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Title: ALTERATIONS IN GROWTH, LIPID METABOLISM AND P:O RATIOS IN MICE FED STERCULIA FOETIDA OIL

Abstract approved: Joseph E. Nixon

Cyclopropenoid fatty acids (CPFA) are natural components of cottonseed oil, a major food oil in the United States. The ability of CPFA to cause abnormal biochemical and physiological effects when fed to laboratory and farm animals has prompted an investigation of their effects on mice.

Between 0.05 and 0.55% CPFA were fed as glycerides of Sterculia foetida oil (SFO, containing 55% CPFA) to mice to determine their effect on certain aspects of growth, lipid metabolism and mitochondrial function.

One-half percent SFO fed to weanling mice caused a small temporary decrease in growth rate when compared to controls. Many mice fed 1% SFO stopped growing and died by the end of a 9-week feeding trial. Mice fed less than 1% SFO, or 1% SFO for shorter periods of time, showed increased liver-to-body-weight ratios,

accumulation of CPFA in adipose tissue and increased ratios of 16:0/16:1, 18:0/18:1 and total saturated to unsaturated fatty acids in liver and depot fat. Erythrocytes from CPFA-fed mice hemolyzed more slowly than erythrocytes from control mice in isotonic-nonelectrolytes, implying an effect of CPFA on membrane lipid composition.

One-half percent SFO fed for 9 to 31 days inhibited almost completely the incorporation of [^{14}C] from labeled palmitate or acetate into liver monounsaturated fatty acids. At the same time, 0.5% SFO retarded the incorporation of label from acetate into $^{14}CO_2$ and total liver lipid, but stimulated twofold the incorporation into liver sterols. The oxidation of labeled palmitate was also reduced. CPFA caused lipid accumulation in livers.

P:O ratios of liver mitochondria from mice fed 1% SFO for 6 to 15 days were 1.06-1.45 while control P:O ratios were 2.30-2.85. The decrease was due to decreased phosphorylation, but increased respiration. The relationship between the observed results and membrane fatty acid composition was discussed.

Alterations in Growth, Lipid Metabolism and P:O Ratios in Mice Fed *Sterculia foetida* Oil

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INTRODUCTION

Cyclopropenoid fatty acids (CPFA) are a natural lipid component of plants of the families Malvaceae and Sterculiaceae. The two most commonly occurring CPFA are sterculic acid (SA) and malvalic acid (MA) found as glycerides in kapok oil and cottonseed oil. Kapok oil is an important food oil in oriental countries as is cottonseed oil in the United States. Both oils are used in the manufacture of cooking oil, vegetable shortening and margarine. Cottonseed flour and meal are processed for human consumption.

Concern over the presence of cyclopropenes in food was stimulated by reports of the occurrence of abnormal physiological effects in animals fed CPFA. Increased liver to body weight ratios, fatty livers, altered lipid metabolism and delayed sexual development with impaired reproduction were reported in laboratory and farm animals fed CPFA.

Of primary concern are reports that CPFA are cocarcinogenic with aflatoxin B_1 in trout. Aflatoxin B_1 may be found in poor quality cottonseed products. The cocarcinogenicity of CPFA has not been unequivocally demonstrated in a mammalian species.

Current research is aimed at defining more completely the metabolism and physiological effects of CPFA in an attempt to understand their cocarcinogenic properties.

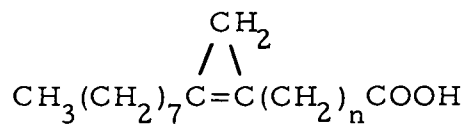
The purpose of the present work was to verify the susceptibility of mice to the effects of dietary CPFA and to note any differences between control and CPFA-fed mice with respect to certain parameters. Alterations in lipid metabolism, membrane function and mitochondrial function were reported previously in various species fed CPFA. The earlier reports prompted similar investigations with mice fed CPFA as glycerides of Sterculia foetida oil.

LITERATURE REVIEW

Occurrence

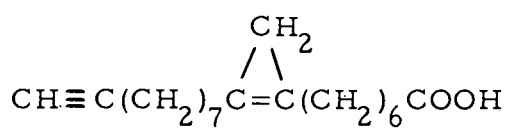
CPFA are distinguished by the inclusion of a disubstituted cyclopropene ring in the aliphatic chain of the fatty acid.

In 1952, Nunn (84) confirmed the structure of SA after its extraction from the seed oil of Sterculia foetida. Another naturally occurring CPFA, MA, was shown by Macfarlane (75) to have a similar structure.



SA, n=7 MA, n=6

Subsequent work (15, 24, 37, 103) verified these structures. A third naturally occurring CPFA is sterculynic acid found as 8% of the fatty acids of oil from Sterculia alta (53).



Sterculynic acid

Phelps et al. (92)and Christie (16) list species of the families Sterculiaceae, Malvaceae, Bombacaceae and Tiliaceae containing CPFA. Three of the more important sources of CPFA are seed oils

of Sterculia foetida (Java olive), Gossypium hirsutum (cotton) and Eriodendron anfractuosum (kapok). Kapok oil, containing 12-14% CPFA, is used in Japan as a major food oil (61). Cottonseed oil (CSO), used in the same manner in the United States, contains 1.1-1.6% MA and 0.4-0.8% SA (16). Cottonseed meal, containing small amounts of CPFA, is widely used as a protein source for livestock (22). Sterculia foetida oil (SFO) containing 45-54% SA and 4-10% MA (16) is a common source of CPFA for chemical and biological investigation.

Johnson et al. (56) and Yano et al. (126) have shown the synthesis of SA and MA in species of Malvaceae to occur by methylation of oleic acid at the Δ^{9-10} position with S-adenosylmethionine. Desaturation at the Δ^{9-10} position of the dihydrosterculic acid formed yields SA. Subsequent α-oxidation may be the route to MA. Intermediate steps in the synthesis of CPFA probably involve phospholipid-bound CPFA intermediates (56), but CPFA are normally isolated in neutral lipid fractions (60).

Christie (16) found, by hydrolysis with pancreatic lipase, that most of the SA of Bombacopsis glabera glycerides was in the β-position.

Analysis

Halphen Test

In 1897, Halphen (45) reported a reaction that appeared to be specific for CSO. The original test involved heating a mixture of 1% sulfur in carbon disulfide with an oil sample in amyl alcohol. The development of a reddish-orange color was assumed to indicate the presence of CSO. In 1956, Faure and Smith (36) found that SA and SFO gave a positive Halphen reaction, but not until 1957 did Macfarlane et al. (74) verify the structure and presence of MA in CSO and show CPFA to be responsible for the color formation observed by Halphen.

The Halphen pigment has not been identified, but the spectrophotometric determination has been developed, notably by Bailey et al. (6) and Hammonds et al. (46), to give quantitative data on the CPFA content of plant and animal lipids. According to Coleman and Firestone (20), their method (a modification of Hammonds' method) has been used to detect and quantitate, respectively, 15 μg CPFA/g oil and 18 μg CPFA/g oil. In a collaborative study by 12 laboratories using his method, Coleman (19) reported close agreement in the determination of 0.00 to 0.19% CPFA in lipid samples. The Halphen test suffers from a lack of precision, a standard is necessary and background is variable depending on the sample source. It is still the

most acceptable method for routine analysis of small amounts of CPFA (18).

Hydrogen Halide Titration

In 1960, Smith et al. (121) reported the occurrence of fatty acids that interfered with the Derbutaki determination of peroxide fatty acids in oils. One of the interfering fatty acids was SA; the cyclopropene ring reacted with hydrogen bromide at nearly the same rate as epoxides. Titration of an oil with hydrogen bromide before and after reduction of the epoxide, but not the cyclopropene ring, gave an indication of the epoxide and CPFA content of the oil. Smith's report, with evidence that hydrogen bromide adds to cyclopropenes in a molar ratio of 1:1 (96), prompted work on a quantitative method for determining CPFA by titration with hydrogen halides. Work by many groups led from the direct titration of CPFA to the method of Rosie and Shone (107), a back titration, recommended by Coleman (18) as the best hydrogen halide method. This method does not give a definite endpoint (90), is not as sensitive as the Halphen test or GLC of CPFA derivatives, and is not recommended for general use (18). It has been used to prepare standards for the Halphen test. Although the hydrogen bromide titration and Halphen test can be used to quantitate CPFA, neither method will distinguish specific acids.

Gas Liquid Chromatography

GLC has been used to separate and quantitate SA and MA in mixtures of fatty acids. Recourt et al. (100) separated the methyl esters directly at reduced column temperature, Shenstone and Vickery (116) reduced cyclopropene to cyclopropane fatty acid methyl esters and Raju and Reiser (96) added methanethiol across the double bond of the cyclopropene ring. Each method was an attempt to quantitate a thermally stable derivative of the CPFA.

Coleman (18) favored the use of the method of Schneider et al. (112) to prepare standards for the Halphen test. Schneider et al. reacted CPFA with silver nitrate in methanol to form thermally stable ether and ketone derivatives separable by GLC. Schneider et al. claim sensitivity to 0.01% after purification of the reaction products by column chromatography.

Nuclear Magnetic Resonance Spectroscopy

Pawlowski et al. (90) have applied NMR to analysis of as little as 1% CPFA in lipid. Samples as small as 5 mg may be used, although 30 mg samples are more desirable. The ratio of terminal methyl protons and cyclopropene ring methylene protons is calculated. A ratio of methyl to methylene protons of 3:2 implies 100% CPFA. The accuracy of the method at 10% CPFA is $\pm$ 0.5%. This method has been used to prepare standards for the Halphen test.

Physiological Effects

Lorenz (73) and Sherwood (118) showed that plants of the family Malvaceae, when fed to hens, caused the pink-white disorder in their refrigerated eggs. Lorenz suggested the causative agent was the Halphen positive compound in the oil of Malvaceous plants. The findings of Shenstone and Vickery (115) supported this suggestion and shortly thereafter SA (77) and MA (117) were shown to cause the pink-white disorder. Deutschman et al. (23) and Nordby (82) found similar results using sterculene and sterculyl alcohol, supporting the idea that the cyclopropene ring was responsible for the discoloration.

Schaible and Bandamer (108) found the pink color to be due to an iron-conalbumin complex formed when iron moved from the yolk to the egg white. Recently (2), ^{59}Fe has been used to verify this point and to show increased retention of iron in hens fed SFO.

Evans et al. (35) reported a selective transfer of three white proteins to the yolk while only one yolk protein moved to the white. Shenstone and Vickery (117) showed that concurrent transfer of water to the yolk kept the protein concentration in the white constant. They also reported increased free proline and glutamic acid in pink-whites. CPFA caused the pH of the yolk and white to converge (82, 117) and altered the size and structure of the yolk (26, 34, 117). The yolk appeared rubbery, especially when cold, and was found to contain a high melting-point fat. This effect was not dependent upon storage.

Subsequent work (1, 2, 4, 29, 34, 55, 110) revealed that ingestion of CPFA increased the ratios of palmitic to palmitoleic and stearic to oleic acids in lipids of yolk, plasma, liver, ovaries, heart and adipose tissue of hens. Also noted were elevations in the level of linoleic acid in yolk and plasma (34) and decreased levels of arachidonic acid in heart lipid (3).

The change in fatty acid composition of egg yolk was shown to be responsible for increased embryo mortality in chicks (5) and quail (29). SFO injected into the 4-day chick embryo was not toxic, but egg yolk from hens fed SFO was. Many investigators (62, 92, 111) have shown that ingestion of CPFA impairs the reproductive process in hens.

Some workers (26, 108) believe the effects of CPFA may be due to the increased permeability of the vitelline membrane. Others (7, 117) suggest a change in the structure of the yolk itself.

Bain (7) has described optical and electron microscope studies of the vitelline membrane of eggs from hens fed 14 mg/kg/day methyl sterculate (MS). He found no visible change in the membrane and suggested that physiochemical organization of the yolk controls diffusion across yolk membranes.

Burley, in two papers (13, 14), described abnormal physical properties of a major egg yolk low-density lipoprotein from hens fed MS. Ultracentrifugation studies indicated increased lipoprotein

interactions and a greater proportion of protein bound to lipid in abnormal yolk. Burley proposed as causes the altered fatty acid composition of yolk lipid and increased protein interaction with other yolk components.

Evans (33) found more lipid associated with the surface of yolk lipovitellines from hens fed CSO than those fed fish oil. CSO-treated yolk lipid contained twice normal levels of stearic acid with a corresponding decrease in oleic and palmitoleic acids. Since the amino acid composition of the control and experimental yolk apoproteins was the same, the more saturated lipids seemed to be responsible for the stronger attractive forces between lipid and protein.

Ferguson (38) reported no alteration in growth rate or efficiency of feed conversion (g food consumed/g weight gained) in rabbits fed 0.5% SFO for 5 weeks. The expected increase in the ratios of 16:0/16:1 and 18:0/18:1 was found for liver and erythrocyte ghost lipids. Levels of plasma cholesterol, liver lipid and liver cholesterol were increased while liver protein concentrations were decreased by CPFA feeding. A 60% incidence of aortic atherosclerosis was found in rabbits fed CPFA with no sign of the disease in controls fed corn oil. With the data of Goodnight and Kemmerer (43) suggesting that CPFA did not decrease the excretion of cholesterol in cockerels, Ferguson concluded that CPFA may stimulate the synthesis of cholesterol.

Braden and Shenstone, as cited by Phelps et al. (92), fed mice purified SA at levels up to 200 mg/kg/day for 6 days and an additional 13 days during mating. Males and females were fed SA in one group and either males or females were fed SA in two additional groups. CPFA had no apparent effect on the proportion of animals mating, their fertility or mean litter size.

Raju and Reiser (97) fed lactating mice 0.2% CPFA as SFO in a fat-free, high carbohydrate diet and assayed hepatic stearoyl-CoA desaturase activity in the pups at weaning and after 30 days on a stock diet. Feeding the stock diet did not restore activity of the desaturase.

The Halphen peak (500 nm) present in pup liver lipids at weaning disappeared after 30 days feeding and apparently gave rise to a second peak (550 nm). Raju and Reiser suggested that the second peak was due to a metabolite of the CPFA ingested via the mother's milk. The activity of sn-glycerophosphate acyl-transferase was elevated at weaning and after 30 days feeding, probably the result of fatty livers in CPFA-fed mice.

CPFA had no effect on the growth rate of the pups.

Two reports (21, 113) concerned primarily with the effect of SFO on reproduction in rats showed that 1% or 2% SFO delayed sexual maturity in females, reduced ovary-oviduct-uterus weights, and reduced growth rate and efficiency of feed conversion. These treatments caused the death of all fetuses or newborn within 48 hours

postpartum. Three percent dietary SFO prevented reproduction.

Miller (79) noted widespread hemorrhaging in the lungs of newborn of dams fed CPFA and ascribed deaths to this defect. Hemorrhaging was believed to be due to capillary and membrane fragility caused by increased levels of saturated fatty acids in these tissues.

Nixon (81) also reported reduced growth rate and efficiency of feed conversion, and elevated liver-to-body-weight ratios for male and female rats fed 2% SFO compared to corn oil controls.

A number of workers (21, 42, 43, 81, 101) have reported CPFA-induced alteration of rat tissue lipids. Increased percent lipid in liver and heart, and increased ratios of 16:0/16:1 and 18:0/18:1 in liver, heart and adipose tissue were noted. Increases in 18:2/20:4 in liver and heart were reported for female (21) and male (101) rats. CPFA accumulate in rat liver, heart, and adipose tissue (21).

Rainbow trout, Salmo gairdneri, have proven to be most susceptible to the effects of CPFA. Dietary levels of 100-200 ppm CPFA have produced the expected increase in ratios of saturated to monoenoic fatty acids with a decrease of 22:6 in liver lipids (106). CPFA were shown to accumulate in triglycerides of depot and intermuscular lipid (105). Increased liver-to-body-weight ratios and fatty livers were reported (104, 122). Histological abnormalities included fatty infiltration, bile duct proliferation and the formation of fibers in liver parenchymal cells (71, 106, 120, 122).

Malevski et al. (76) recently reported no decrease in growth rate for trout receiving 0.5 mg CPFA/kg body wt/day when compared to pair fed controls. Taylor et al. (123) and Malevski et al. (76) noted decreased protein synthesis and normal lipid synthesis from [^{14}C] acetate in the livers of fish fed CPFA.

Sinnhuber et al. (119, 120) reported that 220 ppm dietary CPFA hastened the appearance and increased the incidence and growth of aflatoxin B_1-induced hepatoma in trout. Lee et al. (70, 71) also reported CPFA to be cocarcinogenic with aflatoxin B_1 and, to a lesser degree, with 2-acetylaminofluorine. A second report (69) suggested that SA was a more effective cocarcinogen than MA, and acted by enhancing the growth of abnormal cells.

Lee et al. (68) reported a possible cocarcinogenic effect of CPFA with aflatoxin in rats. In earlier work with rats, Friedman and Mohr (42) found no interaction between aflatoxin and CPFA. Rats seem to be less susceptible than trout to the effects of CPFA.

Effect of CPFA on the Formation of Long-Chain Monoenoic Fatty Acids

The formation of monounsaturated fatty acids occurs through variation of two pathways. The anaerobic dehydrase system is the more primitive of the two and is found in bacteria (32). The aerobic desaturase system is found in various forms in yeast, plant and animal tissue.

The anaerobic dehydrase catalyzes the formation of the cis-β, γ-unsaturated form of decanoyl acyl carrier protein (ACP) or dodecanoyl-ACP. D(-)-β-hydroxy acyl-ACP is an intermediate in each case. Subsequent elongation gives the long-chain monoene; decanoyl-ACP forms cis-vaccenic acid (18:1 Δ^{11}) and dodecanoyl-ACP forms oleic acid (18:1 Δ^{9}) (11, 109). These reactions are closely associated with the soluble fatty acyl synthetase in E. coli (83).

The aerobic desaturase is a microsomal enzyme system dependent on reduced pyridine nucleotides and oxygen (87). The membrane system requires a specific combination of triglyceride, phospholipid and free fatty acids. The degree of saturation of the fatty acids of these lipid components is important for optimum activity (58).

Substrates for this system are palmityl-CoA and stearyl-CoA (49). They are desaturated at the Δ^{9-10} position. Further elongation and desaturation of these and other unsaturated fatty acids is accomplished by other enzyme systems in plants and animals (12, 44).

Workers using yeast, algae, higher plants, hens, mice and rats have shown CPFA to inhibit the desaturation of exogenous [^{14}C] labeled forms of palmitic and stearic acids.

Johnson et al. (54) and Raju and Reiser (98) reported that CPFA inhibit primarily the desaturation of the Δ^{9-10} position of long-chain fatty acids. The varied effects of SA, MA, MS, 2-hydroxy sterculyl alcohol (52), sterculene, sterculyl alcohol and the methyl

ether of the alcohol (82) suggest that the cyclopropene ring is responsible for the inhibition.

Fogerty et al. (40) demonstrated that for hen desaturase inhibition the cyclopropene ring had to involve carbons 9 or 10 of the cyclopropenoid fatty acid chain. Their results suggested that maximum inhibition involved a two-point attachment to the desaturase, one by the fatty acyl functional group and one between the cyclopropene ring and the active site of the enzyme.

Workers with the above plant and animal systems seem to agree that CPFA inhibit the desaturation of palmitic and stearic acids (some examples are given in the next section). In dispute is the effect of CPFA on the synthesis of palmitoleic and oleic acids from acetate and lauric acid.

Donaldson (27), Gurr (44), James (52), and Raju and Reiser (95), using chicks, algae and higher plants, algae, and yeast and rats, respectively, reported little or no inhibition by CPFA of the synthesis of monoenes from [^{14}C] acetate or, in one case (95), [^{14}C] lauric acid.

Alloxan diabetic rats did not use the aerobic desaturase but they were able to synthesize oleic acid when fed a diet high in lauric acid (95).

Raju and Reiser (95) also stated that oleic acid was more rapidly labeled than stearic acid when [^{14}C] acetate was the precursor

in vivo. They suggested that in control rats, approximately 25% of the liver oleate was synthesized via an alternate pathway and that 34% of the oleate derived from [^{14}C] laurate was found in cis-vaccenic acid.

An acute does of CPFA to chicks increased the ratio of specific activities of palmitate to palmitoleate and stearate to oleate, synthesized in vivo from [^{14}C] acetate (27). Chronic dosage (7 days) with SFO decreased these ratios. Donaldson (27) suggested that chronic administration of CPFA induced a repressed alternate pathway to the monoenes. The pathway reportedly involved elongation of a medium-chain monoenoic intermediate.

Donaldson (28) also reported an increase in liver palmitoleate in chicks deficient in biotin. Feeding CPFA to these chicks did not decrease the level of palmitoleate below that of controls, again suggesting the operation of an alternate pathway to palmitoleate.

Workers with algae and higher plants (44, 52) suggested two routes to monoenoic fatty acids. One, for the desaturation of exogenous palmitic or stearic acid involved an acyl transfer from CoA to ACP. Hypothetically, this step was blocked by CPFA. The second route involved endogenous fatty acids synthesized as ACP derivatives and bypassed the acyl transferase. The above workers reported no [^{14}C] oleate synthesis in anaerobic incubations with [^{14}C] acetate.

In most cases the ratio of specific activities of saturated to monoenoic fatty acids was used to express the activity of a desaturating system. Herein lies the basis for criticism by Pearson et al. (91) who, with Coleman and Friedman (21) and Bickerstaff and Johnson (10), found no evidence for an alternate pathway in rats, hens and goats, respectively. Each author found that CPFA inhibited the incorporation of [^{14}C] acetate into oleate.

Pearson et al. (91) dispute the alternate pathway theory on the basis of their own specific activity data and with the argument that the specific activities of substrates and products of the desaturase depend not only on the activity of the enzyme, but on the metabolite pool sizes.

Coleman and Friedman (21) reported continual increases in the mass ratios (by GLC) of the C_{16} and C_{18} saturated to monoenoic fatty acids of rats over 34 weeks of CPFA feeding, indicating that at least the inhibitory effect of CPFA was greater than the capacity of an alternate monoene synthesizing pathway.

Bickerstaff and Johnson (10) found that SA had the expected effect on the fatty acid composition of goat's milk. They also found that SA reduced the incorporation of [^{14}C] acetate into oleate in perfused goat mammary gland.

Evidence for the Mode of Action of CPFA

Kircher (64) showed that MS and sterculene would react with dilute solutions of methyl mercaptan and β-mercaptopropionic acid; sulfhydryls added to the double bond of the cyclopropene ring. Kircher suggested that CPFA could exert their biological effects by reacting with enzyme sulfhydryls.

Fatty Acid Desaturase as a Model

Previously mentioned reports that CPFA increased levels of stearic acid at the expense of oleic acid, and evidence (50) that stearoyl-CoA desaturase was a thiol enzyme prompted studies of the mode of action of CPFA using the desaturase system as a model.

Raju and Reiser (98) reported that rat liver desaturase was partially inhibited by N-ethylmaleimide and iodoacetate, and completely inhibited by p-chloromercuribenzoate (pCMB). The latter was reversible by 2-mercaptoethanol. Various levels of CPFA reduced the concentration of free sulfhydryl groups in solutions of a desaturase preparation, L-cysteine, and reduced glutathione (GSH).

James (52) using Chlorella and Pande and Meade (88) using rats were unable to show a sparing effect by GSH on the CPFA inhibition of desaturation. Pande and Meade found no decrease in sulfhydryl concentration in solutions of GSH or liver microsomes after treatment

with sterculate. They did see *in vivo* inhibition of the desaturase by CPFA. They found no inhibition at low substrate concentrations *in vitro*. Pande and Meade discounted the effects of sulfhydryl binding as nonspecific detergent effects of combined substrate and CPFA.

More recently, Fogerty *et al.* (40) have shown that CPFA at 0.001 mM concentration inhibit 39-48% of the desaturation of 0.1 mM stearic acid in hen liver preparations.

Raju and Reiser (99), in reply to Pande and Meade, reported that mouse liver desaturase was inhibited by 50% in the presence of 0.04 mM potassium sterculate. The substrate concentration was 0.05 mM. Three-tenths mM potassium oleate was required to cause the same degree of inhibition. One-tenth mM CPFA gave 77% inhibition while 0.1 mM oleate gave 11% inhibition. Variations in protein concentration had little effect on the degree of inhibition at 0.04 mM CPFA.

As noted previously (122), most *in vitro* work was performed with CPFA concentrations greater than substrate concentrations. In contrast, Johnson *et al.* (54) and Fogerty *et al.* (40) used substrate to CPFA ratios of 220:1 and 100:1, respectively. Their is probably the only work *in vitro* that approaches physiological reality.

Nixon *et al.* (81) recently reported findings that implicate CPFA in the loss of membrane-associated function in the rat. Noted were a 30% increase in the rate of 0.3 M glycerol induced erythrocyte

hemolysis, complete inhibition of glutathione-induced mitochondrial swelling and a 50% decrease in microsomal codeine demethylase activity. Each of these effects may be related to alterations in membrane fatty acid composition as suggested by 12% and 8% increases in saturated fatty acid of liver mitochondria and microsomes, respectively.

Inhibition of Other Enzyme Activity

Montgomery and Malevski (80), using MS, methyl oleate and pCMB as *in vitro* inhibitors of trout, rabbit and bovine lactate dehydrogenase (LDH), concluded that the inhibition by MS was not primarily due to its detergent effect.

Investigation by others has shown that CPFA inhibit castor bean lipase (85) and yeast alcohol dehydrogenase (98), both sulfydryl enzymes.

Work by Taylor *et al.* (123) showed that in most cases trout fed 100 or 200 ppm CPFA for at least 13 days had decreased activity and specific activity of liver glucose-6-phosphate dehydrogenase (G-6-PDH), NADP-linked isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase. G-6-PDH was particularly sensitive to the effects of CPFA.

Malevski *et al.* (76) have shown CPFA, after 36 days feeding, to decrease the activities of trout liver glutamate dehydrogenase and

alanine-aminotransferase, but not acetyl-CoA carboxylase.

Available evidence suggests that the effects of CPFA may be caused by sulfhydryl binding and/or an alteration in the synthesis of unsaturated fatty acid.

Effects of CPFA on Mitochondria

Struthers (122), in an attempt to locate the primary hepatic lesion caused by CPFA, noted general inhibition of phospholipid phosphate turnover in CPFA-fed trout. Cardiolipin, an important component of inner-mitochondrial membrane, was especially slow to turn over.

Examination of subcellular fractions of livers perfused with [1-^{14}C] oleate indicated that neutral and phospholipids of mitochondria from CPFA-fed fish were very poorly labeled when compared to controls. Control mitochondria phospholipid contained 16 times more label than CPFA-treated mitochondria phospholipid.

The marked effect of CPFA on mitochondrial lipid prompted an investigation of mitochondrial function. Struthers found variable but generally reduced P:O ratios from substrate pairs acetyl-CoA-citrate and pyruvate-glutamate in CPFA liver mitochondrial preparations. CPFA feeding also impaired the ability of mitochondria to oxidize oleate to CO_2. Finally, hepatic levels of ATP were reduced in CPFA-fed fish. The effects of CPFA were accentuated when fed

with protein high in the glucogenic amino acids proline and glutamic acid.

When fed to trout, chickens, rabbits and rats, CPFA are hepatotoxic, alter lipid metabolism and modify the activity of certain enzymes. CPFA are cocarcinogenic with aflatoxin in trout. Humans consume small amounts of CPFA and, at times, aflatoxin. These facts make the study of CPFA in a mammalian system important. The cocarcinogenic effects of CPFA in mice are currently being studied by others in this laboratory.

It is intended that the work reported here will add to the understanding of the effects of CPFA in all species, particularly with respect to alterations in membrane function.

EXPERIMENTAL

Animals

Weanling male and female white Swiss-Webster mice from the breeding colony of the Small Animals Laboratory, Department of Animal Science, Oregon State University were housed individually or in groups in cages with sawdust bedding. Weanlings were fed a standard lab chow for at least 1 week before being fed the semi-purified control and experimental diets. Diet and water were fed ad libitum. Both were changed daily and food consumption was measured each day. Individual or group weights were taken at least once a week.

Diet

The composition of the semipurified diet used in all experiments is shown in Table 1. The diet is essentially that of Krishnarao and Draper (65). The dry premix, vitamin mix and lipid were blended and mixed 1:1 (w/v) with 3.5% agar dissolved in distilled water. The hot mixture was allowed to solidify at 2-4°C. The solid diet was cut into blocks and stored in plastic bags at -30°C until used. The blocks were held at refrigerator temperatures while being used.

Table 1. Composition of dry mouse diet.

Ingredient	Percent of dry diet
Dry Premix	
Cornstarch	30.91
Dextrin[1]	34.41
Protein, Soy[2]	20.00
Cellulose	2.50
Methionine	0.05
$CaCO_3$	2.20
$Ca(H_2PO_4)_2 \cdot H_2O$	2.00
Mineral Mix[3]	0.73
Lipid[4]	5.00
Vitamin Mix[5]	2.20

[1]Cerulose, CPC International, Inc., Englewood Cliffs, New Jersey.

[2]Promine D, Central Soya, Chicago, Illinois.

[3]Mineral Mix composed of the following, in percent: 10.548 NaCl, 22.038 Na_2CO_3, 48.500 K_2CO_3, 14.300 $MgCO_3$, 0.529 $ZnCO_3$, 1.700 $FeSO_4 \cdot 7H_2O$, 0.270 $CuSO_4 \cdot 5H_2O$, 2.110 $MnSO_4 \cdot H_2O$, 0.005 KI.

[4]Control diets (MCO) contained 5% corn oil. Experimental diets (MSF) contained the given percent Sterculia foetida oil (55% CPFA) plus corn oil to provide 5% of the dry diet as lipid. For example, MSF 0.5% contained 0.5% SFO plus 4.5% corn oil, on a dry weight basis.

[5]Vitamin Mix (Vitamin Diet Fortification Mixture, ICN Nutritional Biochemicals, Cleveland, Ohio), in percent: 2.9487 Vitamin A concentrate (200,000 units/g); 0.1638 Vitamin D concentrate (400,000 units/g); 3.2763 Alpha Tocopherol; 29.4867 Ascorbic acid; 3.2763 Inositol, 49.1442 Choline chloride; 1.4743 Menadione; 3.2763 p-Aminobenzoic acid; 2.9487 Niacin; 0.6553 Riboflavin; 0.6553 Pyridoxine hydrochloride; 0.6553 Thiamine hydrochloride; 1.9658 Calcium pantothenate; 0.0131 Biotin; 0.0590 Folic acid; 0.0009 Vitamin B_{12}.

The experimental diet, designated MSF, was formulated by replacing a portion of the corn oil of the control diet, with SFO. MSF 0.5% refers to the experimental diet containing 0.5% SFO and 4.5% corn oil on a dry weight basis. The fatty acid composition of the lipid added to diets MCO, MSF 0.5% and MSF 1% is shown in Table 2.

Experiment 1. Effect of CPFA on Hemolysis of Erythrocytes

Five groups of 10 female mice, all 7 weeks old, were housed as groups and fed diets containing either 0, 0.1, 0.2, 0.5 or 1.0% SFO for 8 weeks.

Triplicate determinations of the maximum rate of hemolysis were made on erythrocytes from each of six or seven mice fed each diet. The rate of hemolysis was followed by recording the change in O.D.$_{600nm}$ per min of a solution containing 10 µl blood in 3 ml of either 0.3 M glycerol-0.05 M Tris-HCl, pH 7.4 or 0.3 M thiourea-0.05 M Tris-HCl, pH 7.4. Blood was taken from the tail with a heparinized micropipet and mixed with the hemolyzing solution in a cuvette held at 25°C in the constant temperature chamber of a recording spectrophotometer. Hemolyzing solution without added blood was used as a reference. Mice used in this experiment were returned to the appropriate dietary group for one additional week before being used in experiment 1a.

Table 2. Dietary lipid fatty acid composition.

Fatty acid	Diet		
	MCO[a]	MSF 0.5%[b]	MSF 1%
16:0	11.2	11.9	12.6
16:1	0.7	0.7	0.7
18:0	2.1	2.1	2.1
18:1	27.3	25.6	23.9
18:2	58.8	54.3	49.8
SA	-	4.8	9.7
MA	-	0.7	1.3

[a]From Nixon et al. (81).

[b]Composition calculated from FA composition of corn oil[a] and SFO from Roehm (104).
MSF 0.5% provides 0.27% of dry diet as CPFA
MSF 1% provides 0.55% of dry diet as CPFA

Experiment 1a. CPFA Accumulation and Effect on Fatty Acid Composition of Liver and Depot Fat

After 9 weeks, all mice fed in experiment 1 were weighed, decapitated, the livers weighed and livers and abdominal fat frozen at -30°C.

Lipid was extracted from tissue samples using a modified Folch procedure (Appendix I). A portion of the lipid extract was transesterified with 14% boron trifluoride-methanol and the fatty acid methyl esters analyzed by GLC (Appendix III).

The percent CPFA in depot fat samples was determined using the Halphen Test (Appendix II). The high background produced during the Halphen Test on liver lipid samples prevented the determination of their CPFA content.

Experiment 2. Effect of CPFA on Conversion of [^{14}C] Labeled Acetate and Palmitate to $^{14}CO_2$ and Lipid

The primary purpose of this series of experiments was to compare control and CPFA-fed mice in their ability to convert [^{14}C] acetate or [^{14}C] palmitate to $^{14}CO_2$. Four-week-old male mice were housed individually and fed the MCO or MSF 0.5% diet. Four experiments were run, three using [^{14}C] acetate and one using [^{14}C] palmitate. Each experiment involved two mice, an MCO-MSF 0.5%

pair, chosen on the basis of equal food consumption up to the time of the experiment. Experiments with [^{14}C] acetate involved three pairs of mice fed 9, 18 and 21 days. The experiment with [^{14}C] palmitate involved one pair of mice fed 31 days.

Before each experiment, mice were fasted 48 hours and refed a fat-free diet (MCO diet with lipid replaced by glucose) for 14 hours (overnight). This was done in an attempt to increase microsomal fatty acyl desaturase activity (51, 86).

Each timed experiment was started by injecting one mouse i. p. with 13.6 μCi [1-^{14}C] acetate (54.6 μCi/μmole, prepared by Doster (30)) in 0.25 ml aqueous solution (three experiments), or 61 μCi [1-^{14}C] palmitate (5.82 μCi/μmole, New England Nuclear, Boston, Mass.) sonicated in 0.5 ml 0.1 M phosphate buffer, pH 7.6 (one experiment). The mouse was immediately placed in one of two metabolism cages, arranged as shown in Figure 1. At a designated time thereafter the second mouse was treated similarly.

Air was drawn through the system by an aspirator and $^{14}CO_2$ was trapped in 40% potassium hydroxide. Radioactivity was recovered only in the 200 ml post-cage trap.

Ten ml aliquots were taken from the trap at timed intervals for 1 hour. Each aliquot was held in a screw-cap vial until $^{14}CO_2$ and total CO_2 could be determined in each sample (Appendices VII and IV,

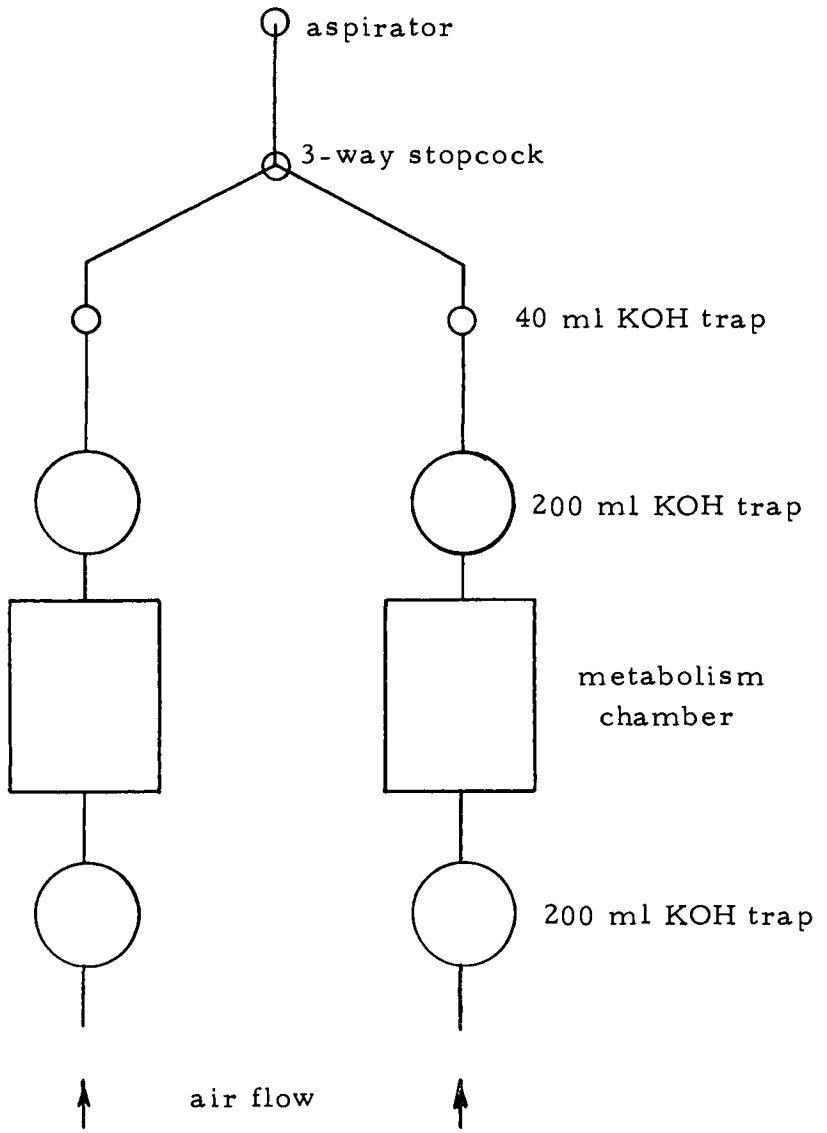


Figure 1. Schematic of apparatus for trapping CO_2.

respectively). Data were used to calculate the specific activity of $^{14}CO_2$ trapped at each time interval.

After the collection of carbon dioxide each mouse was weighed, decapitated, the liver weighed and all tissue frozen in liquid nitrogen and stored at -30°C. [^{14}C] in whole liver was determined by counting a solubilized aliquot of the whole liver homogenate. [^{14}C] associated with total liver lipid was determined on an aliquot of the modified Folch extract (procedures for determining [^{14}C] are described in Appendix VII).

The distribution of [^{14}C] acetate in classes of liver lipid was studied using TLC. A portion of the total liver lipid was concentrated, spotted on a Silica Gel G thin-layer plate (Appendix VI) and developed in two dimensions, first with hexane:diethyl ether:glacial acetic acid, 80:20:1 (v/v/v), and in the second dimension with chloroform: methanol:water, 65:25:4 (v/v/v). The plate was air dried, sprayed with 0.1% dichlorofluorescein in ethanol and lipid spots visualized under U.V. light. Radioactive spots were located with a radiochromatogram scanner (Varian-Berthold Series 6000-20, Varian Aerograph, Walnut Creek, Ca.).

[^{14}C] associated with unsaponifiable liver lipid was determined by counting an aliquot of the hexane extract of the saponification reaction mixture. After TLC separation of the hexane extract, the portion of the total [^{14}C] associated with the spot migrating with

authentic cholesterol was determined by radiochromatogram scanning (Appendix V).

Incorporation of [^{14}C] acetate or [^{14}C] palmitate into saturated and monounsaturated fatty acids was determined after separation of liver lipid methyl esters (Appendix III) on silver nitrate thin-layer plates (Appendix VI). Saturated, monoenoic and dienoic fatty acid methyl esters were separated completely. The portion of the total methyl ester radioactivity associated with saturated and monoenoic fatty acids was found by counting scrapings in Aquasol (Appendix VII).

Experiment 3. Effect of CPFA on P:O Ratios

P:O ratios were determined using mitochondria from male 5-week-old mice housed individually and fed either the MCO or MSF 1% diet. Food consumption data from the first experiment indicated that 1% SFO could be fed successfully for 2 weeks.

Three experiments were performed. Each experiment involved two MCO and two MSF 1% animals chosen on the basis of equal food consumption up to the time of the experiment. Experiments were performed after 6, 12 and 15 days on either diet. Animals were not starved or refed.

Mitochondria were isolated from each liver and used to determine P:O ratios by the method of Clark (17) (Appendix VIII).

Pyruvate and glutamate were used as substrates in each incubation. Incubations for each animal were prepared in triplicate on ice.

The inorganic phosphate content of an aliquot of each incubation mixture was determined before and after oxygen consumption was measured at 37°C with a respirometer (Appendix IX). Phosphate and oxygen uptake data were used to calculate P:O ratios.

Lowry (74) protein determinations (Appendix X) and oxygen consumption data were used to calculate mmoles oxygen consumed per minute per mg mitochondrial protein.

Histology

Selected liver samples were fixed in Bouin's solution, sectioned and stained with hematoxylin and eosin. Other liver samples were fixed in 10% formalin and frozen sections were stained for lipid with Oil-Red-O.

RESULTS AND DISCUSSION

Feeding

An attempt was made to monitor food consumption using the semipurified dry diet described in Table 1. The animals' feeding habits hampered this approach so the diet was presented in solid form.

Mice were weaned on a commercial lab chow for at least 1 week before the semipurified diets were fed. Mice accepted the diets more readily when this procedure was followed.

The average daily consumption (calculated weekly) of control and experimental diets containing up to 0.5% SFO was statistically similar for at least 9 weeks. Some animals did not accept the diet containing 1% SFO; those that did consumed amounts comparable to controls. The MSF 0.5% diet was used in experiments lasting more than 2 weeks.

It should be noted that, in experiments involving a control-experimental pair of mice, the pair was chosen on the basis of equal food consumption up to the time of the experiment. This method of animal selection would be expected to give a minimum difference in the results from each pair of animals; each experimental animal was one of those whose food intake was least affected by CPFA.

Growth

Compared to controls, mice fed 0.5% SFO showed slightly reduced growth rates during the initial growth period (Figure 2). The reduced growth rate for CPFA-fed mice was due primarily to reduced efficiency of feed conversion (10.4 vs. 13.3 g dry diet/g weight gained for controls vs. 0.5% SFO-fed, respectively).

After feeding 3 weeks, 1% SFO caused weight loss in many mice and some died by 9 weeks. Mice with an average food consumption comparable to controls showed normal weight gains and appeared to be in good condition.

Table 3 shows the increase in liver weight, as a percent of body weight, caused by dietary CPFA. Compared to controls, mice fed 0.5% SFO for 9 weeks had increased, but not significantly different, liver-to-body-weight ratios. The increase in liver weight was significant for mice fed 1% SFO ($p < 0.05$) when compared to controls. Mice were sacrificed before harmful effects of dietary CPFA were apparent. Apparently, certain groups of mice were more susceptible than others to the effects of 1% SFO.

Accumulation of CPFA in Tissue

CPFA made up 8% of the adipose tissue lipid from mice fed 1% SFO for 9 weeks. There was a direct relationship (Figure 3) between

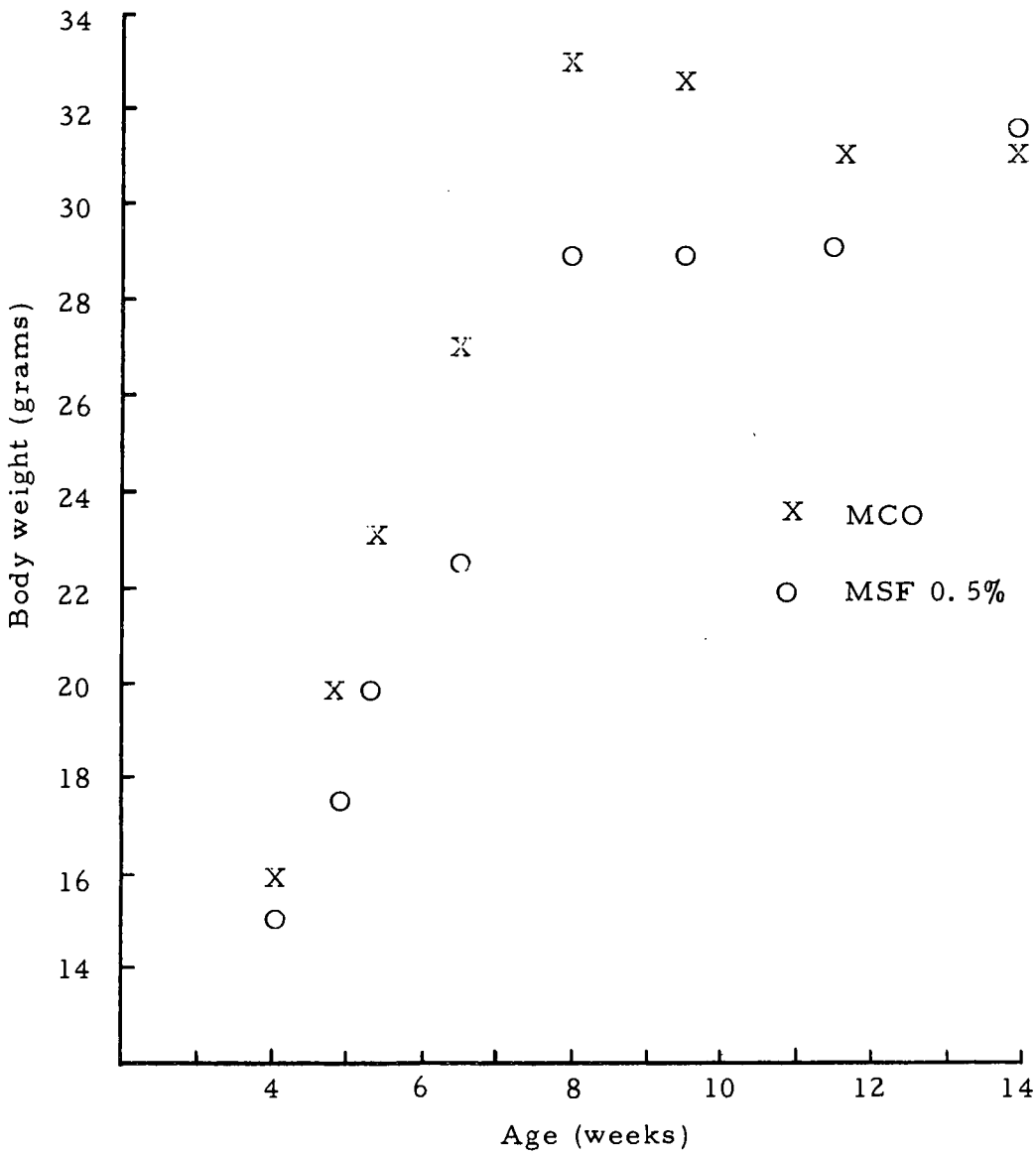


Figure 2. Effect of 0.5% SFO on growth rate of mice.

Table 3. Effect of CPFA on liver weight as a percent of body weight of mice fed 4 and 9 weeks.

Diet	Liver as a percent of body weight	
	4 weeks	9 weeks
MCO	4.51 ± 1.20 (18)[a]	4.02 ± 0.38 (10)
MSF 0.5%	5.36 ± 0.93 (17)	5.03 ± 0.85 (6)
MSF 1%	5.53 ± 0.57 (16)[b]	

[a]Mean ± standard deviation, number of animals in parentheses.

[b]$p < 0.05$

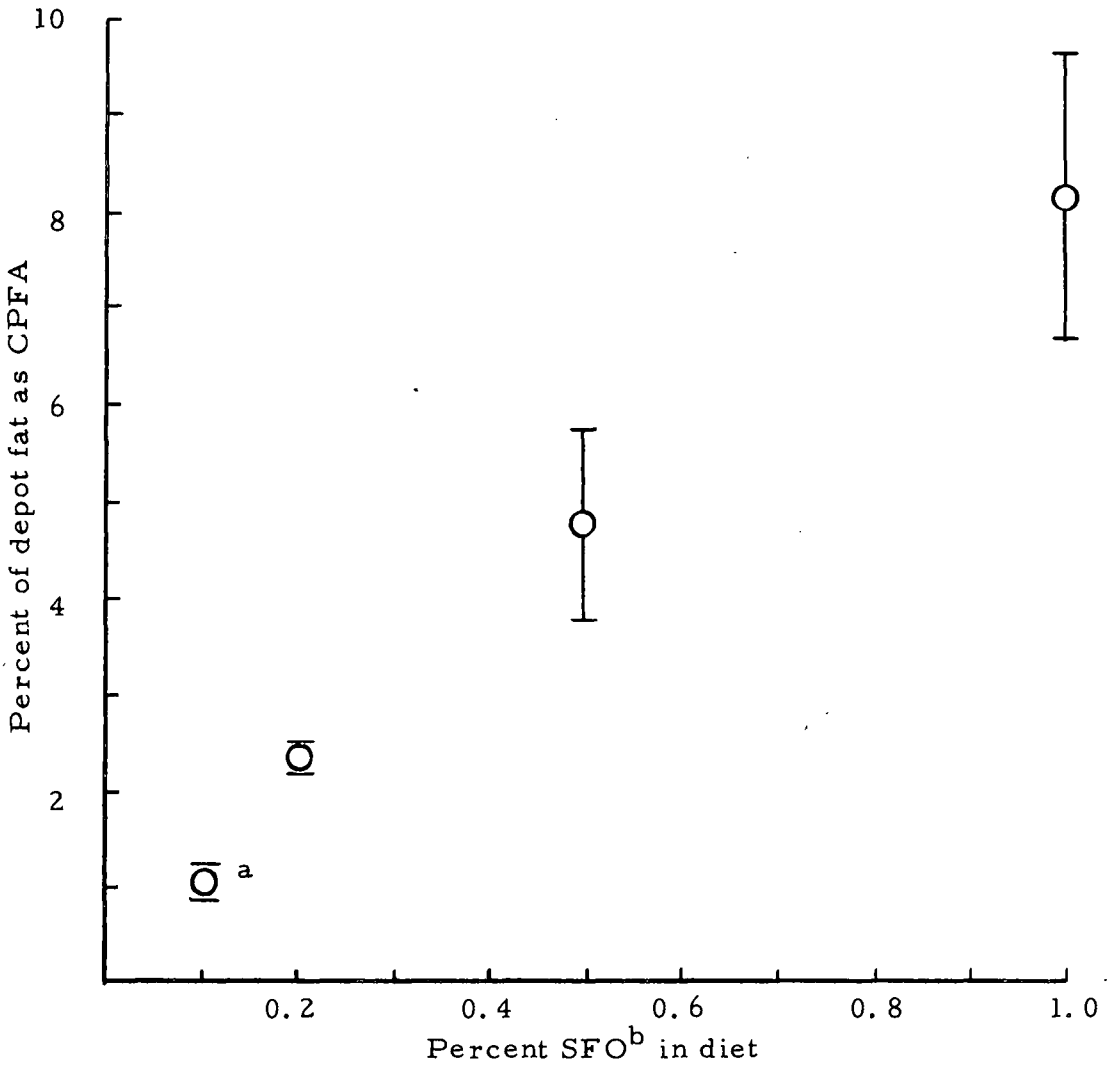


Figure 3. Accumulation of dietary CPFA in mouse depot fat.

[a]Mean ± standard deviation

[b]SFO ≅ 55% CPFA

percent dietary CPFA and the percent CPFA found in body lipid stores. Mobilization of depot lipid may provide a constant supply of CPFA to other tissues. The half-life of depot lipid in the mouse is about 5 days (125).

Effect of CPFA on Fatty Acid Desaturation

Tables 4 and 5 show the average fatty acid composition of liver and depot fat from mice fed up to 1% SFO. The data from experiment 1a are summarized in Table 6.

The increase in dietary CPFA caused an increase in the concentration of saturated fatty acids in both liver and depot fat. As little as 0.1% SFO (about 0.05% CPFA) had a noticeable effect in both tissues.

Table 6 shows that CPFA had a pronounced effect on the ratios of stearate to oleate and palmitate to palmitoleate. These results are in agreement with data of previous workers showing that CPFA increase levels of long-chain saturated fatty acids at the expense of their monounsaturated forms in animals fed CPFA.

The ratio of linoleate to arachidonate was also increased in mice receiving CPFA. The apparent inhibition by CPFA of the conversion of linoleate to arachidonate was noted previously in rats (21).

Table 4. Average percent fatty acid composition of mouse liver lipid.[a]

Diet	% SFO	Fatty acid								
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4
MCO		0.41	19.20	3.21	15.02	20.24	24.49	T[b]	0.37	16.65
MSF	0.1	0.71	20.75	1.95	16.21	20.05	27.57	T	1.04	11.62
MSF	0.2	0.62	24.67	0.76	24.52	14.99	21.68	T	T	12.79
MSF	0.5	1.16	20.47	1.17	16.74	18.39	31.87	0.59	1.42	8.54
MSF	1.0	0.40	22.05	0.76	22.74	12.28	26.54	0.43	0.33	14.15

[a]Average of at least five mice fed each diet for 9 weeks.

[b]Less than 0.25%.

Table 5. Average percent fatty acid composition of mouse depot lipid.[a]

Diet	% SFO	Fatty acid							
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1
MCO		1.19	16.17	6.90	2.82	36.14	35.82	1.71	0.50
MSF	0.1	1.28	19.55	3.46	11.36	30.45	31.04	2.28	0.43
MSF	0.2	1.57	19.05	2.44	15.81	26.02	29.87	1.32	0.43
MSF	0.5	1.34	20.68	2.40	14.92	26.46	30.98	1.63	0.73
MSF	1.0	1.41	23.55	2.11	17.81	25.55	27.94	0.88	0.58

[a]Average of at least five mice fed each diet for 9 weeks.

Table 6. Effect of various levels of dietary CPFA on the level of total saturated fatty acids and the ratios of 16:0/16:1 and 18:0/18:1 in liver and depot fat.[a]

Diet	% SFO	Liver lipid			Depot fat		
		16:0/16:1[b]	18:0/18:1	Total % saturated fatty acids	16:0/16:1	18:0/18:1	Total % saturated fatty acids
MCO		5.98	0.74	34.6	2.34	0.08	20.1
MSF	0.1	10.64	0.74	37.7	5.65	0.37	32.2
MSF	0.2	32.46	1.64	49.8	7.81	0.61	36.4
MSF	0.5	17.50	0.91	38.4	8.62	0.56	36.9
MSF	1.0	28.30	1.85	45.2	11.16	0.70	42.8

[a]Calculated from data, Tables 4 and 5.

[b]Ratio of percents.

CPFA seem to inhibit the conversion of long-chain fatty acids to their more unsaturated forms. If this effect is evident in the fatty acid composition of membranes, CPFA may be associated with alterations in membrane function.

The average fatty acid composition of membrane lipid largely determines the physical properties of the membrane (67, 94). Membrane physical properties and lipid microenvironment affect membrane-associated enzyme activity (31, 63, 89).

Evidence has been provided for the effect of CPFA on membranes. CPFA increased the percent saturated fatty acids in lipid from rat microsomes and mitochondria (81) and rabbit erythrocyte ghosts (38). CPFA altered membrane-bound enzyme activity of rat microsomes and mitochondria (81).

Experiment 1 was designed to determine the rate of hemolysis of mouse erythrocytes in 0.3 M glycerol and thoiurea. These data (Table 7) suggest that dietary CPFA reduced the permeability of the erythrocyte membrane to glycerol or thiourea. Erythrocytes that showed rates of hemolysis significantly slower than controls were taken from mice (dietary groups MSF 0.2% and MSF 1%) that had the highest levels of saturated fatty acids in liver lipid (Table 5). The permeability of membranes to glycerol was shown by McElhaney et al. (78) to be dependent on the cholesterol content and degree of saturation and chain length of the component fatty acids.

Table 7. Effect of dietary CPFA on the rate of erythrocyte hemolysis.[a]

	Diet (fed 8 weeks)				
	MCO	MSF 0.1%	MSF 0.2%	MSF 0.5%	MSF 1.0%
Glycerol	1.04	0.96	0.90[b]	1.04	0.90[b]
Thiourea	3.30	3.11	2.93[b]	3.12	2.80[b]

[a]Rate of hemolysis expressed as change in OD_{600nm} per min in either 0.3 M glycerol-0.05 M Tris-HCl, pH 7.4, or 0.3 M thiourea-0.05 M Tris-HCl, pH 7.4.

[b]Significantly ($p < 0.05$) slower rate of hemolysis when compared to controls (MCO).

Lack of Evidence for a Desaturase Independent Pathway to Monoenes in Mice

Conflicting reports have appeared concerning the ability of CPFA to inhibit the incorporation of [^{14}C] acetate into monoenoic fatty acids. Donaldson (27) and Raju and Reiser (94) suggested a pathway to the long-chain monoenoic fatty acids that was independent of the CPFA inhibited microsomal desaturase. This alternate pathway reportedly involved the elongation of a medium-chain monoene to palmitoleic, oleic or vaccenic acids by a soluble anaerobic system.

One purpose of experiment 2 was to determine the presence or absence of the alternate pathway in CPFA-fed mice. The results (Figure 4) indicated that dietary CPFA greatly reduced the incorporation of exogenous [^{14}C] palmitate and [^{14}C] acetate into long-chain monoenoic fatty acids of mouse liver. Seventeen percent of the dpm associated with control liver fatty acids were found in monoenes when [^{14}C] palmitate was the precursor, and an average of 12% were found in monoenes when [^{14}C] acetate was the precursor. In CPFA-fed animals approximately 1% of the liver fatty acid dpm were found in monoenes when either [^{14}C] palmitate or [^{14}C] acetate was the precursor.

These data are consistent with results of experiment 1a, and suggest that, when fed to mice, CPFA inhibit the formation of long-chain monoenes. We saw essentially no desaturation of a long-chain

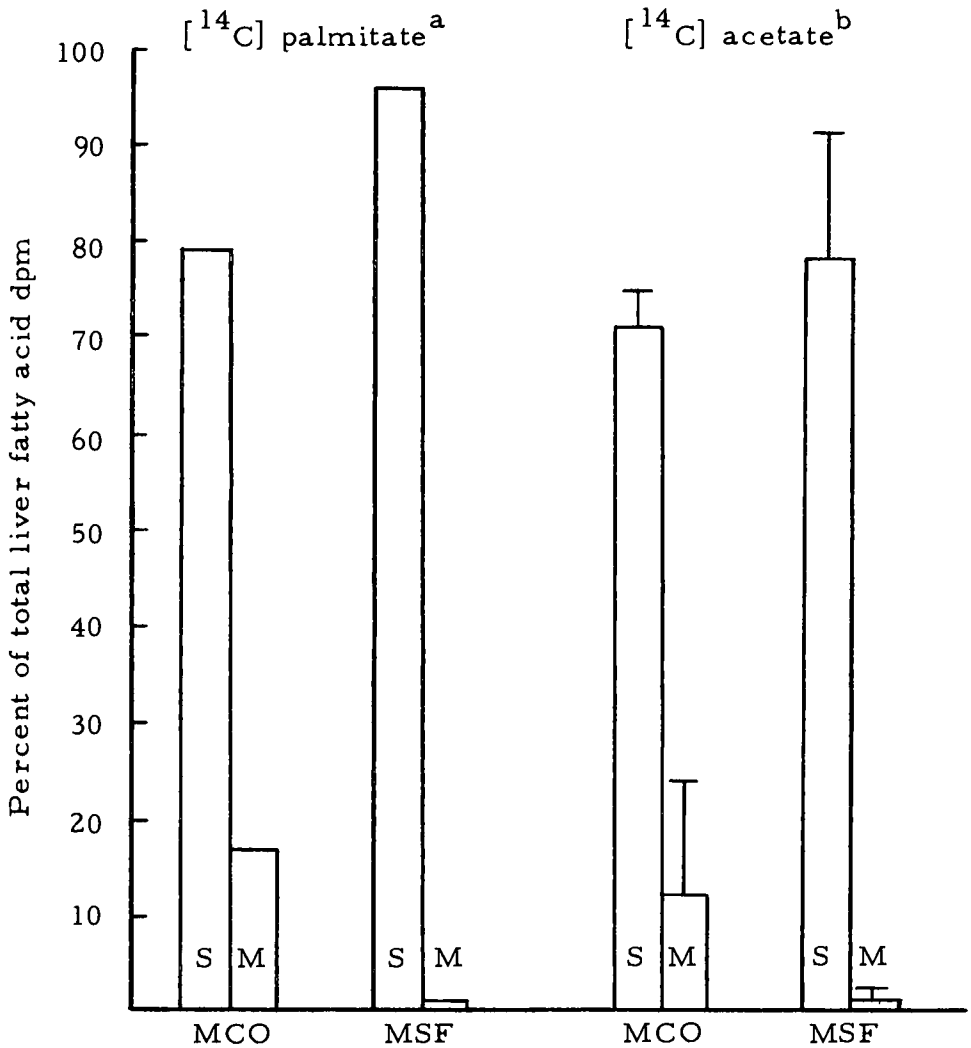


Figure 4. Effect of CPFA on the synthesis of saturated (S) and monounsaturated (M) fatty acids from [^{14}C] acetate and [^{14}C] palmitate.

[a] One experiment

[b] Mean plus standard deviation of three experiments

fatty acid, or elongation of a shorter-chain unsaturated fatty acid with [^{14}C] acetate. These results are consistent with the findings of Pearson et al. (91) and do not support the hypothesized alternate pathway to monoenes. If the more primitive elongation pathway does exist in mice, our experimental techniques have inhibited its expression.

Effects of CPFA on Lipid Synthesis

Additional data from experiment 2, Table 8, show the effect of CPFA on the synthesis of total liver lipids from [^{14}C] acetate and [^{14}C] palmitate, and the accumulation of lipid in each liver. Total liver lipid was more abundant in CPFA-fed mice. Figures 5 and 6 show the accumulation of lipid droplets in liver tissue from selected mice fed 0.5% SFO for 5 weeks. Oil-Red-O was used to stain lipid in frozen liver sections.

Table 8 also shows the specific activity of liver lipid synthesized from [^{14}C] acetate or [^{14}C] palmitate. The specific activity of lipid synthesized with [^{14}C] palmitate was lower in the CPFA-fed animal because of the high percentage of liver lipid. The specific activity of lipid synthesized from [^{14}C] acetate was reduced in the MSF animals because of both lipid accumulation and decreased incorporation of [^{14}C] into lipid. Bartley et al. (9) have shown correlations between the rate of lipid synthesis from [^{14}C] acetate and the

Table 8. Effect of CPFA on the accumulation and specific activity of liver lipid.

	Fed 9 days[a]		Fed 18 days[a]		Fed 21 days[a]		Fed 31 days[b]	
	MCO	MSF 0.5%[c]	MCO	MSF 0.5%	MCO	MSF 0.5%	MCO	MSF 0.5%
mg lipid/liver	35.0	52.5	77.5	95.0	45.0	87.5	23.5	36.0
mg lipid/g liver	25.0	32.6	38.8	52.8	26.0	43.8	12.0	22.0
dpm/mg lipid x 10^{-3}	29.4	4.6	3.6	2.0	5.3	1.8	151.0	102.0
percent of ^{14}C dose recovered in liver lipid	3.4	0.8	0.9	0.6	0.8	0.5	2.6	2.7

[a]Radioactive precursor was [^{14}C] acetate.

[b]Radioactive precursor was [^{14}C] palmitate.

[c]Each experiment involved a control (MCO) - experimental (MSF 0.2%) pair of mice.

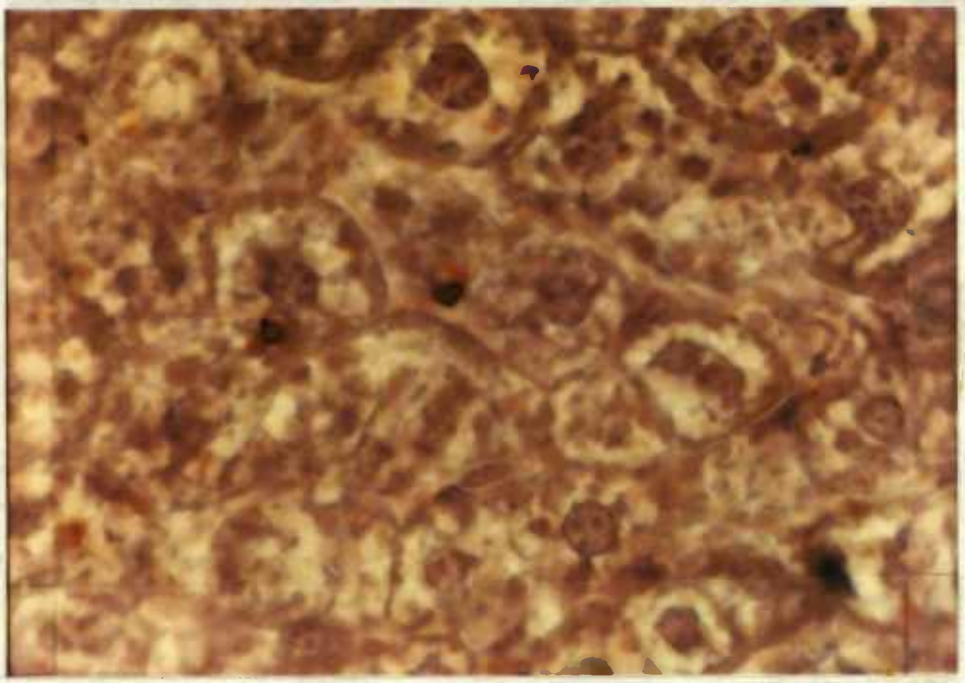


Figure 5. Liver section from control mouse stained for lipid with O-Red-O.

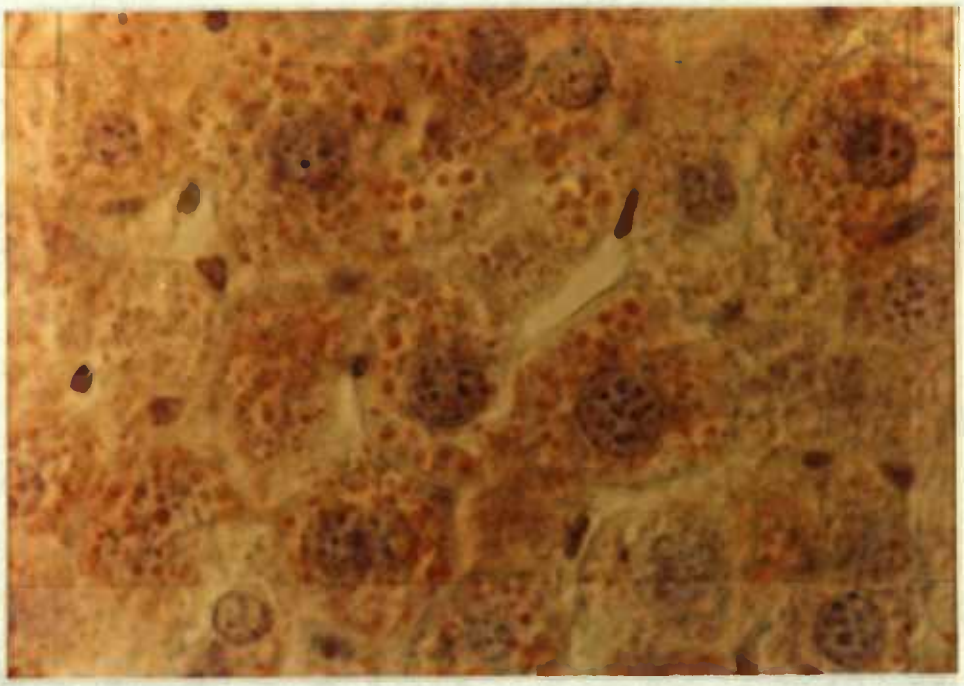


Figure 6. Liver section from mouse fed CPFA stained for lipid with O-Red-O. Shows accumulation of lipid droplets.

activity of five enzymes of lipid synthesis in mice and rats. Although lipid synthesis from acetate appears to be inhibited by dietary CPFA, we know nothing about the dilution of [^{14}C] acetate by endogenous acetate.

Lipid accumulation in the liver is generally caused by one or a combination of the following factors: increased synthesis of lipid in the liver, increased mobilization of lipids from depot tissue to the liver, or decreased excretion of lipid from the liver (72, 125). Assuming that lipid synthesis in the liver was decreased by CPFA, lipid accumulation was due to increased lipid mobilization to and/or decreased lipid excretion from the liver. Decreased excretion of lipid from the liver is linked to reduced liver protein synthesis; lipids are excreted as lipoproteins. There is no evidence for or against decreased liver protein synthesis in mice fed CPFA, but reduced protein synthesis was found in CPFA-fed trout with fatty livers (76). (Data from experiment 3 showed that liver mitochondria protein levels were similar for control and experimental mice.)

CPFA are hepatotoxins as evidenced by their ability to increase liver to body weight ratios and cause fatty livers. Histological evidence in all species has shown CPFA to be hepatotoxic. Hepatotoxins increase lipid mobilization to the liver (125).

Radiochromatogram scans were made of control and experimental liver lipids separated with relatively polar and nonpolar

solvents. When [^{14}C] acetate was the precursor, scans showed no large differences in the distribution of radioactivity between various classes of lipid, with one exception. The percent of total lipid dpm found in sterols was greater in CPFA-fed mice. Table 9 shows this difference as determined by the procedure in Appendix V.

[^{14}C] acetate was more readily used for sterol synthesis in CPFA-fed mice. These data support the results of previous workers (38, 43), who suggested that dietary CPFA caused increased cholesterol synthesis.

Conversion of Labeled Acetate and Palmitate to $^{14}CO_2$

Experiment 2 was designed primarily to detect any obvious difference in the ability of control and experimental mice to convert acetate or palmitate to CO_2.

Table 10 shows the specific activity of $^{14}CO_2$ collected at timed intervals from control and CPFA-fed mice injected with [^{14}C] acetate. In each of three experiments the specific activity of the $^{14}CO_2$ from the control animal reached a maximum greater than that from the experimental animal. In two of three cases the control mouse was able to reach this maximum more rapidly than the experimental mouse. It appears as though control animals incorporated acetate into compounds involved in reactions producing CO_2 more

Table 9. Effect of CPFA on the incorporation of [^{14}C] acetate into liver sterols.[a]

Days fed	MCO	MSF 0.5%
9	1.00	4.13
18	4.56	11.00
21	8.38	9.80
32[b]	1.01	8.87

[a]Expressed as percent of total liver lipid dpm in sterols.

[b]An additional MCO-MSF 0.2% pair of mice was used in verification.

Table 10. Effect of CPFA on the conversion of [^{14}C] acetate to $^{14}CO_2$.[a]

Sample time (min)	Fed 9 days[b]		Fed 18 days		Fed 21 days	
	MCO	MSF 0.5%	MCO	MSF 0.5%	MCO	MSF 0.5%
20	4.51	2.25	7.93	2.62	7.57	3.60
30	7.39	4.82	7.88	5.63	14.20	10.90
45	6.67	5.09	5.05	5.95	8.02	5.63
60	5.00	4.64	5.05	5.81	4.60	3.20

[a] Expressed as specific activity (mCi/mmole x 10^3) of $^{14}CO_2$ trapped up to the given time after i. p. injection of 13.6 μCi [^{14}C] acetate.

[b] Each experiment involved a control (MCO) - experimental (MSF 0.5%) pair of mice.

readily than CPFA-fed animals or those compounds were metabolized more rapidly in control mice.

Similar results (Table 11) were obtained in one experiment with [^{14}C] palmitate. Again, the experimental animal was unable to oxidize the substrate as rapidly as the control animal.

After activation and transfer into the mitochondria, palmitate is cleaved into acetate units for entry into the Krebs Cycle via condensation with oxalacetate. Injected [^{14}C] acetate enters the Krebs Cycle in the same way after activation to acetyl-CoA. Any process hindering the flow of intermediates through the Krebs Cycle would be expected to retard the conversion of palmitate or acetate to CO_2.

Significance of the Observed Effects

CPFA are consistently noted for their ability to inhibit the formation of unsaturated fatty acids. This effect may be significant when related to the fact that variations in the fatty acid composition of a tissue lipid affect the activity of enzymes from that tissue. Membrane-dependent enzymes may be particularly susceptible to changes in the degree of saturation of membrane fatty acids.

The present results suggest an effect of CPFA on mitochondrial enzyme activity. The altered disposition of acetate (retarded oxidation, reduced incorporation into total liver lipid, and increased incorporation into liver sterols) is symptomatic of reduced

Table 11. Effect of CPFA on the conversion of [^{14}C] palmitate to $^{14}CO_2$.[a]

Sample time (min)	Fed 31 days	
	MCO	MSF 0.5%
15	2.39	0.27
30	2.43	0.72
45	2.84	1.89
60	2.70	2.21

[a]Expressed as specific activity (mCi/mmole x 10^3) of $^{14}CO_2$ trapped up to the given time after i.p. injection of 61 μCi [^{14}C] palmitate.

carbohydrate utilization and/or increased mobilization of lipid to the liver (125). The reduced availability of carbohydrate would depress citrate lyase activity and retard operation of the Krebs Cycle (47, 125). The accumulation of fatty acids and the decreased level of cytoplasmic citrate and isocitrate would limit the activity of acetyl-CoA carboxylase, a rate-limiting enzyme of fatty acid synthesis. As a result of reduced oxidation or use in fatty acid synthesis, acetate would be channeled to the formation of β-hydroxy-β-methylglutaryl CoA, the precursor of sterols.

Altered Oxidative Phosphorylation

This experiment was performed to further investigate the possibility that CPFA have an effect on mitochondria.

Table 12 shows the effect of 1% dietary SFO on the capacity of liver mitochondria for oxidative phosphorylation. After 6 to 15 days on 1% dietary SFO, all animals produced a liver mitochondrial fraction with reduced capacity for oxidative phosphorylation. The decrease in the P:O ratio was due primarily to decreased phosphorylation; oxygen consumption per min per mg mitochondrial protein was similar for mitochondrial preparations from control and experimental animals.

Similar results were reported by Haslam et al. (48) and Proudlock et al. (93) for unsaturated fatty acid (UFA) auxotrophs of

Table 12. Effect of CPFA on liver mitochondria P:O ratios and oxygen consumption.

Days on diet	P:O ratio (μ mole P_i/μ atom O)		Oxygen consumption (mmole/min/mg mitochondrial protein x 10^4)	
	MCO	MSF 1%	MCO	MSF 1%
6	2.83[a]	1.09	1.03	1.12
	2.36	1.06	2.60	1.85
12	2.52	1.32	1.30	1.14
	2.30	1.14	1.17	0.72
15	2.85	1.45	1.21	1.02
	2.55	1.38	0.95	0.93

[a]Two MCO and two MSF 1% mice were used in each of three experiments. Triplicate incubations were prepared from the liver of each mouse. Triplicate results were averaged to give each value shown.

Saccharomyces cerevisiae. Inhibition of phosphorylation, but not respiration, paralleled the decrease in mitochondrial UFA. The defect in phosphorylation was purely a lipid lesion reversible by increasing the UFA content of the mitochondria.

Racker's group (59) has demonstrated the requirement for UFA in the phospholipid of preparations capable of performing coupling reactions associated with oxidative phosphorylation.

The maintenance of proper levels of UFA appears to be important to the production of ATP. CPFA may affect oxidative phosphorylation by decreasing the availability of necessary UFA.

SUMMARY

The primary objective of this work was to determine the susceptibility of mice to CPFA fed as glycerides of SFO. After mice were found to be susceptible to SFO, preliminary investigations were used to further define the effects of CPFA.

The susceptibility of mice to dietary CPFA was determined by following parameters used previously in investigations of CPFA-fed animals. Between 0.1 and 1.0% SFO (approximately 0.05-0.5% CPFA) caused an increase in the ratio of long-chain saturated to unsaturated fatty acids from liver and depot lipid. CPFA were shown to accumulate in the lipid stores of mice fed each level of CPFA.

When compared to controls, 5-week-old mice fed 0.5% SFO showed a slightly reduced growth rate due to decreased efficiency of feed conversion. Many mice fed 1.0% SFO stopped growing and died by the end of a 9-week feeding period. Some mice did not seem to be affected adversely by 1.0% dietary SFO. Liver-to-body-weight ratios were increased by feeding 0.5 or 1.0% SFO. Histological examination of livers from mice fed 0.5 and 1.0% SFO showed no characteristic abnormalities other than lipid accumulation.

A possible effect of dietary CPFA on erythrocyte membrane fatty acid composition was demonstrated when erythrocytes from

CPFA-fed mice hemolyzed more slowly than control erythrocytes in isotonic solutions of glycerol and thiourea.

Additional investigation showed that CPFA inhibited the synthesis of long-chain monoenes via desaturation of long-chain saturated fatty acids or by elongation of medium-chain monoenoic fatty acids. These results are in agreement with the report of Pearson et al. (91).

CPFA altered the distribution of acetate through three major pathways for its use. The conversion of the label of [^{14}C] acetate to $^{14}CO_2$ and lipid was reduced and incorporation into sterols was increased in CPFA-fed mice. This pattern of distribution of acetate is symptomatic of the reduced utilization of carbohydrate and/or increased lipid mobilization to the liver (125). Fatty livers were found in CPFA-fed mice.

The observed decrease in the oxidation of labeled palmitate supported the suggestion (122) that CPFA affect the mitochondria. Further evidence for this effect was provided by an experiment showing consistently low P:O ratios in liver mitochondria from mice fed CPFA. Normal respiration with reduced ATP formation suggested the presence of an uncoupler or the lack of necessary unsaturated fatty acids.

SUGGESTIONS FOR FUTURE WORK

Mice appear to be relatively sensitive to the effects of CPFA. Their ease of handling, low maintenance cost and the fact that they are mammals should make mice a good choice as an experimental animal for further investigations.

If an effect of CPFA on membranes is related to altered enzyme activity, it will be important to determine more precisely the effect of CPFA on specific membrane lipid class and fatty acid composition. The relationship between lipid composition and enzyme activity should be determined in a system free of CPFA, since cyclopropenes may directly affect enzyme activity.

Whether CPFA alter enzyme activity directly or through an effect on membranes, their apparent ability to inhibit ATP synthesis deserves more attention. An effect of CPFA on energy metabolism has implications for the control of metabolic pathways. CPFA have already been shown to inhibit enzymes associated with the regulation of glycolysis and the Krebs Cycle in trout (76, 122, 123).

The characterization of a urinary metabolite of SA by Yoss et al. (127) may provide clues to the mechanism of action of CPFA. The metabolite appears to be a dicarboxylic cyclopropane acid resembling intermediates of the Krebs Cycle.

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APPENDICES

APPENDIX I

LIPID EXTRACTION

Lipid was extracted from liver and adipose tissue using a modification of the method of Folch et al. (41).

Tissue was homogenized in 20 volumes (w/v) of chloroform: methanol, 2:1 (v/v) with a Tissumizer model STO homogenizer (Tekmar Co., Cincinatti, Ohio). The homogenate was filtered into a graduated cylinder and the residue rinsed with additional solvent. Lipids in the filtrate were partitioned into the chloroform rich phase by adding 0.2 volumes of 0.5% sodium chloride solution or distilled water. The phases were allowed to separate overnight at 2-4°C, after which the upper phase (methanol-water rich) was removed. The lower phase was filtered through anhydrous sodium sulfate into a pear-shaped flask. The lipid extract was evaporated under vacuum almost to dryness a number of times, each time adding benzene. Lipids were stored in benzene or n-hexane under nitrogen in screw-cap vials at -30°C.

APPENDIX II

HALPHEN TEST

This procedure is essentially that of Hammonds et al. (46).

Between 5 and 100 mg lipid, depending on CPFA concentration, was weighed into a screw-cap tube (Kimax Pyrex, 15 x 415 mm) and mixed with 0. 1 ml 4% morpholine in n-butanol and 4. 9 ml n-butanol. Exactly 1. 0 ml of 1% sulfur in carbon disulfide (prepared just before use) was added under subdued light, the tube was sealed with a teflon-lined cap and the contents thoroughly mixed. The tube was heated in the dark in an oil bath at 110°C for 110 min.

A standard of CPFA glycerides was run with each group of samples.

After heating, the tubes were cooled to room temperature in tap water, 10 ml of n-butanol was added to each tube and the absorbance at 495 nm read on a spectrophotometer.

The % CPFA in a sample was calculated using the following formula:

$$\frac{(\text{wt. standard})\,(A_{495}\ \text{sample})}{(\text{wt. sample})\,(A_{495}\ \text{standard})} \times \%\ \text{CPFA in standard} \times 100$$

$$= \%\ \text{CPFA in sample}$$

APPENDIX III

PREPARATION OF FATTY ACID METHYL ESTERS AND THEIR ANALYSIS BY GAS LIQUID CHROMATOGRAPHY

Transesterification with Borontrifluoride in Methanol

Three to five drops of lipid in a screw-cap test tube (Kimax Pyrex, 15 x 415 mm) were mixed with 1.0 ml benzene, 2.0 ml dry methanol and 1.0 ml 14% (w/v) borontrifluoride in methanol (Applied Sciences Laboratories, State College, Pa.). The tube was sealed with a teflon-lined screw-cap, shaken well and heated in a boiling water bath for 20-25 min. The tube was cooled and about 2 ml saturated sodium sulfate solution plus 10 ml diethyl ether were added. The tube was capped and shaken. After the phases separated, the ether was transferred with a disposable capillary pipette to a second screw-cap test tube. The reaction mixture was extracted again with 10 ml diethyl ether, the ether extracts combined, washed with 10 ml distilled water and transferred to a pear-shaped flask. The solvent was removed almost to dryness.

Fatty acid methyl esters were stored in n-hexane under N_2 in screw-cap vials at $-30^{\circ}C$.

Analysis of Methyl Esters by GLC

The instrument used was an Aerograph model 600-B gas chromatograph with a flame ionization detector. The recorder was a Barber-Coleman Model 8000-2700 with 1 millivolt full-scale sensitivity. Approximately 0.3 μl fatty acid methyl ester was injected and separated on an aluminum column 9 ft by 0.085 in. I.D. packed with 15% DEGS on Anakrom 545A 110/120 (Analabs, Inc., North Haven, Conn.) operated isothermally at 195°C. The injector temperature was 220°C. The carrier gas was nitrogen at a flow rate of 28 ml/min.

Peaks were identified by comparison of retention times with those of a known standard mixture. Peak areas were calculated by disc integrator or triangulation and the percent of each fatty acid expressed as the percent of total peak areas.

APPENDIX IV

DETERMINATION OF TOTAL CARBON DIOXIDE IN POTASSIUM HYDROXIDE TRAPPING SOLUTION

At specified intervals, 10 ml aliquots of 40% potassium hydroxide were taken from carbon dioxide traps and stored approximately 2 hours in screw-cap vials. Samples were taken from each vial for determination of $^{14}CO_2$ (Appendix VII) and total CO_2. At least three determinations of total carbon dioxide were made on solution from each vial.

For each determination 1.0 ml trapping solution was pipetted into the outer well of a Warburg flask with a sidearm. The flask was positioned on the manometer of the respirometer (Gilson Differential Respirometer, model G-20, Gilson Medical Electronics, Middleton, Wisc.) with the operating valve open (see Directions for Operation (25)). After addition of 1.0 ml 4 N sulfuric acid to the sidearm, the sidearm stopper was inserted in the closed position. The flask was equilibrated by shaking (120 oscillations/min) in a 25°C circulating water bath for about 10 min. The manometer valve was closed and the manometer fluid adjusted to the reference mark. Carbon dioxide was released by tipping sulfuric acid into the outer well of the flask. The flask was shaken for an additional 30 min. The total µl carbon dioxide released from 1.0 ml trapping solution was read after

readjusting the manometer fluid to the reference mark. The equivalent μl carbon dioxide at standard temperature and pressure was calculated using the conversion factor defined in the Directions for Operation (25) and Appendix VIII (P_w = 23.8 at 25°C).

Calculation of the total carbon dioxide trapped up to a particular time, included correction for previously removed trapping solution.

APPENDIX V

DETERMINATION OF [^{14}C] IN LIVER STEROLS

The saponification procedure described below is taken from Johnson (57).

Less than 10 mg of liver lipid (known dpm) was mixed with 4.7 ml 95% ethanol and 0.3 ml 33% potassium hydroxide in a capped test tube (Kimax Pyrex screw-cap test tubes, 14 x 415 mm). The mixture was heated in a 55°C water bath for 30 min and cooled. Five ml water was added and the unsaponifiable material extracted with three 10 ml volumes of hexane. Hexane extracts were combined, the hexane removed under vacuum and the unsaponifiable fraction made to 1.0 ml with hexane. An aliquot of this solution was counted in toluene fluor, the rest was evaporated to a small volume and spotted on a Silica Gel G plate. The plate was developed with hexane:diethyl ether:acetic acid, 80:20:1 (v/v/v), air dried and spots visualized with dichlorofluorescein under UV light. Radioactivity was located by a scan (Varian-Berthold series 6000-20 radiochromatogram scanner, Varian Aerograph, Walnut Creek, Ca.) and found to be associated with the spot comigrant with cholesterol.

The [^{14}C] recovered in the aqueous phase of the saponification mixture was determined by counting an aliquot in Aquasol. The recovery of [^{14}C] in the hexane and aqueous phases was always greater

than 95%. [^{14}C] found in sterols was expressed as % of total dpm in liver lipid.

APPENDIX VI

THIN-LAYER CHROMATOGRAPHY

The following procedures were described by Shenstone (114).

Silica Gel G Plates

A slurry of Silica Gel G (EM Laboratories, Inc., Elmsford, N. Y.) in distilled water, 45:85 (w/v) was spread 250 μ thick with a plate spreader (C. Desaga, Heidelburg, W. Germany). The plates were allowed to air dry and were activated before use at 130°C for 45 min. Vertical lanes were scored in each plate before spotting lipids under a stream of nitrogen. Neutral lipids were separated by development in hexane:diethyl ether:glacial acetic acid, 80:20:1 (v/v/v). Polar lipids were separated with chloroform:methanol: water, 65:25:4 (v/v/v).

Chromatography tanks were lined with paper.

After development, spots were visualized by spraying the air dried plate with 0. 1% dichlorofluorescein in ethanol and viewing under UV light.

Silver Nitrate Plates

A slurry of Silica Gel G in 5% silver nitrate solution, 36:80 (w/v) was spread 250 μ thick. Plates were allowed to air dry in the

dark and were activated just before use at 130°C for 30 min. Silver nitrate plates were developed twice in the same direction to at least 15 cm in a paper-lined tank containing petroleum ether:diethyl ether, 92:8 (v/v). The tank was covered to exclude light.

Spots were visualized with dichlorofluorescein and UV light.

APPENDIX VII

LIQUID SCINTILLATION COUNTING PROCEDURES

Fluor Solutions

Toluene: 6 g PPO(2, 5-diphenyloxazole, Sigma Chemical Co., St. Louis, Mo.) plus 50 mg POPOP (1,4-bis[2-(5-phenyloxazole)] benzene, scintillation grade, Nuclear Chicago, Des Plaines, Ill.) per liter of scintillation grade toluene.

Aquasol: a xylene based fluor solution capable of solubilizing organic and aqueous solutions (New England Nuclear, Boston, Mass.). Aquasol forms a clear thixotropic gel with about 25% aqueous solution. Experimentation is necessary to find the proper ratio of aqueous solution to Aquasol so that phases do not separate.

Sample Preparation

Whenever possible, samples were counted in disposable Kimble 1 dram Opticlear glass vials (15 x 45 mm) with polyethylene caps (Kimble, Toledo, Ohio). The vial with sample was placed in a 20 ml screw-cap vial for counting.

Lipids were counted directly in either Aquasol or toluene fluor solution.

Tissue samples were solubilized in NCS tissue solubilizer (Amersham/Searle, Arlington Heights, Ill.) according to instructions.

Generally, wet tissue was digested overnight at about 40°C with five parts NCS (w/v or v/v) in a 20 ml counting vial. The cooled digest was counted in 15 ml toluene fluor.

Carbon dioxide trap samples were counted by mixing 0.2 ml trapping solution with 1.0 ml water and 3.5 ml Aquasol in a disposable vial. The solution was a milky liquid at room temperature and a clear gel at counting temperature, 40°F. Samples were dark-adapted overnight at this temperature before counting.

TLC scrapings were shaken with 15 ml Aquasol in a 20 ml counting vial to extract lipid (66, 124). Samples were counted after the scrapings settled.

All radioactivity is expressed as dpm calculated by means of quench correction curves for toluene and Aquasol fluor solutions.

APPENDIX VIII

DETERMINATION OF P:O RATIOS

P:O ratios were determined using a mitochondrial fraction of mouse liver and the substrate pair pyruvate-glutamate as described by Clark (17). All procedures were performed at 2-4°C unless otherwise stated.

Mitochondrial Preparation

Animals were stunned by a blow to the head, decapitated and the liver excised, weighed and rinsed in oxygenated (95% oxygen-5% CO_2) 0.25 M sucrose-0.001 M EDTA, pH 7.4 (sucrose-EDTA). The liver was minced with scissors and homogenized with a Potter-Elvehjem homogenizer in four volumes (w/v) sucrose-EDTA by six passes with a loosely fitting teflon pestle.

The homogenate was centrifuged (Sorvall model SS-3) 15 min at 600 x g to remove cell debris. The 600 x g supernatant was centrifuged 15 min at 12,000 x g. The resulting mitochondrial pellet was resuspended in a volume (v) of sucrose-EDTA equal to the discarded 12,000 x g supernatant and again centrifuged at 12,000 x g. The pellet was suspended in another volume (v) of sucrose-EDTA and used as the mitochondrial preparation.

Incubation Mixture

Each incubation mixture was prepared in a Warburg flask with a center-well. The following solutions were added to the outer well of each flask: 0.25 ml 0.25 M sucrose-0.001 M EDTA, pH 7.4; 0.5 ml 0.1 M potassium phosphate buffer, pH 7.4; 0.1 ml 0.2 M sodium hydrogen glutamate; 0.1 ml 0.5 M sodium pyruvate (prepared 1 hour before); 1.0 ml cofactor mixture (prepared just before use) containing, in μmoles per ml, 3 ATP, 15 $MgCl_2$, 150 glucose, 0.3 NAD^+, 3 EDTA. A fluted filter paper wick was inserted in the center-well containing 0.3 ml 6 N potassium hydroxide.

Just before the timed experiment was started, 0.5 ml of the mitochondrial preparation was added to each flask and the incubation mixture swirled to disperse all components evenly. The rest of the mitochondrial preparation was frozen for protein determination later (Appendix X). A 0.2 ml aliquot of the incubation mixture was dispersed in 4.8 ml cold 10% trichloroacetic acid (TCA) in a conical centrifuge tube. The next day, after centrifugation (International clinical centrifuge model CL), an aliquot of the TCA was used for phosphate determination (Appendix IX).

At the beginning of the timed experiment, 10 units hexokinase (Hexokinase Type F-300, Sigma Chemical Co., St. Louis, Mo.) in 0.05 ml 0.1% glucose was added to each incubation mixture at 30 sec

intervals. Each flask in turn was sealed and fixed to a designated manometer of the respirometer (Gilson Differential Respirometer, Gilson Medical Electronics, Middleton, Wisc.) (operating valve must be open, see Directions for Operation (25)) and allowed to equilibrate with shaking (120 oscillations/min) in a 37°C circulating water bath for approximately 6 min. Beginning at 6 min, the manometer systems were closed sequentially at 30 sec intervals and the manometer fluid adjusted to the reference mark. The micrometer reading was recorded at this time and at 6 min intervals for up to 54 min, keeping the manometer fluid at the reference mark.

At the end of the experiment, flasks were removed at 30 sec intervals and a second 0.2 ml aliquot of the incubation mixture was dispersed in 4.8 ml cold 10% TCA for phosphate determination.

The barometric pressure (P_b) during the manometric measurement of oxygen consumption was used to calculate the equivalent µl oxygen at standard temperature and pressure (STP) as described in the Directions for Operation (25). The micrometer reading was multiplied by the following factor to give µl oxygen at STP.

$$\frac{(273)\ (P_b - 3 - P_w)}{(t + 273)\ (760)} = \text{multiplying factor}$$

where

P_b = barometric pressure in mm Hg

P_w = 47.1 mm at t = 37°C

t = 37°C

As suggested by Clark (17), oxygen consumption was plotted with time and total consumption determined by extrapolating the curve back to time zero.

To calculated the P:O ratio for each animal, the difference between the average μmoles phosphate of triplicate mixtures before and after incubation was divided by the average μatoms oxygen consumed by those incubation mixtures.

APPENDIX IX

INORGANIC PHOSPHATE DETERMINATION

This and other applications of the Fiske-Subbarow reagent (39) for phosphate determination were described by Bartlett (8).

Fiske-Subbarow Reagent

To approximately 150 ml of 15% sodium bisulfite was added 0.5 g 1-amino-2-naphthol-4-sulfonic acid. This mixture was thoroughly shaken and mixed with 1.3 g sodium sulfite. The clear solution (gentle warming may be necessary) was made to 200 ml, filtered and stored in a brown bottle in a warm part of the room. If kept near 70 °F the reagent is stable for at least 1 week.

Procedure

All glassware was acid washed (dilute HCl) before use to remove phosphate.

To 0.5 ml sample was added with mixing, in the following order: 7.9 ml glass distilled water, 0.4 ml 10 N sulfuric acid and 0.8 ml 2.5% ammonium molybdate solution. At 30 sec intervals 0.4 ml Fiske-Subbarow reagent was added to each tube and after 10 min the absorbance at 660 nm was read using a Beckman DB spectrophotometer. Potassium phosphate at concentrations between 0.02-0.08 μmole/ml was used as a standard.

APPENDIX X

LOWRY PROTEIN DETERMINATION (74)

Reagents

A. 2% sodium carbonate in 0. 1 N sodium hydroxide

B. 0. 5% copper sulfate pentahydrate in 1% sodium tartrate

C. 50 ml A. plus 1 ml B.

D. Commercial Fishers Phenol Reagent 2 N (Folin-Ciocalteau, Fisher Scientific Co., Fair Lawn, N. J.) diluted 1:1 with distilled water

Procedure

Aliquots of mitochondrial preparation were diluted 1:100 with distilled water. One ml of the dilute protein solution was mixed with 5 ml reagent C and held at room temperature for 10 min to allow protein to solubilize. Five-tenths ml reagent D was added and after 30 min at room temperature the absorbance of the solution was read at 700 nm. Bovine serum albumin at concentrations between 25-500 μg/ml was used as a standard.