed to be stained by iodine.

Analysis of Phospholipid Fatty Acid Content

The analysis of the fatty acid composition of choline and ethanolamine phospholipids was performed as described by

Selivonchick and Roots (114). Phospholipids were separated by TLC as described above, the plates were sprayed with 2',7'-dichlorofluorscein and the lipids were viewed under ultraviolet light. Phosphatidyl choline and phosphatidyl ethanolamine spots were scraped from the plates and each subjected to methanolysis. Each sample was mixed with 4 ml of 4% $H_2SO_4$ in methanol (v/v), sealed in nitrogen flushed glass vials and heated at 90°C for 90 minutes. After methanolysis, each sample was diluted with 1 ml of water and the methyl esters were extracted 3 times with 2 ml of hexane. Next, the samples were washed with 1 ml of 5% sodium bicarbonate (w/v) in order to neutralize any remaining acid. The hexane layer was transferred to a test tube, a few grains of sodium sulphate were added to remove any water and the tubes were sealed under nitrogen for 30 minutes. After the samples were dried of water, they were filtered through glass wool into screw cap test tubes and all hexane was evaporated under nitrogen. The samples were suspended in 50 microliters of fresh hexane and subjected to one dimensional TLC. TLC plates were coated with 0.5 mm silica gel G, washed with benzene and activated for one hour at 120°C. The complete 50 microliters of sample were run in one dimension in benzene. The plates were subsequently dried under nitrogen, lipid spots were visualized with 2',7'-dichlorofluorscein as described above and each methyl ester spot was scraped into a test tube. Each sample was then mixed well with 10 ml of hexane/ether (9:1, by volume), filtered through glass wool and evaporated to dryness under nitrogen.

The resulting samples were dissolved in 20 microliters of iso-octane and kept under nitrogen until gas chromatography analysis.

Gas chromatography of the methyl esters was performed using a Varian Aerograph model 1200 gas chromatograph. The relative percentage of each methyl ester was determined with the use of a Hewlett-Packard 3380A integrator and each peak was identified by comparison of its retention time with that of known standards (PUFA #1 and PUFA #2; Supelco, Inc., Bellefonte, PA). The chromatography column was packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc.).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis of protein samples was performed as described by Perdew et al. (115) who developed a modification of the original method of O'Farrell (116).

Isoelectric focussing gels (IEF gels) were formed in glass tubes of size 150 mm x 3 mm inside diameter. The tubes were washed in a chromic acid wash for at least 2 hours, rinsed well with water, dried well in an oven and then coated with Sigmacote (Sigma Chemical Co., St. Louis, MO) in order to facilitate the later removal of the IEF gels from the tubes. A 1 cm parafilm plug was inserted in the bottom of each tube in order to form a lower well for holding lysis buffer during electrophoresis. The IEF gels consisted of 55% urea, 3.8% acrylamide, 0.2% bisacrylamide, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-

propanesulfonate (CHAPS), 1.5% pH 3-10 ampholytes, 0.5% pH 5-7 ampholytes, 0.07% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.01% ammonium persulfate. Immediately after mixing these reagents, the gel solution was injected with a syringe into the glass tubes to a depth of 11.5 cm above the parafilm plug. The gels were overlaid with 8 M urea and allowed to polymerize for 2 hours at which time the urea overlay was replaced with 20 microliters of lysis buffer (3% CHAPS, 1.5% pH 3-10 ampholytes, 0.5% pH 5-7 ampholytes, 0.07 M dithiothreitol and 9.5 M urea). The lysis buffer was overlaid with a small amount of water and the gels were allowed to sit for an additional 2 hours.

After the completion of polymerization the parafilm plugs were removed from the bottom of each tube, the resulting wells were filled with lysis buffer and the wells were then sealed with dialysis tubing (molecular weight cutoff approximately 3,500 from Spectrum Medical Industries, Los Angeles). At this step, care must be taken so as to exclude all air bubbles from the lower well. After replacing the lysis buffer overlay with 25 microliters of fresh lysis buffer, the tubes were placed in an electrophoresis unit which contained an upper reservoir filled with 0.01 M $H_3PO_4$ and a lower reservoir containing degassed 0.02 M NaOH. The electric field applied across the gels was set up so that the cathode was at the top reservoir. The gels were prerun at 200 volts for 15 minutes, then at 300 volts for 30 minutes and finally at 400 volts for 30 minutes.

During the prerun, protein samples were diluted in one volume dispersing buffer (20% glycerol, 0.05 M $Na_2HPO_4$, 0.05 M $NaH_2PO_4$, pH 6.8) and 2 volumes lysis buffer. The samples were next saturated with urea and the pH brought to 4.1 with 3 N HCl. Immediately after the completion of the prerun, the upper and lower reservoirs of the electrophoresis unit were emptied and the protein samples were injected on top of the IEF gels. Gels which would eventually be silver stained were loaded with 200 micrograms of protein while samples for future Western blotting were loaded with 300 micrograms of protein. Each protein sample was next overlaid with 15 microliters of sample overlay solution (0.75% pH 3-10 ampholytes, 0.25% pH 5-7 ampholytes, 0.07 M dithiothreitol, 8 M urea). The upper and lower reservoirs were refilled and a voltage was applied as described above. Electrophoresis was run at 400 volts for 12 hours and then 800 volts for one hour. Gels were removed from the glass tubes by carefully applying air pressure to one end of the tube with a pipette bulb. Gels were equilibrated for 110 minutes in a solution of 10% glycerol, 0.07 M dithiothreitol, 2.3% LDS and 0.625 M Tris-HCl pH 6.8. At this point, IEF gels were either immediately subjected to second dimension electrophoresis or stored at -80°C for future use.

Slab gels used for the second dimension separation of proteins by molecular weight were prepared as described by Broglie, et al. (117). Protein samples were electrophoretically separated on 30 cm by 14 cm by 0.15 cm linear acrylamide slab

gels which ranged from 10% to 15% acrylamide (stabilized with a 5% to 17.5% sucrose gradient). A light acrylamide solution (35 ml of 5% sucrose, 0.375 M Tris-HCl pH 8.8, 10% acrylamide, 0.25% bisacrylamide, 0.1% ammonium persulfate, 0.04% TEMED) and 35 ml of heavy acrylamide solution (17.5% sucrose, 0.375 M Tris-HCl pH 8.8, 15% acrylamide, 0.4% bisacrylamide, 0.1% ammonium persulfate, 0.8% TEMED) were poured into the chambers of a standard gradient maker and the gels were poured by use of a peristaltic pump (10 ml per minute flow rate). The gradients were then overlaid with water saturated butanol and allowed to polymerize for 2 hours. After polymerization, the tops of the gels were rinsed with water and a stacking gel (0.125 M Tris-HCl pH 6.8, 5% acrylamide, 0.14% bisacrylamide, 0.1% ammonium persulfate, 0.1% TEMED) was poured. One well was made in the stacking gel for the inclusion of molecular weight standards.

After polymerization of the stacking gel, an IEF gel was attached to the top of the slab gel with hot 1% agarose in LDS sample buffer. The LDS sample buffer consisted of 2% lithium dodecyl sulphate (LDS; purchased from BDH Chemicals Ltd., Poole, England), 12% sucrose, 50 mM Tris-HCl pH 67.8, 2 mM EDTA, and 0.02 mM dithiothreitol. Upon solidification of the agarose, an electrophoretic chamber was assembled with the lower reservoir filled with a running buffer which consisted of 0.021 M Tris-HCl (pH 8.2) and 0.16 M glycine. The upper running buffer consisted of 0.1% LDS, 1.2 mM EDTA, 0.021 M Tris-HCl (pH 8.2) and 0.16 M glycine. Bio-Rad molecular weight standards were diluted 1:20

in LDS sample buffer that also contained 0.002% bromophenol blue tracking dye. Using a microliter syringe (Hamilton Co., Reno, Nevada), ten microliters of the molecular weight standards were injected into the stacking gel well. Electrophoresis was performed at a constant current of 16 mA per gel and after sufficient migration of the tracking dye, the gels were submitted to either silver staining or Western blotting.

Silver Staining of Gels

Silver staining of O'Farrell gels was performed by the method of Wray et al. (118). Gels were first fixed with 10% trichloroacetic acid (w/v) for 30 minutes and then washed with water for 30 minutes. The gels were then soaked for 3 hours in three changes of 50% methanol containing 0.05% formaldehyde (v/v). At this time the gels were placed in a fresh solution of the methanol-formaldehyde and allowed to soak for 12 to 24 hours. Just prior to staining, the gels were soaked in two changes of water for a minimum of one hour.

Staining solution was prepared by adding dropwise 20 ml of 20% $AgNO_3$ (w/v) to a mixture of 105 ml of 0.36% NaOH and 7 ml of concentrated $NH_4OH$. The resulting solution was brought to 500 ml and used immediately. The gels were placed in the staining solution for 20 minutes with gentle agitation. The gels were then washed three times with water for 5 minutes with gentle agitation. The silver stain was then developed in 500 ml of a solution that containing 2.5 ml of 1% citric acid (w/w) and 0.25

ml of 38% formaldehyde. After suitable color development, the gels were rinsed well in water and then returned to the methanol-formaldehyde solution for 15 minutes. The gels were washed for 15 minutes in water and unwanted background staining was removed with Kodak Rapid Fix (film strength). After the desired amount of background had been removed, the gels were washed twice for 5 minutes in water and then soaked in Kodak Hypo Clearing Agent (film strength) for 30 minutes. The gels were then washed twice in water and returned to the methanol-formaldehyde. Pictures were taken within two days and the gels were stored at 4°C in air tight containers.

Western Blotting and Glycoprotein Detection

Western blotting of O'Farrell gels was performed essentially by the method described by Clegg (119). Gels were put in close contact with nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) and proteins were transferred in a Genie electroblotter (Idea Scientific Company, Corvallis, OR) at 24 volts for 30 minutes. The transfer buffer used consisted of 20% methanol (v/v), 192 mM glycine and 25 mM Tris-HCl (pH 8.3). After completion of the blot, gels were silver stained in order to assess the degree of protein transfer.

For the detection of glycoproteins, the nitrocellulose was first incubated in a phosphate buffered saline (PBS) solution

(10 mM $NaH_2PO_4$ and 150 mM NaCl) that also contained 2.5% bovine serum albumin (BSA, RIA grade). This incubation was conducted at room temperature for 1 hour with gentle agitation. The blots were then incubated for 1 hour in PBS that contained concanavalin A (10 μg/ml), 0.5% Triton X-100 (v/v) and 10 μM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$. The blots were then washed in this same buffer (without the concanavalin A) five times for five minutes each and then incubated with horseradish peroxidase (5 mg/100 ml) in the same buffer for 60 minutes. The blots were washed five times as above and then incubated in a solution of 3,3'-diaminobenzidine (30 mg/100 ml), 0.01% $H_2O_2$ (v/v) in 50 mM sodium acetate (pH 5.0). After suitable color development, the transfers were washed three times in water and dried in the dark.

# RESULTS

## Gross Morphological Observations

During the isolation of hepatic plasma membranes, several differences were observed between the livers of control and CPFA treated fish. Livers that were isolated from control-fed trout had relatively sharp edges around the perimeter of each lobe and were dark reddish purple in color. In comparison, livers from CPFA-fed trout were rather swelled with rounded edges, pale tan in color and much firmer in texture. Also noted during the isolation procedure was the fact that CPFA treated livers were much more difficult to Dounce homogenize than control livers.

## Electron Microscopy of Plasma Membranes

Electron microscopy did reveal a visual difference between hepatic plasma membranes prepared from control-fed and CPFA-fed trout. As shown in Figure 1, control-fed fish yielded a more homogeneous plasma membrane preparation than CPFA-fed fish. The control preparation (Figure 1A) consisted mainly of relatively large membrane sheets along with a few smaller membrane fragments. In comparison, plasma membranes isolated from trout fed 50 ppm CPFA (Figure 1B) contained fewer membrane sheets, a greater number of small membrane fragments and a number of swirled membrane vesicles which appear to be absent from the control plasma membranes. Hepatic plasma membranes isolated

from fish fed 300 ppm CPFA (Figure 1C) appeared even less homogeneous than the membranes isolated from fish fed 50 ppm CPFA. Once again, these experimental membranes contained fewer membrane sheets and a greater number of small membrane fragments and vesicles as compared to control plasma membranes.

Analysis of Marker Enzyme Activities

The assay of succinate dehydrogenase activity was utilized to detect the presence of mitochondria. Results from a typical succinate dehydrogenase assay are presented in graph form in Figure 2. These data were generated from the analysis of a whole liver homogenate isolated from control fish. In the trial depicted by Figure 2, 0.5 mg hepatic protein was used in the assay and the succinate dehydrogenase activity was calculated to be 0.021 micromoles of DCPIP reduced per minute per milligram of protein. Succinate dehydrogenase activity of whole liver homogenates from CPFA-fed trout was not significantly different from control values. Purified plasma membranes isolated from both control and CPFA fish contained no detectable succinate dehydrogenase activity.

Presented in Table 1 is a summary of the analysis of glucose-6-phosphatase, an enzyme known to be associated with the endoplasmic recticulum. Livers from trout fed CPFA exhibited a lowered activity of glucose-6-phosphatase in both whole liver homogenates and purified plasma membranes. The enrichment of this enzyme due to the purification process was slightly higher

for control plasma membranes, but both control and experimental enrichment values were close to 1.0.

Data concerning the plasma membrane marker 5'-nucleotidase are also presented in Table 1. The activity of this enzyme was approximately 0.015 micromoles of phosphate liberated per minute per milligram of protein for both control and experimental whole liver homogenates. A 41x fold enrichment of 5'-nucleotidase activity was observed in plasma membranes purified from control fish while the enrichment of this enzyme activity in membranes from CPFA-fed fish was 31x fold.

Analysis of Plasma Membrane Phospholipids

The phospholipid composition of plasma membranes isolated from control-fed and CPFA-fed rainbow trout are shown in Table 2. Trout fed control diets, 50 ppm CPFA diets and 300 ppm CPFA diets exhibited very similar phospholipid profiles. As trout were exposed to increasing levels of CPFA, a gradual increase in choline phospholipids and a gradual decrease in serine phospholipids were observed. However, both of these observed trends were inadequate to demonstrate a true statistical difference.

Constituent Fatty Acids of Ethanolamine and Choline Phospholipids

The fatty acid composition of ethanolamine and choline phospholipids isolated from hepatic plasma membranes is depicted

in Tables 3 and 4. The most noticeable effect of the CPFA diets was observed on the 18-carbon length fatty acids. Both ethanolamine and choline phospholipids exhibited an increase in stearic acid (18:0) content and a decrease in oleic acid (18:1) content due to CPFA insult. This effect on the 18-carbon fatty acids resulted in a marked increase in the ratio of stearate:oleate as indicated in the tables. CPFA treatment tended to decrease the content of both palmitic acid (16:0) and palmitoleic acid (16:1) of both phospholipids tested. Other tendencies observed included a decrease of 20:1 n-9 fatty acid and an increase of 22:6 n-3 fatty acid due to dietary CPFA. It should also be noted that the unsaturation index of total fatty acids tested was slightly increased by CPFA treatment, due to the high levels of 22:6 n-3.

Two-Dimensional Gel Electrophoresis of Plasma Membranes

Two-dimensional gel electrophoresis revealed several differences between the protein content of control and CPFA treated plasma membranes. The most obvious difference between the two was found in the high molecular weight region. While control samples (Figure 3A) contained no proteins heavier than 66,000 daltons in molecular weight, plasma membranes from CPFA-fed fish (Figure 3B) contained several proteins in the molecular weight range of 66,000 to 180,000 daltons. As Figure 3 illustrates, a family of six proteins with molecular weight of 75,000 daltons and iso-electric point (pI) ranging from 5.0 to

5.5 was observed only in plasma membranes isolated from CPFA-fed fish. The distinct pattern of these proteins has also been observed by two-dimensional electrophoresis in both control and CPFA treated microsomes. CPFA treated plasma membranes additionally contained a few proteins which ranged in molecular weight from 120,000 to 180,000 daltons and ranged in pI from 4.5 to 5.0. One protein with a molecular weight of 150,000 daltons and a pI of 5.9 also was detected in CPFA treated plasma membranes.

Most of the proteins contained in both the control and CPFA samples were found in the molecular weight range of 21,000 to 66,000 daltons and the pI range of 4.0 to 6.0. Comparison of proteins in this area revealed very similar electrophoretic patterns. CPFA treated plasma membranes did contain a few proteins in this molecular weight range with a pI greater than 6.0 that were absent in the control samples.

The electrophoretic patterns for proteins under 21,000 daltons in molecular weight were quite different between control and CPFA plasma membranes. Few low molecular weight proteins were observed in CPFA membranes. In comparison, control membranes contained those proteins found in CPFA membranes plus additional proteins not discernable on the CPFA gels.

Western Blotting and Glycoprotein Detection of Plasma Membranes

As was the case in two-dimensional electrophoresis, most of the glycoproteins observable by Western blotting were in the

molecular range of 21,000 to 66,000 daltons and the pI range of 4.0 to 6.0. Electrophoretic patterns in this area of the blots were similar between control and CPFA membranes. Once again, CPFA membranes contained high molecular weight proteins not present in control membranes (Figure 4). The family of six proteins of molecular weight 75,000 daltons that were observed in the two-dimensional gels proved to be glycoproteins. Additionally, a family of four glycoproteins of molecular weight 80,000 daltons and pI 6.8 were present only in CPFA plasma membranes. As in the case of the 75,000 dalton family, the 80,000 dalton family has been previously identified as being associated with microsomal membranes. Furthermore, a family of three glycoproteins of molecular weight 180,000 daltons and pI of 4.5 to 5.0 were present only in CPFA plasma membranes.

## DISCUSSION OF RESULTS

Data collected during the course of this study indicate that dietary CPFA alter the hepatic system of rainbow trout in a variety of ways. The presence of enlarged, firm, pale livers can be caused by a wide range of toxins and is a typical indication of hepatic damage (120). Since CPFA have previously been shown to induce this response in trout (40,41), the altered liver morphology observed during the present study is an expected occurrence. Another expected result was also observable on the subcellular level. The decreased activity of whole liver glucose-6-phosphatase observed in the present study is predictable since previous investigators have reported that dietary CPFA inhibit the microsomal activity of this enzyme in trout (44). The immediate cause of the decreased glucose-6-phosphatase activity is currently not known, but may be due to either a lowered enzyme content or a diminished capacity of enzyme catalysis due to alterations in the lipid environment.

The analysis of phospholipid fatty acids indicates that the inhibition of fatty acyl desaturase has resulted in differences between the lipid content of control and CPFA plasma membranes. The increase in stearic acid and decrease in oleic acid are well-known consequences of CPFA exposure. Although CPFA have been reported to cause an increase in the ratio of palmitic acid:palmitoleic acid in trout liver triacylglycerols and

phospholipids (40), such was not the case for hepatic plasma membranes. The observed decrease of palmitoleic acid is a predictable result of desaturase inhibition. However, CPFA plasma membranes expressed a decrease in palmitic acid instead of an increase of this saturated fatty acid. The decrease of palmitic acid content may be due to a combination of factors. First of all, the recent finding that dietary CPFA decrease acetyl-CoA carboxylase content (50) can be strongly implicated. Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is in turn reacted upon by the fatty acid synthase enzyme complex to ultimately form palmitic acid. Since acetyl-CoA carboxylase is considered to be the key regulatory enzyme in the de novo synthesis of long chain fatty acids (121), it seems reasonable that the effects of a lowered carboxylase content would be most apparent as a decrease in the major product of fatty acid synthase, namely palmitic acid. Extending this scenario further, the decreased acetyl-CoA carboxylase content would also be responsible for the slight decrease observed in myristic acid (14:0) and would also contribute to the decrease observed in palmitoleic acid. A second factor that could be responsible for the decreased palmitic acid content is that palmitic acid may be sacrificed in order to replenish the depleted oleic acid pool. Such a mechanism requires that palmitic acid first be elongated to stearic acid. In this scheme stearic acid would then be changed to oleic acid, but the inhibition of the desaturase would hinder

the production of oleic acid. This scheme of events suggests that the observed increase in stearic acid should be somewhat larger than the observed decrease in oleic acid. Such an imbalance is present in CPFA plasma membrane choline phospholipids, which are rich in palmitic acid (see Table 4).

CPFA plasma membranes showed an increase in the phospholipid content of docosahexaenoic acid (22:6 n-3). Roehm et al. (40) have previously reported that dietary CPFA cause a decrease of this fatty acid in hepatic triacylglycerols. These investigators therefore suggested that CPFA may inhibit the biosynthesis of long chain polyunsaturated fatty acids. The increase of docosahexaenoic acid in plasma membrane phospholipids, however, may indicate that this fatty acid is actually transferred from the triacylglycerol fraction to the plasma membrane phospholipid fraction. Such a transfer would maintain a high unsaturation index in order to compensate for the consequences of desaturase inhibition and thereby prevent a decrease in membrane fluidity.

During the isolation of plasma membranes, the possibility of contamination from other subcellular components was of special concern. For this reason, the activities of marker enzymes were assayed. The complete lack of succinate dehydrogenase activity in the purified plasma membranes indicates that mitochondrial contamination was inconsequential. Furthermore, cardiolipin, a mitochondrial phospholipid, was not observed in any of the plasma membrane lipid extracts examined.

The low levels of glucose-6-phosphatase activity suggest that microsomal contamination was minimal, while the comparable enrichment values of this enzyme indicate that microsomal contamination was nearly equal between control and CPFA plasma membranes. The high enrichment values observed for 5'-nucleotidase activity show that the isolation procedure was valid for both control and CPFA membranes.

The results obtained from electron microscopy and protein electrophoresis are of special interest because both procedures yielded data that could be interpreted as evidence that CPFA plasma membranes were contaminated with microsomal membranes. The presence of membrane vesicles and the appearance of certain protein families are characteristic of microsomes. However microsomal contamination does not seem to be a problem, as evidenced by the comparably low levels of glucose-6-phosphatase activity in control and CPFA plasma membranes. The question emerges, therefore, as to how these peculiarities arise in the CPFA plasma membranes.

Plasma membranes have indeed been previously observed to form membrane vesicles as a result of isolation procedures (121). Such vesicles are formed by either the process of endocytosis or exocytosis. When plasma membranes form vesicles via exocytosis, contents of the cytoplasm are trapped within the vesicles. Similarly, cytoplasmic material is trapped within the lumen of microsomal vesicles (122). It is therefore possible that proteins common to both hepatic microsomes and

CPFA plasma membranes are actually cytoplasmic in origin. Of additional interest are the conditions under which plasma membranes form vesicles. It has long been known that plasma membranes isolated from a wide variety of cell types tend to form vesicles and small membrane fragments as the homogenizing force is increased (121). These morphological characteristics are an accurate description of CPFA plasma membranes, yet both control and CPFA plasma membranes were subjected to the same homogenization procedure. The possibility arises, therefore, that exposure to CPFA has resulted in structurally weakened plasma membranes. Consequently, the isolation procedure which is suitable for control membranes may be too harsh for the efficient isolation of CPFA plasma membranes as membrane sheets.

Although the presence of vesicles in CPFA plasma membranes indicates that the differences observed in electrophoretic patterns may be due to cytoplasmic proteins, the possibility remains that exposure to CPFA actually causes additional proteins to be incorporated into the plasma membrane. Such may well be the case for the 80,000 dalton family of glycoproteins observed in the CPFA Western blots. In addition to being identified in microsomal membranes, these glycoproteins remain associated with microsomes under conditions which remove the lumenal contents of vesicles (123). This data implies that these glycoproteins are intrinsic membrane proteins in nature. Furthermore, these glycoproteins have previously been shown to be sensitive to CPFA insult, since their presence in microsomal

membranes is altered by exposure to dietary methyl sterculate (123). Similarly, evidence exists that suggests that the 75,000 dalton family of glycoproteins may also be sensitive to CPFA exposure (123). The biological consequences of introducing these additional proteins into the plasma membrane are unknown, but such surface proteins have been shown to be important to the processes of intercellular adhesiveness, intercellular communication, control of cellular growth by contact inhibition and cellular antigenicity (121,124).

The differences between control and CPFA electrophoretic patterns in the low molecular weight region of two-dimensional gels is also of interest. The fewer number of low molecular weight proteins observed in CPFA gels may truly be an indication of a loss of plasma membrane proteins due to dietary CPFA. Such a protein loss would be expected to be accompanied by a loss of membrane function. However, the greater number of high molecular weight proteins associated with CPFA plasma membranes could be masking the presence of these low molecular weight proteins.

Figure 1. Electron micrographs reveal morphological differences between plasma membranes isolated from trout exposed to A) 0, B) 50, and C) 300 ppm dietary CPFA. Control plasma membranes consist mainly of membrane sheets (double arrows, 1A) while CPFA membranes contain more small membrane fragments and membrane vesicles (single arrows, 1C). The 5 micron marker is valid for all three micrographs.

FIGURE 1.

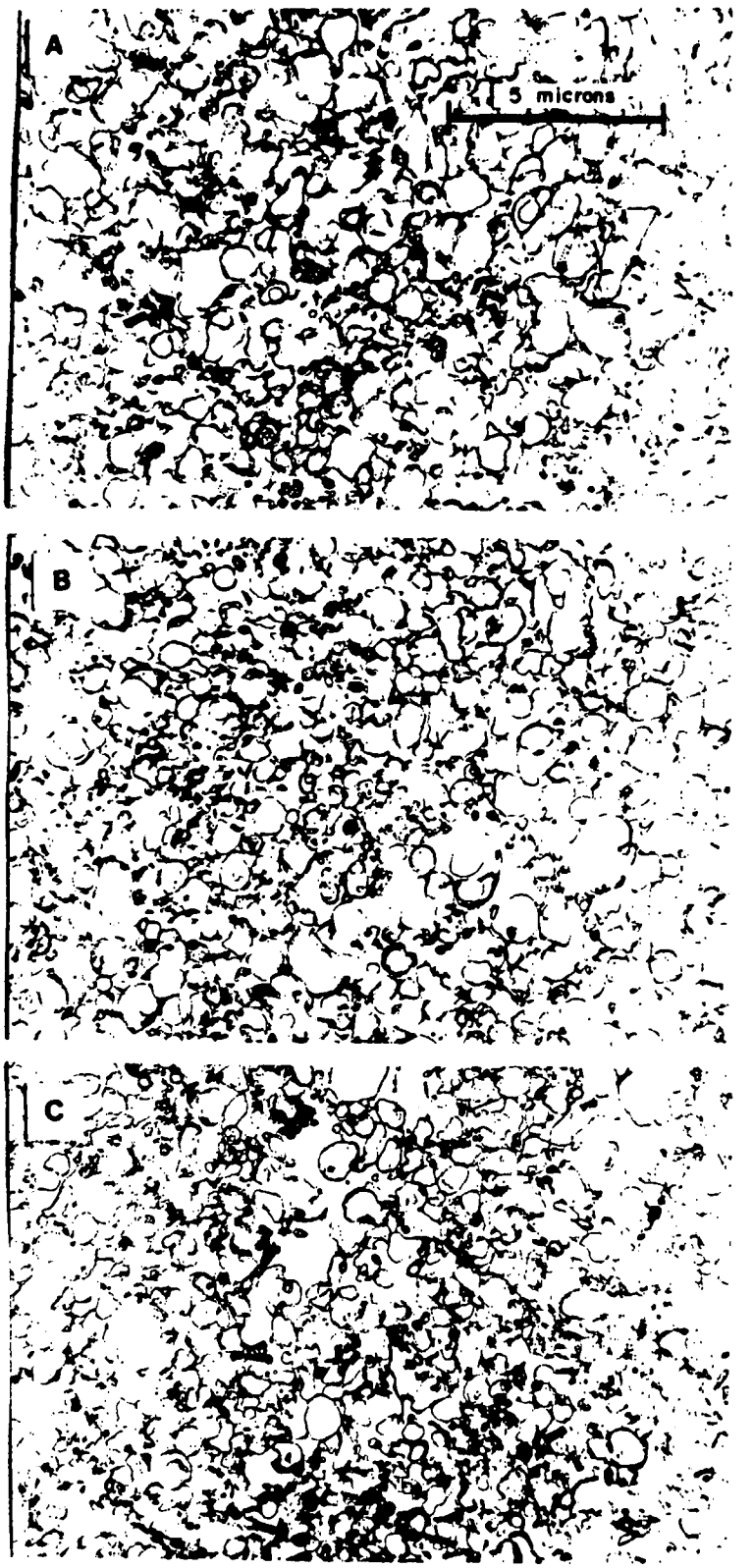


Figure 2. A graph generated from data from a typical succinate dehydrogenase assay depicts a change in optical density (600 nm) over time. In this instance, 0.5 mg of control whole liver homogenate was assayed to reduce 0.021 micromoles DCPIP per minute per mg protein.

FIGURE 2.

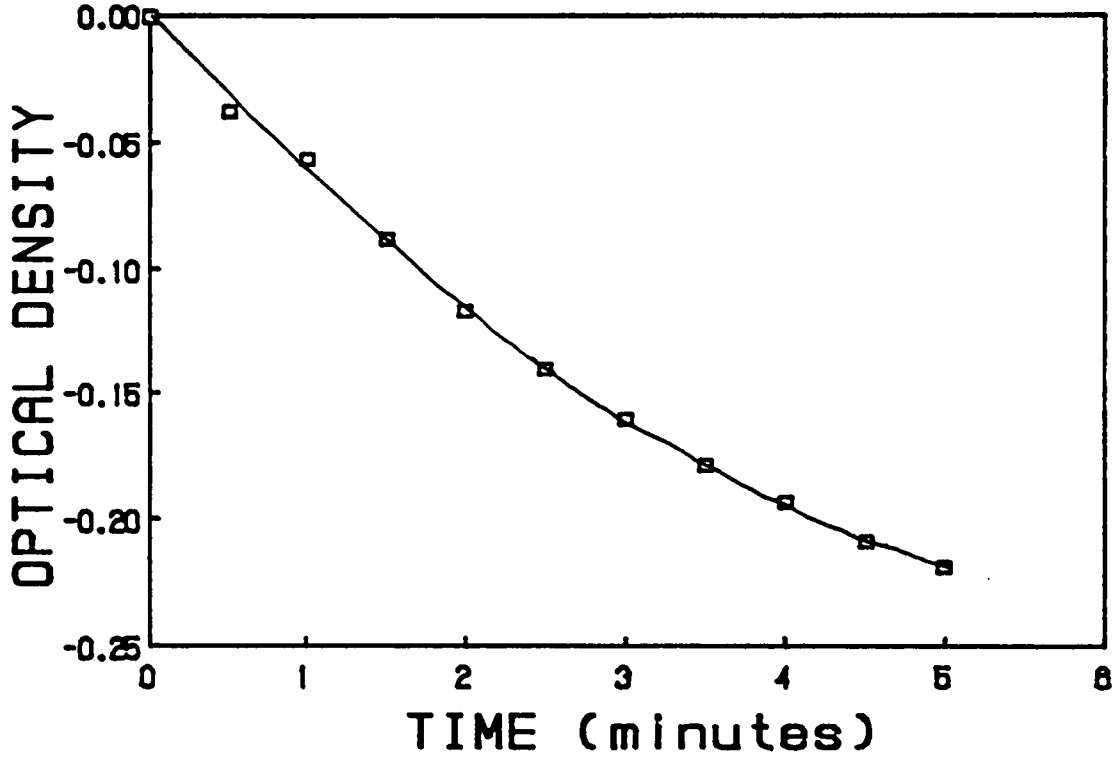

0.00
-0.05
-0.10
-0.15
-0.20
-0.25
OPTICAL DENSITY
0
1
2
3
4
5
6
TIME (minutes)

Figure 3. Two-dimensional gel electrophoresis reveals differences between the protein content of A) control and B) CPFA treated plasma membranes. High molecular weight proteins, present only in CPFA plasma membranes, include a family of 6 proteins of molecular weight 75 kilodaltons (kd, arrow) and a 180 kd family (rectangle). Control plasma membranes contain more proteins under 21 kd in molecular weight.

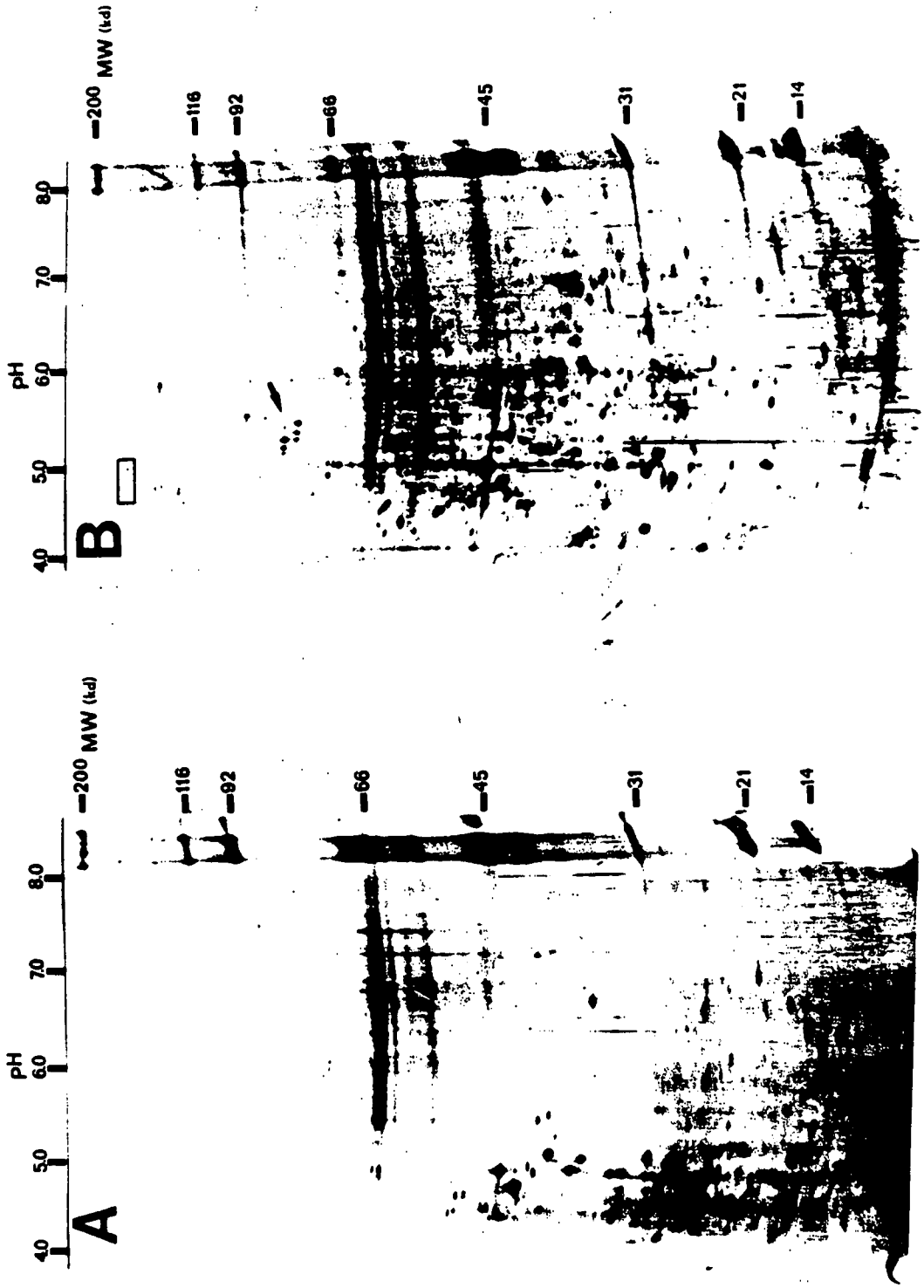



FIGURE 3.

Figure 4. Western blotting followed by concanavalin A-peroxidase staining indicates that plasma membranes isolated from A) control-fed and B) CPFA-fed trout differ in glycoprotein content. An 80 kd family of glycoproteins (circle) is present only in CPFA plasma membranes. The rectangle and arrow correspond to the same proteins highlighted in Figure 3.


FIGURE 4.

TABLE 1

ENZYME ANALYSIS OF WHOLE LIVER HOMOGENATES AND OF HEPATIC PLASMA MEMBRANES FROM RAINBOW TROUT FED 0 AND 300 PPM CYCLOPROPENOID FATTY ACID

| Enzyme Assay | Dietary CPFA | Homogenate Activity[a] | Plasma Membrane Activity[a] | Enrichment Value[b] |
|---|---|---|---|---|
| Glucose-6-Phosphatase | 0[c] | .0498 ± .0064* | .0728 ± .0287 | 1.4 |
| | 300[d] | .0344 ± .0028* | .0264 ± .0137 | 0.7 |
| 5'-Nucleotidase | 0[c] | .0147 ± .0044 | .586 ± .128 | 41 |
| | 300[d] | .0148 ± .0023 | .454 ± .107 | 31 |

a All activity values are expressed as micromoles of phosphate liberated per minute per milligram of protein (± standard deviation).

b Enrichment values are calculated as enzyme activity in plasma membranes divided by enzyme activity in whole liver homogenates.

c n = 4 assays

d n = 3 assays

* Values are significantly different at $p < .05$.

TABLE 2

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES FROM RAINBOW TROUT ON CONTROL, 50 PPM AND 300 PPM CYCLOPROPENOID FATTY ACID[a]

| | PERCENT OF TOTAL LIPID PHOSPHOROUS | | |
|---|---|---|---|
| PHOSPHOLIPID | CONTROL | 50 PPM | 300 PPM |
| Choline Phospholipids | 53.2 (51.6-54.9) | 54.9 (49.5-58.6) | 57.5 (54.1-60.4) |
| Ethanolamine Phospholipids | 17.7 (15.6-19.3) | 18.7 (17.8-20.4) | 16.8 (14.2-18.1) |
| Inositol Phospholipids + Sphinogomyelin | 16.5 (15.9-17.6) | 17.0 (15.9-19.2) | 16.7 (15.4-17.9) |
| Serine Phospholipids | 7.4 (6.5-9.2) | 6.0 (5.7-6.2) | 4.6 (4.1-5.1) |
| Phosphatidic Acid | 1.2 (trace-1.3) | trace (trace-1.9) | trace (trace-trace) |

[a] Plasma membranes were prepared from at least six fish livers. Results are from three separate preparations and the ranges of values are presented in parentheses.

TABLE 3

COMPOSITION OF CONSTITUENT FATTY ACIDS (w%) OF ETHANOLAMINE PHOSPHOLIPIDS FROM LIVER PLASMA MEMBRANES OF RAINBOW TROUT FED DIFFERENT LEVELS OF CYCLOPROPENOID FATTY ACID[a]

| | ETHANOLAMINE PHOSPHOLIPIDS | | |
|---|---|---|---|
| ACYL GROUPS | CONTROL | 50 PPM | 300 PPM |
| 14:0 | 0.94 ± 0.72 | 0.24 ± 0.19 | 0.60 ± 0.53 |
| 16:0 | 12.24 ± 1.38 | 9.30 ± 1.19 | 8.63 ± 0.26 |
| 16:1 | 3.86 ± 1.55 | 2.86 ± 1.53 | 2.77 ± 1.47 |
| 18:0 | 9.29 ± 0.87[c] | 13.72 ± 0.94[c] | 17.84 ± 1.02[c] |
| 18:1 n-9 | 28.07 ± 5.56 | 22.73 ± 2.26 | 19.74 ± 4.33 |
| 20:1 n-9 | 6.11 ± 1.10 | 5.80 ± 0.73 | 4.82 ± 1.08 |
| 20:5 n-3 | 4.47 ± 1.37 | 4.81 ± 0.97 | 4.70 ± 0.94 |
| 22:6 n-3 | 27.84 ± 4.09 | 36.23 ± 5.92 | 37.20 ± 4.40 |
| 18:0/18:1 | 0.33 | 0.60 | 0.90 |
| U.I.[b] | 227 | 273 | 274 |

[a] Acyl group values represent the mean (±sd) of duplicate analysis from at least three experiments. Plasma membranes were prepared from at least six livers pooled.

[b] U.I. = unsaturation index defined as (number of double bonds in each fatty acid) x (mol % of each fatty acid).

[c] Corresponding values are significantly different from control values ($p < .01$).

TABLE 4

COMPOSITION OF CONSTITUENT FATTY ACIDS (w%) OF CHOLINE PHOSPHOLIPIDS FROM LIVER PLASMA MEMBRANES OF RAINBOW TROUT FED DIFFERENT LEVELS OF CYCLOPROPENOID FATTY ACID[a]

| | CHOLINE PHOSPHOLIPIDS | | |
|---|---|---|---|
| ACYL GROUPS | CONTROL | 50 PPM | 300 PPM |
| 14:0 | 1.51 ± 0.40 | 1.32 ± 0.06 | 1.04 ± 0.42 |
| 16:0 | 32.01 ± 3.41 | 26.05 ± 2.59 | 24.30 ± 1.85 |
| 16:1 | 6.93 ± 0.68[c] | 4.06 ± 0.18[c] | 4.20 ± 0.25[c] |
| 18:0 | 5.52 ± 1.49[d] | 13.72 ± 0.18[d] | 15.57 ± 2.11[d] |
| 18:1 n-9 | 21.42 ± 3.27 | 15.91 ± 0.78 | 17.41 ± 2.55 |
| 20:1 n-9 | 2.74 ± 1.02 | 1.85 ± 0.14 | 1.51 ± 0.12 |
| 20:5 n-3 | 3.54 ± 0.31 | 4.48 ± 0.18 | 5.46 ± 0.88 |
| 22:6 n-3 | 22.71 ± 1.80 | 29.14 ± 3.31 | 26.52 ± 3.20 |
| 18:0/18:1 | 0.26 | 0.86 | 0.89 |
| U.I.[b] | 185 | 219 | 210 |

[a] Acyl group values represent the mean (±sd) of duplicate analysis from at least three experiments. Plasma membranes were prepared from at least six livers pooled.

[b] U.I. = unsaturation index defined as (number of double bonds in each fatty acid) x (mol % of each fatty acid).

[c,d] Corresponding values are significantly different from control values ($p < .01$).

## III. REFERENCES


1. Macfarland, J.J., Shenstone, F.S., and Vickery, J.R. 1957. Malvalic acid and its structure. Nature. 179:830-831.

2. Nunn, J.R. 1952. The structure of sterculic acid. J. Chem. Soc. 9:313-318.

3. Turner, R.B., Goebel, P., Mallon, B.J., Doering, W.E., Coburn, J.F., and Pomerantz, M. 1968. Heats of hydrogenation. VIII. Compounds with three- and four-membered rings. J. Am. Chem. Soc. 90:4315-4322.

4. Greenberg, A., and Harris, J. 1982. Cyclopropenoid fatty acids. J. Chem. Ed. 59:539-543.

5. Carter, F.L. and Frampton, V.L. 1964. Review of the chemistry of cyclopropene compounds. Chem. Rev. 64:497-525.

6. Christie, W.W. 1970. Cyclopropane and cyclopropene fatty acids. In: Topics in Lipid Chemistry, Vol. 1. Edited by F.D. Gunstone. pp. 1-49. Logos Press Ltd., London.

7. Faure, P.K. 1956. Sterculic acid and its Halphen reaction. Nature. 178:372-373.

8. Harris, J.A., Magne, F.C., and Skau, E.L. 1964. Methods for the determination of cyclopropenoid fatty acids. IV. Application of the step-wise HBr titration method to the analysis of refined and crude cottonseed oil. J. Am. Oil Chem. Soc. 41:309-311.

9. Bailey, A.V., Boudreaux, G.J., and Skau, E.L. 1965. Methods for the determination of cyclopropenoid fatty acids. VI. A direct infrared absorption method. J. Am. Oil Chem. Soc. 42:637-638.

10. Schneider, E.L., Sook, P.L., and Hopkins, D.T. 1968. Gas-liquid chromatographic analysis of cyclopropenoid fatty acids. J. Am. Oil Chem. Soc. 45:585-590.

11. Pawlowski, N.E., Nixon, J.E., and Sinnhuber, R.O. 1972. Assay of cyclopropenoid lipids by nuclear magnetic resonance. J. Am. Oil Chem. Soc. 49:387-392.

12. Loveland, P.M., Pawlowski, N.E., Libbey, L.M., Bailey, G.S., and Nixon, J.E. 1983. HPLC analysis of cyclopropenoid fatty acids. J. Am. Oil Chem. Soc. 60:1786-1788.

13. Altenburger, E.R., Berry, J.W., and Deutschman, A.J. 1970. Synthesis of methyl sterculate. J. Am. Oil Chem. Soc. 47:77-78.

14. Pawlowski, N.E., Lee, D.J., and Sinnhuber, R.O. 1972. Synthesis of 1,2-dialklcyclopropenes, methyl malvalate, and methyl sterculate. J. Am. Oil Chem. Soc. 37:3245-3248.

15. Pawlowski, N.E., Nixon, J.E., Lee, D.J., and Sinnhuber, R.O. 1973. Synthesis of C-14 labelled methyl sterculate. J. Labelled Comp. 10:45-48.

16. Shenstone, F.S., Vickery, J.R., and Johnson, A.R. 1965. Studies on the chemistry and biological effects of cyclopropenoid compounds. J. Agr. Food Chem. 13:410-414.

17. Kawase, Y., Niizuma, H., and Yasuda, K. 1968. Improvement of foaming tendency of kapok seed oil by catalytic hydrogenation. J. Am. OIl Chem. Soc. 45:852-854.

18. Phelps, R.A., Shenstone, F.S., Kemmerer, A.R., and Evans, R.J. 1965. A review of cyclopropenoid compounds: Biological effects of some derivatives. Poult. Sci. 44:358-394.

19. Mattson, F.H. 1973. Potential toxicity of food lipids. In: Toxicants Occurring Naturally in Foods, Second Edition. pp. 189-209. National Academy of Sciences, Washington, D.C.

20. Berry, S.K. 1980. Cyclopropene fatty acids in Gnetum gnemon (L.) seeds and leaves. J. Sci. Food Agric. 31:657-662.

21. Sherwood, R.M. 1928. The effect of various rations on the storage quality of eggs. Texas Agr. Expt. Sta. Bull. 376:12.

22. Lorenz, F.W., Almquist, H.J., and Hendry, G.W. 1933. Malvaceous plants as a cause of "pink white" in stored eggs. Science. 77:606.

23. Masson, J.C., Vavich, M.G., Heywang, B.W., and Kemmerer, A.R. 1957. Pink discoloration in eggs caused by sterculic acid. Science. 126:751.

24. Shenston, F.S., and Vickery, J.R. 1959. Substances in plants of the order malvale causing pink whites in stored eggs. Poult. Sci. 38:1055-1070.

25. Schaible, P.J., and Bandemer, S.L. 1946. Composition of fresh and storage eggs from hens fed cottonseed and non-cottonseed rations. III. Iron content. Poult. Sci. 25:451-452.

26. Schaible, P.J., and Bandemer, S.L. 1946. Composition of fresh and storage eggs from hens fed cottonseed and non-cottonseed rations. V. Cause of discoloration. Poult. Sci. 25:456-459.

27. Schneider, D.L., Kurnick, A.A., Vavich, M.G., and Kemmerer, A.R. 1962. Delay of sexual maturity in chickens by Sterculia foetida oil. J. Nutr. 77:403-407.

28. Kemmerer, A.R., Heywang, B.W., Vavich, M.G. and Sheehan, E.T. 1965. Effect of cottonseed oil on egg hatchability. Poult. Sci. 44:1315-1317.

29. Kemmerer, A.R., Heywang, B.W., and Lowe, B.W. 1967. Effect of cottonseed oil on egg production and egg quality. Poult. Sci. 46:1165-1167.

30. Schneider, D.L., Vavich, M.G., Kurnick, A.A., and Kemmerer, A.R. 1961. Effect of Sterculia foetida oil on mortality of the chick embryo. Poult. Sci. 40:1644-1648.

31. Donaldson, W.E., and Fites, B.L. 1970. Embryo mortality in quail induced by cyclopropene fatty acids: Reduction by maternal diets high in unsaturated fatty acids. J. Nutr. 100:605-610.

32. Schneider, D.L., Sheehan, E.T., Vavich, M.G., and Kemmerer, A.R. 1968. Effect of Sterculia foetida oil on weanling rat growth and survival. J. Agr. Food Chem. 16:1022-1024.

33. Miller, A.M., Sheehan, E.T., and Vavich, M.G. 1969. Prenatal and postnatal mortality of offspring of cyclopropenoid fatty acid-fed rats. Proc. Soc. Exp. Biol. Med. 131:61-66.

34. Nixon, J.E., Eisele, T.A., Hendricks, J.D., and Sinnhuber, R.O. 1977. Reproduction and lipid composition of rats fed cyclopropene fatty acids. J. Nutr. 107:574-583.

35. Nixon, J.E., Eisele, T.A., Wales, J.H., and Sinnhuber, R.O. 1974. Effect of subacute toxic levels of dietary cyclopropenoid fatty acids upon membrane function and fatty acid composition in the rat. Lipids. 9:314-321.

36. Scarpelli, D.G. 1975. Preliminary observations on the mitogenic effect of cyclopropenoid fatty acids on rat pancreas. Cancer Res. 35:2278-2283.

37. Scarpelli, D.G. 1974. Mitogenic activity of sterculic acid, a cyclopropenoid fatty acid. Science. 185:958-960.

38. Roehm, J.N., Lee, D.J., Sinnhuber, R.O., and Polityka, S.D. 1971. Deposition of cyclopropenoids in the tissue lipids of rainbow trout fed methyl sterculate. Lipids. 6:426-430.

39. Eisele, T.A., Parker, R.S., Yoss, J.K., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. 1979. Incorporation of label from [9,10-methylene-$^{14}$C] sterculic acid in rainbow trout, Salmo gairdneri. Lipids. 14:523-528.

40. Roehm, J.N., Lee, D.J., Wales, J.H., Polityka, S.D., and Sinnhuber, R.O. 1970. The effect of dietary sterculic acid on the hepatic lipids of rainbow trout. Lipids. 5:80-84.

41. Malevski, Y., Montgomery, M.W., and Sinnhuber, R.O. 1974. Liver fat and protein metabolism in rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. J. Fish. Res. Board Can. 31:1093-1100.

42. Struthers, B.J., Wales, J.H., Lee, D.J., and Sinnhuber, R.O. 1975. Liver composition and histology of rainbow trout fed cyclopropenoid fatty acids. Exp. Mol. Path. 23:164-170.

43. Sinnhuber, R.O., Wales, J.H., Ayres, J.L., Engebrecht, R.H., and Amend, D.L. 1968. Dietary

factors and hepatoma in rainbow trout (Salmo gairdneri). I. Aflatoxins in vegetable protein feedstuffs. J. Natl. Cancer Inst. 41:711-718.

44. Scarpelli, D.G., Lee, D.J., Sinnhuber, R.O., and Chiga, M. 1974. Cytoplasmic alterations of hepatocytes in rainbow trout (Salmo gairdneri) induced by cyclopropenoid fatty acids. Cancer Res. 34:2984-2990.

45. Malevski, Y., Wales, J.H., and Montgomery, M.W. 1974. Liver damage in rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. J. Fish. Res. Board Can. 31:1397-1400.

46. Selivonchick, D.P., Williams, J.L., and Schaup, H.W. 1981. Alteration of liver microsomal proteins from rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. Lipids. 16:211-213.

47. Eisele, T.A., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. 1978. Effects of dietary cyclopropene fatty acids on the mixed function oxidase system of the rainbow trout. J. Environ. Path. Toxicol. 1:773-778.

48. Eisele, T.A., Coulombe, R.A., Williams, J.L., Shelton, D.W., and Nixon, J.E. 1983. Time- and dose-dependent effects of dietary cyclopropenoid fatty acids on the hepatic microsomal mixed function oxidase system of rainbow trout. Aquatic Toxicology. 4:139-151.

49. Morrissey, M.T. 1983. The effects of cyclopropenoid fatty acids on structural components of microsomal membranes. Ph.D. Thesis. Oregon State University.

50. Perdew, G.H., Schaup, H.W., Becker, M.M., Williams, J.L., and Selivonchick, D.P. 1986. Alterations in the synthesis of proteins in hepatocytes of rainbow trout fed cyclopropenoid fatty acids. Biochim. Biophys. Acta. 877:9-19.

51. Taylor, S.L., Montgomery, M.W., and Lee, D.J. 1973. Liver dehydrogenase levels in rainbow trout, Salmo gairdneri, fed cyclopropenoid fatty acids and aflatoxin $B_1$. J. Lipid Res. 14:643-646.

52. Reiser, R., Stevenson, B., Kayama, M., Choudhury, R.B.R., and Hood, D.W. 1963. The influence of dietary fatty acids and environmental temperature on

the fatty acid composition of teleost fish. J. Am. Oil Chem. Soc. 40:507-513.

53. Ellis, N.R., Rothwell, C.S., and Pool, W.O. 1931. The effect of ingested cottonseed oil on the composition of body fat. J. Biol. Chem. 92:385-398.

54. Cook, L.J., Scott, T.W., Mills, S.C., Fogerty, A.C., and Johnson, A.R. 1976. Effects of protected cyclopropene fatty acids on the composition of ruminant milk fat. Lipids. 11:705-711.

55. Tinsley, I.J., Wilson, G., and Lowry, R.R. 1982. Tissue fatty acid changes and tumor incidence in C3H mice ingesting cottonseed oil. Lipids. 17:115-117.

56. Raju, P.K., and Reiser, R. 1973. Hepatic stearoyl-CoA desaturase activity in mice as affected by early postnatal dietary cyclopropene fatty acids. J. Nutr. 103:904-907.

57. Reiser, R., and Raju, P.K. 1964. The inhibition of saturated fatty acid dehydrogenation by dietary fat containing sterculic and malvalic acids. Biochem. Biophys. Res. Commun. 17:8-11.

58. Raju, P.K., and Reiser, R. 1967. Inhibition of fatty acyl desaturase by cyclopropene fatty acids. J. Biol Chem. 242:379-384.

59. Evans, R.J., Bandemer, S.L., Anderson, M., and Davidson, J.A. 1962. Fatty acid distribution in tissues from hens fed cottonseed oil or Sterculia foetida seeds. J. Nutr. 76:314-319.

60. Abou-Ashour, A.M., and Edwards, H.M. 1970. Effect of dietary Sterculia foetida oil on pink-white discoloration and fatty acid distribution in stored eggs. J. Nutr. 100:757-766.

61. Johnson, A.R., Pearson, J.A., Shenstone, F.S., and Fogerty, A.C. 1967. Inhibition of the desaturation of stearic to oleic acid by cyclopropene fatty acids. Nature. 214:1244-1245.

62. Allen, E., Johnson, A.R., Fogerty, A.C., Pearson, J.A., and Shenstone, F.S. 1967. Inhibition by cyclopropene fatty acids on the desaturation of stearic acid in hen livers. Lipids. 2:419-423.

63. James, A.T., Harris, P., and Bezard, J. 1968. The inhibition of unsaturated fatty acid biosynthesis in plants by sterculic acid. Eur. J. Biochem. 3:318-325.

64. Einerson, M.A., Schaup, H.W., and Selivonchick, D.P. 1983. Effect of cyclopropenoid fatty acids on membrane components of liver from rainbow trout (Salmo gairdneri). J. Am. Oil Chem. Soc. 60:746.

65. Struthers, B.J., Lee, D.J., and Sinnhuber, R.O. 1975. Altered lipid metabolism in livers of rainbow trout fed cyclopropenoid fatty acids. Exp. Mol. Path. 23:181-187.

66. Nixon, J.E., Yoss, J.K., Eisele, T.A., Pawlowski, N.E., and Sinnhuber, R.O. 1977. Metabolism and tissue distribution of label from [9,10-methylene-$^{14}$C] sterculic acid in the rat. Lipids. 12:629-634.

67. Jeffcoat, R., and Pollard, M.R. 1977. Studies on the inhibition of the desaturases by cyclopropenoid fatty acids. Lipids. 12:480-485.

68. Evans, R.J., Davidson, J.A., and Bandemer, S.L. 1961. Fatty acid and lipide distribution in egg yolks from hens fed cottonseed oil or Sterculia foetida seeds. J. Nutr. 73:282-290.

69. Johnson, A.R., Fogerty, A.C., Pearson, J.A., Shenstone, F.S., and Bersten, A.M. 1969. Fatty acid desaturase systems of hen liver and their inhibition by cyclopropene fatty acids. Lipids. 4:265-269.

70. Fogerty, A.C., Johnson, A.R., and Pearson, J.A. 1972. Ring position in cyclopropene fatty acids and stearic acid desaturation in hen liver. Lipids. 7:335-338.

71. Tennent, D.M., Zanetti, M.E., Siegel, H., Gunther, K.W., and Ott, W.H. 1959. The influence of selected vegetable fats on plasma lipid concentrations and aortic atheromatosis in cholesterol-fed and diethylstilbestrol implanted cockerels. J. Nutr. 69:283-288.

72. Goodnight, K.C. Jr., and Kemmerer, A.R. 1967. Influence of cyclopropenoid fatty acids on the cholesterol metabolism of cockerels. J. Nutr. 91:174-178.

73. Ferguson, T.L., Wales, J.H., Sinnhuber, R.O., and Lee, D.J. 1976. Cholesterol levels, atherosclerosis and liver morphology in rabbits fed cyclopropenoid fatty acids. Fd. Cosmet. Toxicol. 14:15-18.

74. Matlock, J.P. 1985. Altered lipid metabolism and impaired clearance of cholesterol in plasma of Swiss-Webster mice fed cyclopropenoid fatty acids. Ph.D. Thesis. Oregon State University.

75. Yoss, J.K., Nixon, J.E., Pawlowski, N.E., Lee, D.J., and Sinnhuber, R.O. 1974. Metabolism of $^{14}C$-sterculic acid in the rat. Fed. Proc. 33:248.

76. Almquist, H.J., Lorenz, F.W., and Burmester, B.R. 1934. Relation of depot fat to egg yolk fat in laying hens. J. Biol. Chem. 106:365-371.

77. Altenburger, E.R., Berry, J.W., Reid, B.L., and Deutschman, A.J. 1971. Study of carbon-14 labelled sterculic acid in chickens. Poult. Sci. 50:646-648.

78. Lorenz, F.W, Almquist, H.J., and Hendry, G.W. 1933. Malvaceous plants as a cause of "pink white" in stored eggs. Science. 77:606.

79. Eisele, T.A, Yoss, J.K., Nixon, J.E., Pawlowski, N.E., Libbey, L.M., and Sinnhuber, R.O. 1977. Rat urinary metabolites of [9,10-methylene-$^{14}C$] sterculic acid. Biochim. Biophys. Acta. 488:76-87.

80. Wood, R., and Reiser, R. 1965. Cyclopropane fatty acid metabolism: Physical and chemical identification of propane ring metabolic products in the adipose tissue. J. Am. Oil Chem. Soc. 42:315-320.

81. Chung, A.E. 1966. Metabolism of cyclopropane fatty acids oxidation of cis-9,10,-methylene hexadecanoic and cis-9,10-methylene octadecanoic acids by rat liver mitochondria. Biochim. Biophys. Acta. 116:205-213.

82. Sinnhuber, R.O., Wales, J.H., and Lee, D.J. 1966. Cyclopropenoids, co-carcinogens for aflatoxin-induced hepatoma in trout. Fed. Proc. 25:555.

83. Sinnhuber, R.O., Lee, D.J., Wales, J.H., and Ayres, J.L. 1968. Dietary factors and hepatoma in rainbow

trout (Salmo gairdneri). II. Cocarcinogenesis by cyclopropenoid fatty acids and the effect of gossypol and altered lipids on aflatoxin-induced liver cancer. J. Nat. Cancer Inst. 41:1293-1301.

84. Lee, D.J., Wales, J.H., Ayres, J.L., and Sinnhuber, R.O. 1968. Synergism between cyclopropenoid fatty acids and chemical carcinogens in rainbow trout (Salmo gairdneri). Cancer Res. 28:2312-2318.

85. Lee, D.J., Wales, J.H., and Sinnhuber, R.O. 1971. Promotion of aflatoxin-induced hepatoma growth in trout by methyl malvalate and sterculate. Cancer Res. 31:960-963.

86. Sinnhuber, R.O., Lee, D.J., Wales, J.H., Landers, M.K., and Keyl, A.C. 1974. Hepatic carcinogenesis of aflatoxin $M_1$ in rainbow trout (Salmo gairdneri) and its enhancement by cyclopropenoid fatty acids. J. Natl. Cancer Inst. 53:1285-1288.

87. Hendricks, J.D., Sinnhuber, R.O., Nixon, J.E., Wales, J.H., Putnam, G.B., Loveland, P.M., Masri, M.S., and Hsieh, D.P.H. 1978. Carcinogenicity of aflatoxin $Q_1$ to rainbow trout and its potentiation by cyclopropene fatty acids. Fed. Proc. 37:451.

88. Hendricks, J.D., Sinnhuber, R.O., Nixon, J.E., Wales, J.H., Masri, M.S., and Hsieh, D.P.H. 1980. Carcinogenic response of rainbow trout (Salmo gairdneri) to aflatoxin $Q_1$ and synergistic effect of cyclopropenoid fatty acids. J. Natl. Cancer Inst. 64:523-527.

89. Schoenhard, G.L., Hendricks, J.D., Nixon, J.E., Lee, D.J., Wales, J.H., Sinnhuber, R.O., and Pawlowski, N.E. 1981. Aflatoxicol-induced hepatocellular carcinoma in rainbow trout (Salmo gairdneri) and the synergistic effects of cyclopropenoid fatty acids. Cancer Res. 41:1011-1014.

90. Hendricks, J.D. 1982. Chemical carcinogenesis in fish. In: Aquatic Toxicology. Edited by L.J. Weber. pp. 149-211. Raven Press, NY.

91. Sinnhuber, R.O., Hendricks, J.D., Putnam, G.B., Wales, J.H., Pawlowski, N.E., Nixon, J.E., and Lee, D.J. 1976. Sterculic acid, a naturally occurring cyclopropene fatty acid, a liver carcinogen to rainbow trout, Salmo gairdneri. Fed. Proc. 35:505.

92. Hendricks, J.D., Sinnhuber, R.O., Loveland, P.M., Pawlowski, N.E., and Nixon, J.E. 1980. Hepatocarcinogenicity of glandless cottonseeds and cottonseed oil to rainbow trout (Salmo gairdneri). Science. 208:309-311.

93. Lee, D.J., Wales, J.H., and Sinnhuber, R.O. 1969. Hepatoma and renal tubule adenoma in rats fed aflatoxin and cyclopropenoid fatty acids. J. Natl. Cancer Inst. 43:1037-1044.

94. Nixon, J.E., Sinnhuber, R.O., Lee, D.J., Landers, M.K., and Harr, J.R. 1974. Effect of cyclopropenoid compounds on the carcinogenic activity of diethylnitrosamine and aflatoxin $B_1$ in rats. J. Natl. Cancer Inst. 53:453-458.

95. Carroll, K.K., and Khor, H.T. 1971. Effects of level and type of dietary fat on incidence of mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz(a)anthracene. Lipids. 6:415-420.

96. Swenson, D.H., Lin, J.K., Miller, E.C., and Miller, J.A. 1977. Aflatoxin $B_1$-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins $B_1$ and $B_2$ to rat liver DNA and ribosomal RNA in vivo. Cancer Res. 37:172-181.

97. Voss, S.D., Shelton, D.W., and Hendricks, J.D. 1982. Effects of dietary Aroclor 1254 and cyclopropene fatty acids on hepatic enzymes in rainbow trout. Arch. Contam. Toxicol. 11:87-91.

98. Williams, D.E., and Buhler, D.R. 1983. Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin $B_1$ to aflatoxin $B_1$-2,3-epoxide. Cancer Res. 43:4752-4756.

99. Loveland, P.M., Nixon, J.E., Pawlowski, N.E., Eisele, T.A., Libbey, L.M., and Sinnhuber, R.O. 1979. Aflatoxin $B_1$ and aflatoxicol metabolism in rainbow trout (Salmo gairdneri) and the effects of dietary cyclopropene. J. Environm. Pathol. Toxicol. 2:707-718.

100. Bailey, G.S., Taylor, M.J., Loveland, P.M., Wilcox, J.S., Sinnhuber, R.O., and Selivonchick, D.P. 1984. Dietary modification of aflatoxin $B_1$ carcinogenesis: mechanism studies with isolated hepatocytes from rainbow trout. In: Use of Small Fish Species in

Carcinogenicity Testing. Edited by K.L. Hoover. pp. 379-385. NIH publication No. 84-2653.

101. Pardee, A.B. 1971. The surface membrane as a regulator of animal cell division. In Vitro. 7:95-104.

102. Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., and Putnam, G.B. 1977. Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. Ann. N.Y. Acad. Sci. 298:389-408.

103. Lutz, F. 1973. Isolation and some characteristics of liver plasma membranes from rainbow trout. Comp. Biochem. Physiol. 45B:805-811.

104. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

105. Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Research. 26:31-43.

106. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biology. 17:208-213.

107. Khouw, B.T., and McCurdy, H.D. 1969. Tricarboxylic acid cycle enzymes and morphogenesis in Blastocladiella emersonii. J. Bacteriology. 99:197-205.

108. Swanson, M.A. 1955. Glucose-6-phosphatase from liver. Methods in Enzymology II. Pp. 541-543.

109. Michell, R.H., and Hawthorne, J.N. 1965. The site of diphosphoinositide synthesis in rat liver. Biochem. Biophys. Res. Commun. 21:333-338.

110. Fiske, C.H., and Subbarow, Y. 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66:375-400.

111. Bligh, E.G., and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. and Physiol. 37:911-917.

112. Rouser, G., Fleischer, S., and Yamamoto, A. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. Lipids. 5(5):494-496.

113. Bartlett, G.R. 1959. Phosphorous assay in column chromatography. J. Biol. Chem. 234:466-468.

114. Selivonchick, D.P., and Roots, B.I. 1977. Lipid and fatty acyl composition of rat brain capillary endothelia isolated by a new technique. Lipids. 12(2):165-169.

115. Perdew, G.H., Schaup, H.W., Selivonchick, D.P. 1983. The use of zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. Anal. Biochem. 135:453-455.

116. O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

117. Broglie, R.M., Hunter, C.N., Delepelaire, P., Neiderman, R.A., Chua, N-H., and Clayton, R.K. 1980. Isolation and characterization of the pigment-protein complexes of Rhodopseudomonas sphaeroides by lithium dodecyl sulfate/polyacrylamide gel electrophoresis. Proc. Nat. Acad. Sci. USA. 77:87-91.

118. Wray, W., Boulikas, T., Wray, V.P., and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118:197-203.

119. Clegg, J.C.S. 1982. Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to Arenavirus and Flavivirus proteins. Anal. Biochem. 127:389-394.

120. Plaa, G.L. 1986. Toxic responses in the liver. In: Toxicology, the Basic Science and Poisons. Third Edition. Edited by C.D. Klaassen, M.O. Amdur, and J. Doul. pp. 286-309. MacMillan Publishing Company, New York, NY.

121. Steck, T.L., and Wallach, D.F.H. 1970. The isolation of plasma membranes. In: Methods in Cancer Research. Volume V. Edited by H. Busch. pp. 93-153. Academic Press, New York, NY.

122. Perdew, G.H. 1985. Alteration in levels and synthesis of proteins in trout hepatocytes due to dietary cyclopropenoid fatty acid. Ph.D. Thesis. Oregon State University.

123. Perdew, G.H., Schaup, H.W., Williams, D.E., Buhler, D.R., and Selivonchick, D.P. 1988. Alterations in hepatic microsomal protein levels in rainbow trout fed cyclopropenoid fatty acids analyzed by two-dimensional gel electrophoresis. Can. Biochem. Cell Biol. 66:138-143.

124. Reithmeier, R.A.F. 1985. Assembly of proteins into membranes. In: Biochemistry of Lipids and Membranes. Edited by D.E. Vance and J.E. Vance. pp. 503-558. Benjamin/Cummings Publishing Company, Menlo Park, CA.