AN ABSTRACT OF THE THESIS OF

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in Food Science and Technology presented on 4 December 1978

Title: COMPARATIVE EFFECTS OF STERCULIC AND MALVALIC ACIDS ON THE HEPATIC MICROSOMAL CYTOCHROME P-450 SYSTEM OF RAINBOW TROUT *(Salmo Gairdneri)*

Abstract approved: _____
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Cyclopropenoid fatty acids (CPFA) are structurally unique compounds found in many seed oils, including cottonseed and kapok which are used as food sources. Their demonstrations of carcinogenic and co-carcinogenic effects as well as other physiological disorders have led to research designed to elucidate their biological and chemical activities. Included in this quest in information regarding CPFA effects on microsomal cytochrome P-450 drug-metabolizing enzyme systems, the induction or inhibition of which affects the metabolism of many endogenous and exogenous substances.

Malvalic and sterculic acids are the two major cyclopropenoid fatty acids. For the first time, they have been isolated and assessed in separate feeding trials. Rainbow trout *(Salmo gairdneri)* fed semipurified diets of 40, 50, 60 or 70% protein were subjected to 20-day feeding trials of 50 ppm or 300 ppm methyl sterculate or methy malvalate. On day 21, microsomal fractions of liver homogenates were prepared and analyzed for indicators of microsomal enzyme activity: cytochrome P-450 and cytochrome b_5 content, cytochrome c reductase activity, aromatic hydrocarbon hydroxylase activity, and microsomal protein content.

Cytochrome P-450 content was significantly lower in sterculate samples than in control or malvalate samples. Cytochrome c reductase activity decreased significantly at all levels of malvalate and sterculate. Microsomal protein content was relatively constant at all dietary variations. No significant differences were observed for cytochrome b_5 content or aromatic hydrocarbon hydroxylase activity; standard deviation for these values were large and no conclusions were drawn. Level of dietary protein showed few significant effects on the parameters measured, although trout on control diets and 300 ppm sterculate diets tended to have greater P-450 content and cytochrome c reductase activity at 40 and 50% protein than at 60 and 70% protein.

Comparative Effects of Sterculic and Malvalic Acids on the Hepatic Microsomal Cytochrome P-450 System of Rainbow Trout *(Salmo Gairdneri)*

by

Marcia Lynn Bailey

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

June 1979

APPROVED:

Professor of Food Science and Technology
in Charge of Major

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Date thesis is presented 4 December 1978

Typed by the researched for Marcia Lynn Bailey

ACKNOWLEDGEMENTS

I wish to thank Dr. Joseph Nixon for the advice, assistance and patience which he extended throughout this project. Thanks also go to Dr. Jerry Hendricks for his valuable assistance and to Ted Will, John Casteel and Mark Witham for their help with fish handling. Special gratitude is expressed to Dr. Norman Pawlowski for his constant support and for sharing his research perspectives with me.

Public Health Service Grants CA 25766 and ES 01926 partially supported this investigation.

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INTRODUCTION

Since 1928 the cause of certain physiological disorders in farm animals has been attributed to cyclopropenoid fatty acids (CPFA). These compounds, unique in structure with a three-membered unsaturated ring in the middle of a fatty acid chain, are present in the seed oils of cotton and other plants of the order Malvales. Sterculic and malvalic acids, the major cyclopropenoid fatty acids, are present in small quantities in two major food oils.

In 1966 CPFA were discovered to be co-carcinogens when fed with aflatoxin B_1 to rainbow trout. Since that time, extensive research has been carried out with the findings that CPFA are co-carcinogens with certain other chemicals, and that they are carcinogens in their own right. Other effects noted include reproductive abnormalities and alterations of lipid metabolism.

Several species of animals and plants exhibit inhibition of fatty acid desaturation due to CPFA, with a resulting increase in the ratio of saturated to unsaturated fatty acids in tissue lipids The hepatic drug-metabolizing, or mixed function oxidase system has a lipid requirement and is found in the lipid-rich membranes

of endoplasmic reticulum. It is a well-supported theory that lipid alterations have direct effects on membrane composition and functionality. There may be a crucial relationship between the altered lipid composition caused by CPFA and the activation or inhibition of metabolism of drugs, chemicals and carcinogens. For this reason, the hepatic microsomal cytochrome P-450 system, the site of metabolism of many endogenous and exogenous substances, has been examined. Preliminary studies in the laboratory have indicated that the microsomal enzyme system of trout is depressed by dietary CPFA. The study reported here was undertaken to expand on these findings and to delineate the different effects, if any, of the two CPFA, malvalic and sterculic acids. In addition, dietary protein levels, which have been shown to influence tumor incidence and microsomal enzyme activity, were varied in order to study a possible secondary influence on the physiological implications of cyclopropenoid fatty acids.

REVIEW OF LITERATURE

Occurrence of Cyclopropenoid Fatty Acids

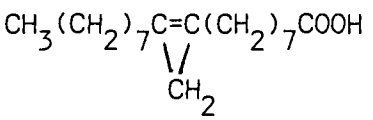
The presence of a highly strained, 1,2-disubstitued ring in the center of the long chain fatty acid makes cyclopropenoid fatty acids (CPFA) unique. They are of additional interest in light of the fact that two CPFA, malvalic and sterculic acids, are present in some common food sources. Seed lipids from many plants of the order Malvales contain CPFA. Lists of species containing CPFA have been compiled by Shenstone et al. (1959), Phelps et al. (1965) and Christie (1970): of these, cotton *(Gossypium hirsutum)* and kapok *(Eriodendron anfractuosum)* provide important food oils. Cottonseed oil, popular in the United States, is used in cooking oils, salad oils, shortenings and margarines. Cottonseed flour and meal contain small amounts of CPFA and are used as a protein source and in animal feed, respectively. In addition, cottonseed "nuts" were recently approved in the U.S. for promotion and consumption as a snack item and for use in baked products. Crude cottonseed oil has been reported to contain from 0.4- 0.8% sterculic acid and from 1.1- 1.6% malvalic acids (Christie, 1970). Kapok oil is used extensively as a food oil in many Eastern countried and contains as much as 14% CPFA (Kawase et al., 1968). *Sterculic foetida* (Java olive) oil contains 45- 54% sterculic acid and 4- 10% malvalic acid; *Hibiscus syriacus* oil contains 2.2- 3.4%

sterculic acid and 13.6- 16.3% malvalic acid (Shenstone and Vickery, 1961; Christie, 1970). These seed oils are used for experimental purposed in determining the chemical and biological properties of CPFA.

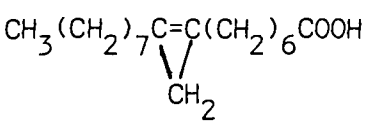
Structures and Assays

The chemical structures of malvalic and sterculic acids are shown below. They differ by only one carbon in their fatty acid chains and have nearly identical physical and chemical properties.

sterculic acid



malvalic acid



The structure of sterculic acids was elucidated by Nunn in 1952; in 1957, MacFarlane et al. reported the structure of malvalate. Cyclopropenes are highly reactive, having a ring strain energy of 53 kcal/mole (Turner et al., 1974). Their stability is increased with increased substitution (Srinivasan, 1971). Carter and Frampton (1968) and Christie (1970) have reviewed the chemistry of cyclopropene compounds.

Methods for cyclopropene assays have been reviewed by Coleman (1970). The Halphen test (Halphen, 1897) is the oldest and most frequently used assay. It is based on an unidentified pigment reaction specific for a substituted cyclopropene ring structure (Brooke and Smith, 1957). This test is quick and simple but is not precise, a standard is necessary, and background in natural oils is usually quite high. Hydrogen halide titration of CPFA, originated by Smith (1960), is not as sensitive as the Halphen test and, like the Halphen, does not distinguish specific acids.

Gas-liquid chromatography is difficult because high column temperatures destroy the cyclopropene ring, forming several rearrangement products. Attempts to modify the structure for GLC analysis have been made (Shenstone and Vickery, 1965; Raju and Reiser, 1966), the most successful of which was that reported by Schneider et al. (1968) in which CPFA are reacted with silver nitrate in methanol to form derivatives which are stable on the gas chromatograph.

Pawlowski et al. (1972) have utilized nuclear magnetic resonance spectroscopy for CPFA assay. This method is quick, simple, nondestructive, direct and allows the operator to detect impurities. Samples can be as small as 5 mg but because of background must have reater than 1% CPFA content. Unfortunately this method involves considerable instrumentation expense.

High pressure liquid chromatography has recently been used successfully for determining the amount of CPFA in relatively pure samples with high CPFA content (Loveland, 1978). This procedure is highly accurate and differentiates between malvalic and sterculic acids.

Physiological Effects

The physiological effects on humans of cyclopropene-containing compounds are not known, but many manifestations of their biological activity have been observed in animals. As early as 1930, observations of pink discoloration in egg whites from hens fed cottonseed meal were reported. This pink pigment, determined later to be a iron-conalbumin complex (Schaible and Bandemer, 1946), was reported in 1933 by Lorenzy et al. to be caused by a Halphen-reactive substance present in cottonseed meal. Confirmation that this phenomenon was due to the presence of cyclopropenoid fatty acids was made (Masson et al., 1957; Nordby et al, 1962), and it is commonly believed that structural changes in the egg's vitelline membrane caused by CPFA were responsible for increased permeability to iron, which was then able to pass from the yolk to the white (Schaible and Bandemer, 1946; Evans et al., 1962).

Other effects from CPFA have been reviewed by Phelps et al. (1965) and include reduced egg hatchability, delayed sexual maturity in young chickens and rats, and increased prenatal and postnatal mortality of offspring of rats fed 1- 2% *Sterculia foetida* oil. Schneider et al. (1968) found CPFA from *Sterculia foetida* oil to be toxic to young male rats at dietary levels of .35%; these workers also noted fetal liver damage, particularly fatty infiltration and necrosis, widespread hemorrhaging, and renal tubule and glomeruli degeneration. Nixon et al. (1974) fed 2% *Sterculia foetida* oil to rats and reported observations of retarded growth, elevated organ to

body weight ratios, increased saturation of tissue lipid and alteration of certain membrane functions. In addition, high serum cholesterol in response to CPFA has been reported in chickens (Goodnight and Kemmerer, 1967), New Zealand rabbits (Ferguson, 1974) and mice (Nixon, 1976).

Reports were made as early as 1916 (Eckles and Palmer, 1916) that butter from cows fed cottonseed meal was of a sticky consistency and had a high melting point. Higher melting fats in response to cottonseed meal rations were subsequently reported for egg yolks (Sherwood, 1928) and carcass fat in pigs (Ellis et al., 1931), and attributed to higher stearic acid content. Evans et al. (1962) reported increased stearate: oleate ratios in plasma, liver, ovaries, depot lipid and egg yolks from hens fed cottonseed oil or *Sterculia foetida* seeds. Roehm et al. (1968) and Roehm et al. (1970) fed *Sterculia foetida* oil to trout and found both stearate: oleate and palmitate: palmitoleate ratios to be increased. In 1964, Raju and Reiser noted inhibition of desaturation of dietary stearate to oleate in rats fed *Sterculia foetida* oil. This inhibition did not occur when ^{14}C- acetate was used as a precursor instead of ^{14}C- stearate, and this held true for all species tested, including chicks (Donaldson, 1967), tobacco leaf discs and *Chlorella vulgaris* (James et al., 1968), egg yolk lipids (Pearson et al., 1972) and trout (Roehm et al., 1968; Roehm et al., 1970). Kircher (1964) suggested that the biological activity of CPFA, particularly desaturase inhibition, is due to a reaction with sulfhydryl groups on proteins. Raju and Reiser (1974)

further postulated that CPFA bind to a sulfhydryl group on the desaturase enzyme itself. This theory, however, has not been substantiated and is highly controversial.

Outbreaks of liver cancer among hatchery trout fed aflatoxin-contaminated cottonseed meal led to research in which Sinnhuber et al. (1968) found CPFA to be potent co-carcinogens when fed with aflatoxin B_1 to rainbow trout. Co-carcinogenic effects were observed at levels of 20 ppm (Lee et al., 1971). In addition, Sinnhuber et al. (1976) and Hendricks (1978) have reported carcinogenic effects as well, at levels as low as 15 ppm, based on sterculic acid content of *Sterculia foetida* oil. Histological observations made by these researchers inlcude hepatocyte swelling and necrosis, pale, blotchy livers, hyperplasia of epithelia and connective tissue of bile ducts and blood vessels, and hypertrophied, hyperchromatic nuclei and cytoplasmic striations of parenchymal cells (Malevski et al, 1974a; Struthers et al., 1975a). Malevski et al. (1974b) reported a decrease in protein synthesis in trout fed 0.5 mg CPFA/kg body weight/day. After only a few days on a CPFA diets, Struthers et al. (1975b) observed increased liver lipids and reduced conversion of ^{14}C- oleic acids to $^{14}CO_2$. Decreased lipid synthesis from ^{14}C- acetate in trout fed CPFA was reported by Taylor et al. (1973) and Malevski et al.)1974b).

Different physiological effects have been reported for sterculic and malvalic acids, but this information is not based on effects of isolated CPFA. Because *Sterculia foetida* oil has a higher percentage

of sterculic acid, it has been considered to be an indicator of sterculate activity, whereas *Hibiscus syriacus* oil is considered an indicator of malvalate activity. Both oils, however, contain both malvalic and sterculic acids. The method used to purify CPFA from these oils, described by Kircher (1964), is a urea adduct crystallization and does not separate the two CPFA from each other. Lee et al. (1971), using this method, reported aflatoxin-induced hepatoma growth in trout to be significantly increased by feeding methyl sterculate, while no tumor promotion was seen with methyl malvalate. Johnson et al. (1969) noted greater inhibition of fatty acid desaturation in hen livers with sterculate than with malvalate. Later, Lee et al. (1972) conducted feeding trials of aflatoxin B_1 with methyl sterculate, methyl malvalate or one of several synthesized cyclopropenoid compounds. Trout fed methyl sterculate produced significantly more hepatomas, indicating the probability of a specific requirement for the sterculate molecule.

Cytochrome P-450 System (Mixed Function Oxidase)

The hepatic mixed function oxidase (MFO) system is located in the membranes of endoplasmic reticulum in intact animals; it is isolated in the centrifugation-created microsomal fraction of liver homogenates. It requires NADPH and O_2 and consists of the following basic components:

1) Cytochrome P-450, a hemeprotein, named for its maximum absorption at 450 nm; it is the substrate and oxygen

binding site;

2) NADPH cytochrome c reductase, a flavoprotein which transports electrons from NADPH to cytochrome P-450;
3) Cytochrome b_5, possibly a second electron donor to cytochrome P-450 and incolved in some but not all microsomal NADPH-dependent reactions; and
4) Phosphatidlycholine, the active component of the lipid fraction, essential for electron transfer from NADPH to cytochrome P-450.

Together these components function as an electron transport system responsible for biotransformation reactions, particularly oxidations, reductions, hydrolyses and conjugations (Gillette, 1963). Substrates of this system include xenobiotics such as drugs and environmental contaminants, and some endogenous substances such as steroids, bile and fatty acids (Lu, 1976). The *in vivo* duration and degree of biological activity of these substrates would be altered by a change in the rates of their biotransformation; such a change could be caused by inhibition or induction of the microsomal enzymes by many possible factors, such as current or prior exposure to other MFO substrates. Other factors that can alter MFO activity include age, nutrition, species and stress (Hopkins and West, 1976; Wad and Norred, 1972).

In vitro xenobiotic metabolism by the MFO system of fish has been well documented and described (Adamson, 1967; Dewaid and Henderson, 1968; Schoenhard, 1974; Malins, 1977; Bend et al.,

1977). Ahokas et al. (1977) have published an extensive summary of metabolic biotransformation reactions in fish. The MFO system of fish is similar to that of mammals, requiring oxygen, NADPH and cytochrome P-450 (Bend et al., 1974; Papahadjopoulos, 1974), but some differences also exist. Microsomal drug metabolizing enzymes of fish have lower temperature optima than mammalian systems: 29 C compared to 37 C in rats (Buhler and Rasmusson, 1968). Cytochrome P-450 and its reducing enzyme NADPH cytochrome c reductase are found at lower levels in fish than in mammals (Chan et al., 1967; Bend et al., 1974; Ahokas et al., 1976). One compound which induces P-450 MFO activity in mammals, phenobarbital, fails to do so in fish (Bend et al., 1973). Aflatoxin B_1, a potent carcinogen, has proved to be an inducer of MFO activity in rainbow trout (Schoenhard, 1974; Schoenhard et al., 1976), and one of its metabolites, aflatoxicol, is also toxic and carcinogenic (Schoenhard et al., 1976).

The effect of CPFA on the membrane-bound desaturase enzyme suggests that other CPFA effects observed may also be due to alterations in membrane composition and functionality, and consequently on MFO system activity. Nixon et al. (1974) found an inhibition of induction of mitochondrial swelling by reduced glutathione and a 50% decrease in microsomal codeine demethylase activity in rats fed 2% *Sterculia foetida* oil. Eisele et al. (1978) fed 300 ppm CPFA to rainbow trout and reported decreased in cytochrome P-450 content, microsomal protein, cytochrome c reductase activity, and

b_5 content. In contrast, benzo(*a*)pyrene hydroxylase activity was increased.

Dietary protein also has a role in microsomal enzyme activity, but its effects are not easily predictable because of conflicting evidence. In 1952, increased toxicity of certain drugs was associated with protein deficiency by Drill. Campbell and Hayes (1976) have reviewed evidence of decreased xenobiotic biotransformation in protein-deficient animals.

Lee et al. (1975) reported that the level of casein in the diet of trout did not influence aflatoxin-induced hepatomas. But a different study at the same laboratory (Lee et al., 1978) showed differences caused by both level and type of protein; high levels of fish protein concentrate produced more hepatomas than high or low levels of casein, both of which produced more than low levels of fish protein concentrate. Both of the investigations involved 12 month feeding trials. In agreement with these observations, Schoenhard (1974) found that trout fed a low level of fish protein concentrate had a reduced conversion of aflatoxin B_1 to a toxic metabolite.

Stott and Sinnhuber (1978a) noted a decrease in cytochrome P-450 content in trout fed increased dietary casein and an increase with increased fish protein concentrate. They also observed a decrease in hepatic epoxide hydrase activity with increased fish protein concentrate. Higher aldrin epoxidation activity occurred with lower protein intake (Stott and Sinnhuber, 1978b), and trout fed a low level of casein were more susceptible to the MFO-activated insecticide chlordane (Mehrle et al., 1974).

In unpublished data, Nixon et al. noted an influence of protein on the carcinogenic and histologic effects of CPFA. At 50 ppm CPFA, casein caused four to five times more histologic damage and three times higher hepatoma incidence that fish protein concentrate. At the same level of CPFA, when the casein level was increased from 38% to 49.5%, the histologic damage was increased nearly 50%, and hepatoma incidence more than doubled. This phenomenon did not occur with fish protein concentrate.

Protein has definitely been implicated as an influencing factor in tumor incidence, and CPFA appears to potentiate its effects. However, the etiology of the responses to these variable is not understood, and the interrelationship of CPFA and dietary protein has not been defined.

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COMPARATIVE EFFECTS OF STERCULIC AND MALVALIC ACIDS ON THE HEPATIC MICROSOMAL CYTOCHROME P-450 SYSTEM OF RAINBOW TROUT *(SALMO GAIRDNERI)*

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ABSTRACT

Sterculic and malvalic acids, the two major cyclopropenoid fatty acids (CPFA), were isolated and assessed in separate feeding trials for the first time. Young rainbow trout were fed semi-purified diets of 40, 50, 60 or 70% protein in conjunction with 50 parts per million (ppm) or 300 ppm methyl sterculate or methyl malvalate for 20 days. Hepatic microsomal fractions were obtained and analyzed for indicators of mixed function oxidase activity.

Cytochrome P-450 content was significantly lower in sterculate samples than in control or malvalate samples. Cytochrome c reductase activity was depressed significantly at all levels of malvalate and sterculate. Microsomal protein content was relatively constant at all dietary variations. No significant differences were observed for cytochrome b_5 content or for aromatic hydrocarbon hydroxylase activity; standard deviations for these values were large and no conclusions were drawn. Level of dietary protein showed few significant effects, although trout on control diets and 300 ppm sterculate diets tended to show greater P-450 content and cytochrome c reductase activity at 40 and 50% protein than at 60 and 70% protein.

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) occur naturally in seed oils of plants belonging to the family Malvales. Cottonseed and kapok oils contain CPFA and are used as food sources. Phelps et al. (1965) have reviewed physiological disorders caused by CPFA, including retarded growth in rats and chicks, delayed sexual development in female rats, pink discoloration of egg whites and altered lipid metabolism in mammalian and avian species. In more recent studies, Sinnhuber et al. (1968) reported the co-carcinogenicity of CPFA in rainbow trout and later found them to be carcinogenic as well (Sinnhuber et al., 1976). The inhibition of acyl desaturase by CPFA has been demonstrated by several groups (Raju and Reiser, 1967; Johnson et al., 1969; Roehm et al., 1968). This causes the stearate: oleate ratio in tissue lipids to rise.

Because alterations in lipid composition cause variations in membrane permeability (Kogl et al., 1960; March et al., 1966), it is important to determine if there exists a correlation between CPFA-caused lipid changes and changes in the membrane-bound cytochrome P-450 enzyme system which is responsible for biotransformations of many endogenous and exogenous substances, including the activation of at least some carcinogens (Czygan et al., 1974). Preliminary studies in this laboratory (Eisele et al., 1978) indicated that the microsomal enzyme system in trout is depressed by dietary CPFA.

The level and type of dietary protein have been shown to influence aflatoxin-induced tumor incidence in rainbow trout (Lee et al., 1978). High levels of fish protein concentrate produced more hepatomas than did low levels and more than either high or low levels of casein; but low levels of fish protein concentrate produced fewer tumors than either level of casein. Nixon (1978) reported that trout fed 50 ppm CPFA demonstrated a three-fold increase in hepatomas when casein was fed, compared with fish protein concentrate. This was even more pronounced at higher levels of casein. In addition, the protein level has been reported to alter cytochrome P-450 content and hepatic epoxide hydrase activity in trout (Stott and Sinnhuber, 1978).

In a few studies, different physiological manifestations have been attributed to the two major CPFA, sterculic and malvalic acids. Sterculic acids has been reported to have greater biological activity and in essence to be responsible for the deleterious effects observed (Johnson et al., 1969; Lee et al., 1971; 1972). In the past, researchers have used *Sterculia foetida* oil as an indicator of sterculate activity and *Hibiscus syriacus* oil for malvalate activity, because these oils have higher concentrations of those respective CPFA. However, malvalate and sterculate have not previously been isolated and assessed separately. This study, then, had three obectives: 1) to expand upon preliminary findings of CPFA effects on the microsomal cytochrome P-450 system of trout: 2) to study a possible influence of dietary protein level on CPFA effects; and 3) to elucidate the different effects, if any, of malvalic and sterculic acids on these parameters.

EXPERIMENTAL

Isolation and Separation of CPFA

The separation of malvalic and sterculic acids was accomplished by the method of Pawlowski (1978), not previously published. Transesterfied *Hibiscus syriacus* oil was subjected to a simple vacuum distillation to provide a clean source of methyl malvalate for further purification. Methyl sterculate was obtained in essentially the same manner from *Sterculia foetida* seed extract.

Methyl malvalate was rectified on a 7mm x 46 mm stainless steel spinning band column under a vacuum of 0.004 mm Hg. The vacuum was obtained with a Welch Duo-Seal pump in good condition but well broken in. Pump oil was changed after each simple distillation or after three runs on the spinning band column. After each oil change, the oil and hose lines were devasses by running the system closed for 24 hours before turning on the column.

The column pot was stirred rapidly and heated with an oil bath. So that the distillation would remain adiabatic, the oil bath, column, head and all interfaces were completely wrapped with insulation. Column and head heaters were applied to suppress heat loss from the low density vapor. With a pot temperature of 130 C, column 114 C and head 97 C, distillation commenced. Starting with 30 ml of methyl esters, 10 to 15 ml of material were distilled over a period of four to five days, slowly raising the temperature of all components to maintain a reasonable distillation rate. The fact

that methyl malvalate has a slightly lower boiling point and faster distillation rate than methyl sterculate made differential distillation possible; as fractions of distillate were obtained, they were analyzed by nuclear magnetic resonance and high pressure liquid chromatography (Loveland, 1978) to assess purification and concentration of the now separate CPFA.

Animals and Diets

Mt. Shasta strain rainbow trout *(Salmo gairdneri)* were fed the 60% protein, semi-purified diet described by Lee et al. (1967), until they attained three months of age. At this time they were placed on diets containing 40, 50, 60 or 70% protein (casein plus 8% gelatin). The variations in diet ingredients are shown in Table 1.

Beginning at four months of age, 20 day feeding trials were begun in which trout from each diet were fed the same protein level plus either 50 ppm or 300 ppm mehtyl sterculate or methyl malvalate. The purified oils obtained from ditillation were mixed with salmon oil so that 1 gm of total oil contained 100 mg of either methyl malvalate or methyl sterculate. These were incorporated into the diet lipid to make 50 ppm or 300 ppm, and the remaining lipid required in each diet was made up with regular salmon oil. In all, twenty different diets were used (Table 2). Between 30 and 40 trout were fed each diet for each 20-day feeding trial conducted.

Feeding trials were conducted over a period of five months, and in this time trout grew from an average weight of 1.5 gm at the beginning to approximately 23 gm at the conclusion.

Preparation of Tissue

On day 21 of the feeding trials, trout were killed by a cranial blow and their livers immediately exised. Livers from trout on the same diet (30-40 livers) were combined and homogenized in 6 volumes (w/v; 1:6) 0.15 M KCl- 0.01 M potassium phosphate buffer, Ph 7.4. The homogenates were centrifuged for 15 minutes at 200 x g and then for 30 minutes at 9500 x g. The resulting post-mitochondrial supernatant was centrifuged at 105,00 x g for 60 minutes. The microsomal pellet obtained was resuspended in 0.1 M potassium phosphate buffer pH 7.4 so that 6.0 ml contained the equivalent of 1.0 gm liver.

Assays

Protein content of microsomes was determined by the method of Lowry et al. (1951) as modified by Miller (1959). Cytochrome P-450 content, b_5 content, and cytochrome c reductase activity were determined using a Beckman Acta CIII spectrophotometer with a scattered transmission accessory. The assay and calculations were done according to the procedure described by Mazel (1971).

Aromatic hydrocarbon hydroxylase assay utilized the incubation mixture of Pedersen et al. (1974), and consisted of the following, added in this order: 2.5 ml 0.1 phosphate buffer, pH 7.5; 0.7 mg NADPH in 0.5 ml H_2O; 1.0 ml resuspended microsomes; 0.025 mg, (275,000 dpm), ^{3}H-7,8-benzy(α)pyrene.

The radioactive mixture was incubated for twenty minutes in a metabolic shaker at 28.5 C, taking care to shield from excessive light. The reaction was terminated with 3.0 ml 0.25 N KOH in 50% ethanol. A few mg NaCl were added followed by 10 ml hexane. The test tubes were shaken for two minutes and then centrifuged at 3000 rpm for 10 minutes to break the emulsion. At this point, the test tubes were placed in a dry ice/acetone bath which quickly froze the bottom (aqueous) layer. The top layer (hexane) was decanted and the interface rinsed with 1 ml hexane. After the aqueous layer thawed, 1 ml of each phase was measured into a counting vial with 3 ml of the appropriate fluor (Aquasol* or toluene) and counted. The amount of polar products formed from metabolism of the benzo(α)-pyrene by the microsomes added to the incubation were calculated on the assumption that metabolic products of benzo(α)pyrene are polar and remain in the aqueous phase (DePierre et al., 1975). The proportion of dpm remaining in the aqueous phase of each sample was compared with a blank value that was obtained from the aqueous phase of an incubation containing no microsomes. Calculations were made from the following equation:

$$\frac{\text{Total aqueous dpm, sample}}{\text{Total dpm, aqueous + hexane}} - \frac{\text{Total aqueous dpm, blank}}{\text{Total dpm, aqueous + hexane}}$$

$$= \text{per cent metabolized}$$

*New England Nuclear, Boston

Results

Cytochrome P-450 content at each level of dietary casein and CPFA is shown in Table 3. At each level of casein, P-450 content decreased at 300 ppm sterculate compared with 300 ppm malvalate and with controls. For data from combined protein levels, both 300 ppm and 50 ppm sterculate are significantly lower than controls and both levels of malvalate. There is no significant difference between controls and malvalate. Casein level effects (across rows) show greater values at 40 and 50% than at 60 and 70% for controls and 300 ppm sterculate.

Cytochrome c reductase activity, represented in Table 4, decreased significantly at all levels of malvalate and sterculate for combined protein levels, as well as at each individual level. For 300 ppm sterculate, 40 and 50% casein diets exhibited greater reductase activity than 60 and 70%. This was also true for the controls, but was not statistically significant.

Microsomal protein content was fairly consistent for all dietary variations at approximately 3 mg protein/ml microsomes. The efficiency and weight gain figures for the diets in the last two feeding trials are shown in Table 5. For controls and 300 ppm sterculate, the 60% protein diet proved to be the best for efficiency, but for 300 ppm malvalate, the 70% protein was more efficient. Forty and 50% protein diets showed consistently lower efficiency than 60 and 70% protein.

Microsomal content of b_5 was very inconsistent and had large standard deviations, revealing no trand whatsoever.

Although the results were not statistically significant, controls exhibited a higher level of aromatic hydrocarbon hydroxylase activity than did all levels of CPFA, for combined protein level values. However, the activity seen in this assay was consistently very low and showed large standard deviations. Because the results were so erratic, the validity of the assay needed to be confirmed, and so the following experiment was designed: trout were injected with aromatic hydrocarbon hydroxylase inducers: 100 mg/kg of either Araclor 1242 (a polychlorinated biphenyl) or 7,8-benzoflavone. Three days later the radioactive benzo(α)pyrene hydroxylase assay was carried out using microsomes from these trout. The results showed a three-fold increase in metabolic products from Araclor 1242-treated trout and a two-fold increase in products from 7,8-benzoflavone-treated trout, compared with controls, indicating that the assay was indeed perfomring.

Discussion

It is of interest that both cytochrome P-450 content and cytochrome c reductase activity decreased with diets containing 300 ppm sterculate, while 300 ppm malvalate resulted only in decreased reductase activity; the quantity of cytochrome P-450 remained unchanged. This would suggest a change caused by malvalate that is perhaps not as extreme as that seen with sterculate. The decreases observed for CPFA-fed trout support the data reported by Eisele et al. (1978), although they observed decreased microsomal protein and increased aromatic hydrocarbon hydroxylase activity, which does not agree

with this study.

Increased microsomal enzyme activity has been associated in some cases with a decrease of chemical carcinogenesis (Wattenber, 1975). If the converse is also true, then the decreases in activity reported here are compatible with the fact that cyclopropenoid fatty acids potentiate the carcinogenicity of aflatoxin B_1 and other known carcinogens. A decrease in aromatic hydrocarbon hydroxylase activity would seem to be consistent with these observations, but we cannot draw any conclusions from the data in this study about CPFA effects on this enzyme.

Because trout used in this study were young and growing rapidly, responses of the mixed function oxidase system may have been changing with age, and this could have been a factor in the relatively large standard deviations we obtained over the five month trial period.

The small size of the livers prohibited perfusion, and hemoglobin was believed to be an interference with the b_5 assay, as it absorbs light at the same frequency as b_5. Washing the microsomes by centrifuging them a second time at 105,000 x g after they have been resuspended may be a way to prevent this from occurring. In fact, we attempted this with one sample and obtained a significantly lower value for b_5 than with a duplicate sample which had not been washed.

The higher values for P-450 content and cytochrome c reductase activity at lower casein levels correlates with the report by Mehrle et al. (1974) that trout on low casein diets were more susceptible

to chlordane, a chemical that is activated by the mixed function oxidase system. Stott and Sinnhuber (1978) also observed decreased P-450 content with increasing dietary protein when casein was the source, but increased P-450 with increasing protein when fish protein concentrate was the source. These findings are not readily understandable, although there may be a correlation between a high stress diet and an increase in mixed function oxidase activity. In this case, a low casein diet would appear to be high stress, because of higher values for cytochrome P-450 and cytochrome c reductase activity. A high level of fish protein concentrate would also be high stress, as it produced more hepatomas with aflatoxin B_1 than did low levels of fish protein concentrate and more than casein (Lee et al., 1978). But the findings of Nixon (1978) indicate that a higher level of casein, when combined with CPFA, is more deleterious to the trout than is a lower level of casein.

The mode of action for the co-carcinogenicity of cyclopropenoid fatty acids remains a mystery. Evidence implicating CPFA as substrates of the mixed function oxidase system is not available, and since CPFA appear to depress MFO activity, they are apparently not inducers. The fact that they alter lipid structure and metabolism strongly suggests that membrane integrity may be impaired, and some evidence supports this (Nixon et al., 1974). This may be the route to altered activity of the membrane-bound cytochrome P-450 system and consequently to differential metabolism of carcinogens. If this is true, the potentiation of aflatoxin B_1 carcinogenesis by CPFA may be in response to a decreased rate of metabolism to less toxic

metabolites, due to the decreased activity of the microsomal enzymes which we have seen occur in this study. In fact, it has been shown that the production of aflatoxicol, aflatoxin M_1 and other metabolites of aflatoxin B_1 is inhibited in trout by dietary CPFA (Loveland, 1978). Whether or not this might lead to an increased interaction of the carcinogen with DNA is not known, but surely exists as a possibility.

ACKNOWLEDGEMENTS

This investigation was supported in part by Public Health Service Grants ES 00550 and ES 01926 from the Division of Environmental Health Service. We thank Dr. Norman Pawlowski for supplying methyl malvalate and methyl sterculate.

TABLE 1

COMPOSITION OF DIETS

Diet ingredients	Protein levels 40%	50%	60%	70%
Casein	32	42	52	62
Gelatin	8	8	8	8
Dextrin	21.3	21.9	12.5	3.1
α-cellulose	13.7	8.1	10.5	12.9
mineral mix	1.0	1.0	1.0	1.0
carboxymethyl-cellulose	1.0	1.0	1.0	1.0
choline chloride	1.0	1.0	1.0	1.0
vitamin mix	2.0	2.0	2.0	2.0
salmon oil (includes CPFA where applicable)	20.0	15.0	12.0	9.0
	100.0	100.0	100.0	100.0

Numbers represent % of diet, by weight

Diets are isocaloric

TABLE 2

CPFA AND PROTEIN COMBINATIONS IN FEEDING TRIALS:

TWENTY DIETS

		50 ppm		300 ppm	
Protein Level	0 CPFA	Sterculate	Malvalate	Sterculate	Malvalate
40%	1	5	9	13	17
50%	2	6	10	14	18
60%	3	7	11	15	19
70%	4	8	12	16	20

TABLE 3

Effect of CPFA on cytochrome P-450 content* at different levels of dietary casein

Level CPFA	Casein Level 40%	50%	60%	70%	Combined Protein Levels
0 CPFA	.242 ± .134(3)+	.265 ± .103(4)^{ab}	.167 ± .056(12)	.226 ± .185(4)^{a}	.204 ± .105(23)^{ab}
50ppm Malvalate	.171 ± .074(2)	.202 ± .207(2)	.083	.129 ± .043(2)	.168 ± .111 (7)^{d}
300ppm Malvalate	.297 ± .206(3)	.090 ± .087(3)^{a}	.307 ± .226(2)	.174 ± .103(6)	.191 ± .132(14)^{c}
50ppm Sterculate	.151 ± .008(2)	.128 ± .054(2)	.129 ± .005(2)	.130 ± .019(2)	.134 ± .024 (8)^{bcd}
300ppm Sterculate	.137 ± .071(2)	.118 ± .069(3)^{b}	.109 ± .056(4)	.091 ± .055(8)^{a}	.105 ± .056(17)

* nmoles P-450/mg microsomal protein

+ mean ± standard deviation (number of samples, 30-40 livers/sample)

In each column, the same superscripts indicate a significant difference ($P < .05$), t test

TABLE 4

Effect of CPFA on cytochrome c reductase activity* at different dietary casein levels

Level CPFA	Casein Level 40%	50%	60%	70%	Combined Protein Levels
0 CPFA	44.95 ±20.62(3)+	46.90 ±12.14(4)^{abc}	39.41 ±10.94(12)^{ab}	37.95 ±19.38(3)^{ab}	41.32 ±13.09(22)^{abcd}
50ppm Malvalate	11.09 (1)	31.55 ±28.85(2)	20.47 ± 5.70 (2)^{b}	30.57 ±13.46(2)^{c}	25.18 ±15.43 (7)^{a}
300ppm Malvalate	34.02 ±32.66(2)	12.65 ± 8.29(3)^{a}	23.63 ±25.11 (2)	27.44 ±15.14(5)	23.24 ±17.04(12)^{b}
50ppm Sterculate	14.98 (1)	17.30 ± 1.90(2)^{b}	13.84 ± 9.13 (2)	15.90 ± 2.88(2)^{bc}	15.82 ±15.43 (7)^{c}
300ppm Sterculate	30.04 ±12.26(4)	36.00 ± 4.95(4)^{c}	25.82 ±10.17 (4)^{a}	20.61 ± 5.33(8)^{a}	25.76 ± 9.35(20)^{d}

* nmoles cytochrome c reduced/min/mg protein

+ mean ± standard deviation (number of samples, 30-40 livers per sample)

In each column, the same superscripts indicate a significant difference ($P < .05$), t test

TABLE 5

EFFICIENCY AND WEIGHT GAIN,

20-DAY FEEDING TRIAL

		Protein Level			
		40%	50%	60%	70%
0 CPFA:	EFFICIENCY*	37.49± 8.59(2)‡	22.93± 2.98(2)	53.41± 12.81(7)	43.07± 12.77(2)
	WEIGHT GAIN+	2.65± .28	1.65± .03	3.45± 1.47	4.46± 1.07
300 ppm Malvalate:	EFFICIENCY	34.84 (1)	22.37 (1)	39.50 (1)	53.12± 5.20(6)
	WEIGHT GAIN	3.23	1.65	3.96	2.99± 1.18
300 ppm Sterculate:	EFFICIENCY	27.68± .65 (2)	34.13± 3.74(2)	44.89± 5.22(2)	36.00± 14.78(4)
	WEIGHT GAIN	2.78± 1.91	2.46± .51	4.29± .23	1.91± .64

* Efficiency: $\frac{\text{weight gained}}{\text{weight diet consumed}} \times 100$

\+ Weight gain, grams

‡ mean± standard deviation (number of samples, 30- 40 fish per sample)

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APPENDIX I

Effects of dietary casein on P-450 content* at different levels of CPFA

Level Casein	CPFA Level					
	0 CPFA	50ppm Malvalate	300ppm Mal.	50ppm Sterculate	300ppm Ster.	Combined CPFA
40%	.242 ±.134 (3)+	.171 ±.074 (3)	.297 ±.206 (3)	.151 ±.008 (2)	.137 ±.071 (2)	.211 ±.127 (12)[a]
50%	.265 ±.103 (4)[a]	.202 ±.207 (2)	.090 ±.087 (3)	.128 ±.054 (2)	.118 ±.069 (3)	.174 ±.118 (14)
60%	.167 ±.056(12)[a]	.083 (1)	.307 ±.226 (2)[a]	.129 ±.005 (2)	.109 ±.056 (4)	.150 ±.061 (21)[a]
70%	.226 ±.185 (4)	.129 ±.043 (2)	.174 ±.103 (6)[a]	.130 ±.019 (2)	.091 ±.055 (8)	.145 ±.106 (22)

* nmoles cytochrome P-450/mg microsomal protein

+ mean ±standard deviation (number of samples, 30-40 livers per sample)

In each column, the same superscripts indicate a significant difference ($P<.05$, t test)

APPENDIX II

Effects of dietary casein on cytochrome c reductase activity* at different CPFA levels

Level Casein	CPFA Level					
	0 CPFA	50ppm Malvalate	300ppm Mal.	50ppm Sterculate	300ppm Ster.	Combined CPFA
40%	44.95 ±20.62(3)†	11.09	34.02 ±32.76(2)	14.98 (1)	30.04 ±12.26(4)[a]	31.69 ±19.04 (11)
50%	46.90 ±12.14(4)	31.55 ±28.85(2)	12.68 ± 8.29(3)	17.30 ±1.90(2)	36.00 ± 4.95(4)[b]	30.45 ±17.72 (15)
60%	39.41 ±10.94(12)	20.47 ±5.70(2)	23.63 ±25.11(2)	25.17 ±16.02(2)	25.82 ±10.17(4)	30.58 ±13.12 (23)
70%	37.95 ±19.38(3)	30.57 ±13.46(2)	27.44 ±15.14(5)	15.90 ± 2.88(2)	20.61 ± 5.53(8)[ab]	27.16 ±14.54 (21)

*nmoles cytochrome c reduced/min/mg microsomal protein

†mean ± standard deviation (number of samples, 30-40 livers/sample)

In each column, the same superscripts indicate a significant difference, ($P < .05$), t test

APPENDIX III

Effects of CPFA on cytochrome c reductase activity in relation to P-450 content*

Level CPFA	Casein Level 40%	50%	60%	70%	Combined Protein Levels
0 CPFA	302.91 ±59.22(2)+	269.33 ± 94.43(4)	257.01 ± 84.20(12)	283.00 ± 69.11(3)	269.05 ± 77.81 (22)
50ppm Malvalate	47.64 ± 2.92(2)	230.29 ±139.81(2)	317.69 ±169.37(2)	197.36 ± 77.21(2)	198.24 ± 136.36 (8)
300ppm Malvalate	221.18 ±78.57(3)	86.97 ± 38.59(2)	148.67 ±189.14(3)	240.61 ±136.13(6)	194.80 ± 130.78 (14)
50ppm Sterculate	229.76±179.10(2)	152.21 ± 79.11(2)	244.45 ±189.07(2)	122.84 ± 4.27(2)	187.31 ± 116.50 (8)
300ppm Sterculate	276.39±104.06(3)	574.54 ±369.05(2)	253.93 ± 67.54(2)	276.69 ±147.96(6)	314.30 ± 183.10 (14)

*nmoles cytochrome c reduced/min/nmole P-450

+mean ± standard deviation (number of samples, 30-40 livers per sample)

APPENDIX IV

Effects of CPFA on cytochrome b_5 content* at different dietary casein levels

Level CPFA	Casein Level 40%	50%	60%	70%	Combined Protein Levels
0 CPFA	.152 ± .005 (3)+	.172 ± .028 (4)	.089 ± .041 (12)	.133 ± .079 (3)	.119 ± .053 (22)
50ppm Malvalate	.086 ± .070 (2)	.111 ± .076 (2)	.026 (1)	.110 ± .115 (2)	.095 ± .067 (2)
300ppm Malvalate	.209 ± .104 (3)	.109 ± .105 (3)	.160 ± .125 (3)	.105 ± .054 (6)	.137 ± .091 (15)
50ppm Sterculate	.088 ± .035 (2)	.122 (1)	.093 ± .008 (2)	.062 ± .024 (2)	.087 ± .027 (7)
300ppm Sterculate	.125 ± .075 (3)	.137 ± .029 (3)	.168 ± .069 (4)	.124 ± .031 (8)	.136 ± .050 (18)

*nmoles b_5/mg microsomal protein

+mean ± standard deviation (number of samples, 30-40 livers per sample)

APPENDIX V

EFFECT OF CPFA ON BENZO(α)PYRENE HYDROXYLASE ACTIVITY* FOR COMBINED LEVELS OF PROTEIN

0 CPFA	1.62 ± 2.78 (55)+
50 ppm Malvalate	1.13 ± 1.68 (12)
300 ppm Malvalate	1.14 ± 1.93 (31)
50 ppm Sterculate	0.76 ± 1.01 (13)
300 ppm Sterculate	1.10 ± 1.83 (49)

*% of incubated benzo(α)pyrene converted to aqueous, or hydroxylated, form.

+mean ± standard deviation (number of samples)

APPENDIX VI

Effect of pretreatment on benzo(a)pyrene hydroxylase activity

Injected 3 days prior to sampling with 100 mg/kg:*	% benzo(a)pyrene converted to aqueous product
Araclor 1242	14.93 5.32 (8)[ab]
7,8-Benzoflavone	7.40 5.70 (3)[a]
Control	4.25 .98 (4)[b]

* Araclor 1242 and 7,8-benzoflavone were injected with dimethylsulfoxide (DMSO) as vehicle. Control received DMSO only.

+ Mean standard deviation (number of samples, 15-20 livers/sample)

The same superscripts indicate a significant difference ($P < .05$, t test)

APPENDIX VII

Determination of Cytochrome P-450 and Cytochrome b_5

The extinction coefficients for cytochrome P-450 and cytochrome b_5 are 91 mM^{-1} and 171 mM^{-1}, respectively.

1. Place 2.0 ml of the resuspended microsomes in matched cuvettes. Determine the baseline on the Beckman Spectrophotometer at 500 nm. Use the special scattered transmission accessory for this assay.
2. Add a few mg of sodium dithionite to the sample cuvette only. Record the difference spectrum of the reduced b_5 by scanning from 500 to 410 nm.

$$\frac{(\Delta O.D.)(1000)}{(171)(\text{mg protein in 2 ml sample})} = \text{nmoles cyt } b_5\text{/mg microsomal protein}$$

3. Bubble carbon monoxide gently through the reference and sample cuvettes for 20 seconds.
4. Record the spectrum from 500 to 410 nm.

$$\frac{(\Delta O.D.)(1000)}{(91)(\text{mg protein})} = \text{nmoles P-450/mg microsomal protein}$$

APPENDIX VIII

Determination of Cytochrome c Reductase

Solution I: NADPH 5.7 mg
KCN 9.75 mg
Nicotinamide 366 mg
Dissolve in 100 ml 0.05 N K-PO_4 buffer pH 7.6 containing 10^{-3} M EDTA.

Solution II: Cytochrome c 3.68 mg/ml in H_2O
Type III or IV
Sigma Chemical, St. Louis

Solution III: KCN 9.75 mg
Nicotinamide 366 mg
Dissolve in 100 ml 0.05 M K-PO_4 buffer pH 7.6 containing 10^{-3} M EDTA.

The molar extinction coefficient for cytochrome c is 19.1 mM^{-1}.

Procedure

Tube 1: 2.0 ml Solution I
Tube 2: 2.0 ml Solution III
Incubate these for 8 minutes at 25 C.
Tube 1: Add 0.5 ml Solution II
Tube 2: Add 0.5 ml Solution II
Incubate for two additional minutes
Tube 1: Add 0.1 ml microsomes and 0.4 ml H_2O
Tube 2: Add 0.1 ml microsomes and 0.4 ml H_2O
Mix and pour into matched cuvettes.
Read the change in absorbance at 550 nm for about 3 minutes. Chart settings: 1 nm/sec
50 nm/inch

Total volume = 3.0 ml in each cuvette

Calculations:

$$\frac{(\Delta O.D./min)(3.0\ ml)}{(19.1)(mg\ microsomal\ protein)}$$

= nmoles cytochrome c reduced/min/mg microsomal protein

APPENDIX IX

Toluene Fluor

To make toluene fluor, start with scintilaation grade toluene. Add the following:

50 mg POPOP/liter
6 gm PPO/liter

For a one gallon jug, add:

190 mg POPOP
22.8 gm PPO

POPOP is 1,4-bis(2-(5-phenoloxazolyl))benzene
Nuclear-Chicago

PPO is 2,5-diphenyloxazole
Sigma Chemical