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Title: PLASMA CHOLESTEROL LEVELS, ATHEROSCLEROSIS AND LIVER DAMAGE IN RABBITS FED CYCLOPROPENOID FATTY ACIDS

Abstract approved: D. J. Lee

In this five-week feeding study male New Zealand rabbits were fed diets containing cyclopropene fatty acids (CPFA), diets containing cholesterol, and diets with both CPFA and cholesterol added to test the effect of CPFA on cholesterol metabolism and atherosclerosis induction. When CPFA-fed animals were compared with control animals, they tended to have higher plasma cholesterol levels, higher liver cholesterol levels and a high incidence of aortic atherosclerosis. Control animals had no atherosclerosis. A similar pattern was seen when animals fed cholesterol were compared with those fed both cholesterol and CPFA. Mean triglyceride levels were higher in CPFA-fed rabbits than in controls.

A wide range of sensitivity to the CPFA was observed. Some animals had plasma cholesterol levels eight to ten times normal after three weeks of feeding; others showed only slight changes after five

weeks. Histological examination of liver cells from CPFA-fed animals showed evidence of alteration in cellular morphology and, in some cases, extensive damage. In some liver cells of CPFA-fed rabbits, subcellular organelles appear to be aligned in fiber-like structures. The most extreme signs of toxicity were noted in livers of rabbits fed CPFA and cholesterol.

After five weeks on experimental diets, adipose tissue of the rabbits contained approximately 2% CPFA. Ratios of 16:0 to 16:1 and 18:0 to 18:1 fatty acids increased in liver lipid and erythrocyte ghost lipid after CPFA feeding. Over the five-week study there were no differences in rate of weight gain or in feed conversion ratios in CPFA-fed animals.

Plasma Cholesterol Levels, Atherosclerosis and Liver Damage in Rabbits Fed Cyclopropenoid Fatty Acids

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PLASMA CHOLESTEROL LEVELS, ATHEROSCLEROSIS AND LIVER DAMAGE IN RABBITS FED CYCLOPROPENOID FATTY ACIDS

INTRODUCTION

The cyclopropenoid fatty acids (CPFA), sterculic and malvalic, occur naturally in most plants of the order Malvales. Seed oil of the cotton plant (Gossypium hirsutum) contains a small amount of both the 18 carbon malvalic acid and the 19 carbon sterculic acid. The cyclopropene ring is a highly strained and reactive moiety, but the naturally-occurring acids are stable as the glycerol esters. In most experimental animals, intact cyclopropene rings can be detected in adipose, liver and other tissues after CPFA are fed.

The presence of these unusual fatty acids in a major food oil makes them of interest to food scientists and toxicologists. The discovery in 1966 that CPFA can act as a potent co-carcinogen in rainbow trout has stimulated interest in the study of these lipids.

There have been many reports of adverse physiological effects occurring when oils containing CPFA or pure sterculic acid have been fed to animals. Abnormalities in reproductive processes and alterations in lipid metabolism were the main effects noted in early investigations. In more recent work, the effects of CPFA feeding have been studied extensively in rats and trout. It has been well

established that the ratio of saturated to unsaturated fatty acids increases in both species after CPFA feeding. Other physiological changes which occur include fatty infiltration of the liver and striking morphological changes in liver cell structure. CPFA do not appear to act as a co-carcinogen in rats as they do in rainbow trout.

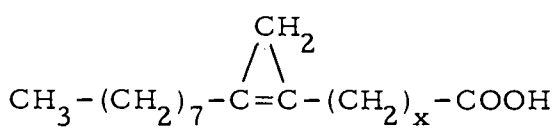
One interesting physiological effect of CPFA feeding has been largely overlooked. A paper published in 1966 described how CPFA could alter cholesterol metabolism in cockerels. CPFA-fed cockerels had increased serum cholesterol levels and a significant increase in incidence of atherosclerosis.

Preliminary studies in this laboratory showed that New Zealand rabbits were extremely sensitive to dietary CPFA. This suggested that rabbits might be a good experimental animal for studies of CPFA and their effects on cholesterol metabolism and atherosclerosis induction in mammals. This report describes the semi-purified diet developed as a vehicle for feeding CPFA and the pronounced effects of CPFA on cholesterol levels, atherosclerosis induction and liver morphology in rabbits over a five-week exposure.

LITERATURE REVIEW

Cyclopropenoid Fatty Acids, Occurrence and Analysis

The Halphen test, developed in 1897 to detect cottonseed oil in other vegetable oils (42), is one of the most common methods of detecting cyclopropenoid fatty acids (CPFA) in oils or fats. It has been shown that the Halphen reaction, described in a later section, is specific for a substituted cyclopropene ring structure (12). Faure (32) reported in 1956 that sterculic acid gave a positive Halphen test. One year later Macfarlane et al. (62) showed malvalic acid also was Halphen-positive. These two fatty acids, sterculic and malvalic, are the most common naturally-occurring substances known to contain the cyclopropene structure. Sterculic acid was first isolated by Nunn (73); malvalic acid was isolated and characterized by Macfarlane et al. (62). The structures of both acids have been confirmed by several authors (12, 28, 62, 95).



Malvalic acid	X=6
Sterculic acid	X=7

In a 1965 review, Phelps et al. (79) lists 46 species of plants

containing oils which give positive Halphen reactions. Nearly all the plants listed by Phelps are of the order Malvales. The plants of this order which are important sources of food oils are cotton (Gossypium hirsutum) and kapok (Bombax oleogineum).

The most important food oil in the United States containing CPFA is cottonseed oil. This oil ranks third in consumption behind soybean and corn oils and is used in salad oils and dressings, cooking oils, vegetable shortenings and margarines. CPFA content of crude cottonseed oil ranges from 0.56 to 1.5% (8) for malvalic acid and 0.3 to 0.5% for sterculic acid (95). Cottonseed meal also contains small amounts of CPFA and is used as a protein source in animal feeds. Kapok oil is a major food oil in Japan. Production in 1969 was 10,000 tons and it is used in margarine, shortenings and cooking oils (52).

Another oil containing CPFA is Sterculia foetida (Java olive) seed oil. This oil contains approximately 49% sterculic acid and 6% malvalic acid (85) and is often used as an experimental source of these acids.

The review of Phelps and co-authors mentioned previously contains the best available account of plant species containing CPFA. They also discuss the findings of studies concerned with the biological effects of CPFA ingestion by animals. A more recent review of cyclopropene and cyclopropane fatty acids can be found in a book by Christe (19) published in 1970, which discusses the occurrence,

isolation, structural determination, analysis and synthesis of both compounds. A general review of CPFA was written by Struthers (101) in 1973.

The Halphen Test

The Halphen test (42) originated when it was discovered that a red pigment developed when cottonseed oil, sulfur, carbon disulfide and alcohol were heated together. It was shown many years later that fatty acids containing a substituted cyclopropene ring gave a positive Halphen test (12).

The original procedure has been modified many times in order to develop a reliable method for determining CPFA content of oils. Bailey et al. (9) in 1965 described a spectrophotometric method for determining CPFA content of cottonseed oils based on the Halphen reaction. In 1971, Hammonds et al. (43) published details of a modification of Bailey's method which has proven sensitive and reliable.

In 1972, Coleman and Firestone (23) studied a closed-tube system similar to the one of Hammonds et al. and reported lower limits of sensitivity to be 18 and 15 μg CPFA/g oil for quantitative and qualitative analyses, respectively. Coleman (22) in a 1973 report demonstrated good agreement in the results of 12 laboratories analyzing nine samples containing 0 to 0.19% CPFA. All labs used the method of Coleman and Firestone.

The only non-cyclopropenoid compound known to give a positive Halphen test was ethyl-2, 3-decadienoate (51). This compound or closely related compounds are not known to occur naturally. The few naturally-occurring allenic compounds are fungus metabolites.

HBr-HCl Titrations

Hydrogen bromide titration was developed as a method of CPFA analysis after it was noted sterculic acid interfered with the Durbetaki titration of oxygen (33). One mole of CPFA reacts slowly but quantitatively with one mole of HBr in glacial acetic acid. Generally, samples are dissolved in glacial acetic acid and benzene, then titrated with 0.1N HBr in glacial acetic acid using a crystal violet indicator.

In 1968, Feuge et al. (33) described the HBr titration as the simplest and most convenient of analytical methods available for CPFA. Since 1968 little research has been done on the HBr method, and the Halphen spectrophotometric method has been adopted as the easiest and most precise at low levels of CPFA.

Hydrogen chloride also reacts in a quantitative manner with the cyclopropene ring (63). The analytical method developed using the HCl titration is tedious and appears to be inferior to procedures using hydrogen bromide.

Nuclear Magnetic Resonance

Pawlowski et al. (77) have developed a method to measure CPFA at levels of 1 to 100% using nuclear magnetic resonance (NMR). The authors state that the NMR method gives results which compare well with those obtained using the Halphen method. At 10% CPFA, the accuracy of this method is $\pm$ 0.5%. Accurate analysis of samples containing less than 1% CPFA is not possible.

Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) is one of the strongest analytical tools available. Attempts to develop a GLC method for CPFA determination have met with limited success. At the high temperatures necessary in GLC analysis of lipids, the cyclopropene ring is broken and several rearrangement products are formed (104). Several attempts have been made to alter the ring so it will be stable during analysis. Shenstone et al. (96) demonstrated hydrogenation of the cyclopropenes prevented decomposition on the GLC column. Other modifications of the cyclopropene ring have included reaction with low molecular weight sulfhydryls (81) and reaction with silver nitrate followed by hydrohalogenation (50). Schneider et al. (91) reacted CPFA with silver nitrate in methanol to form the ether and ketone derivatives. They claim these products can be successfully separated by GLC on 15% diethyl glycol succinate columns and

samples containing from 0.1% to 100% CPFA can be analyzed. However, these methods have not replaced the Halphen procedure, mainly due to lack of accuracy at low levels of CPFA.

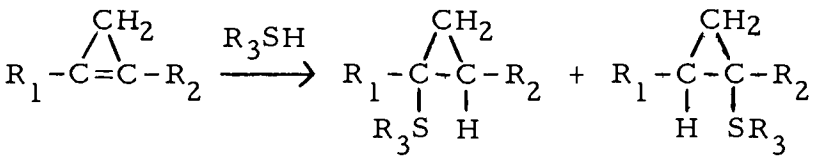
Chemistry of CPFA

The CPFA are unique in that they contain a reactive three-membered ring which includes a double bond. This gives the molecule some unusual properties. Carter and Frampton (17) reviewed the chemistry of cyclopropenoid compounds in 1962.

Hydrogenation of the cyclopropene to the cyclopropane cannot be carried out under normal conditions without destroying the ring. Palladized calcium carbonate has been used successfully to hydrogenate sterculic acid (73, 96). Hydrohalogenation occurs rapidly and quantitatively, and is the basis for several methods of CPFA analysis (33, 63).

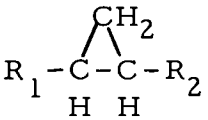
Nunn (73) reported a high molecular weight polymer was formed from sterculic acid held at room temperature. In 1960 Rinehart and co-workers (84) reported that this polymerization occurred with rearrangement of the cyclopropene ring.

In 1964 Kircher (53) demonstrated the *in vitro* addition of simple sulfhydryls to methyl sterculate without ring opening. He suggested this type of addition might occur *in vivo* and may be related to the biological activity of CPFA.



Ory and Altschul (74) found that sterculic acid could inhibit a microbial lipase enzyme and they describe sterculic acid as a general sulfhydryl reagent. They reported no inhibition of the SH-containing lipase if cysteine was added to the reaction mixture.

Cyclopropane fatty acids are similar to cyclopropene fatty acids and occur in many species of bacteria (20). The most common example of a cyclopropane fatty acid is lactobacillic acid (cis-11, 12-methylene octadecanoic acid). A general formula for cyclopropanes is shown below:



The distribution (108) and biosynthesis (109) of cyclopropene and cyclopropane fatty acids in plants were discussed in 1972 by Yano et al. Wood and Reiser (107) studied the metabolism of cyclopropane fatty acids in rats. They concluded that these fatty acids did not affect lipid metabolism the way CPFA do and accumulation of 3, 4-methylene dodecanoic acids in adipose tissue suggests the inability of β -oxidation to proceed past the cyclopropane ring. These results were confirmed by Chung (20).

Physiological Effects

Interest in the physiological effects of CPFA ingestion grew out of the observation that eggs from chickens fed plants of the order Malvales or oils from those plants developed a pink discoloration of the white after storage. This was demonstrated in 1928 by Sherwood (97) for cottonseed oil and meal, and by Lorenz *et al.* (60) for *Malva parviflora*.

Lorenz *et al.* (60) suggested in 1933 that the pink white disorder might be caused by the Halphen-reactive material in the cottonseed meal or oil. Shenstone and Vickery (95) isolated the Halphen-positive material from several plants and identified them as two cyclopropenoid fatty acids, malvalic and sterculic acid. These acids fed in daily doses of 25 mg or less produced the typical symptoms of the pink white disorder.

Shenstone and Vickery (95) also showed that the hydrogenation of malvalic and sterculic acids to the corresponding cyclopropanes eliminated their Halphen reactivity and prevented them from causing pink whites in hens eggs. Nordby *et al.* (72) showed through the use of esters, ethers, alcohols and hydrocarbons that the terminal group of sterculic acid was not critical in the Halphen reaction or the pink white disorder. This work helped establish that the cyclopropene ring was the active moiety in the pink white disorder.

Schaible and Bandemer (89) determined that the pink color was due to the combination of iron, from the yolk, with an egg white protein, conalbumin. They assumed the diffusion occurred because of an alteration in permeability of the egg vitelline membrane due to CPFA ingestion.

As early as 1926 it was reported that cottonseed meal in animal rations could cause the animals to produce fat with a high melting point. Deuel (26) reviewed several papers on this subject in 1955. A 1931 paper by Ellis et al. (29) reported the storage fat of pigs fed rations containing at least 4% cottonseed oil contained more stearic and linoleic acids and less oleic acid. Other studies (15) have shown that cows fed a ration containing 6% cottonseed oil produced milk fat with a significantly elevated level of stearic acid. This milk fat produced butter with a sticky, putty-like consistency.

In 1961 Evans et al. (30) described the effects of CPFA on egg lipids. They found that the stearic acid concentration of yolk lipids was increased with a nearly equal decrease in oleic acid. Linoleic acid increased slightly. The magnitude of change in stearic:oleic acid ratio in egg lipid fractions was in the following order: sterol > triglyceride > di- and monoglyceride. Only small changes were seen in cephalin and lecithin fractions. Evans et al. (31) also reported increased stearic:oleic acid ratios in lipids from plasma, liver, ovaries, heart and adipose tissue when hens were fed cottonseed oil

or S. foetida seeds. There was a marked increase of linoleic acid in plasma and ovaries. A 1970 report (1) showed blood plasma fatty acid ratios change more rapidly than egg lipid fatty acid ratios in S. foetida-fed hens. At 0.1% S. foetida oil, plasma stearic acid levels were twice normal levels after two days.

Altenburger et al. (5) studied the distribution of ^{14}C-sterculic acid in chickens. They reported that in one chicken given an intravenous dose of methylene-^{14}C-sterculic acid and held on control diet for five days, the largest concentration of label was recovered in skin fat and abdominal fat. Approximately 3% of the administered activity was recovered as labeled CO_2.

In 1964 Reiser and Raju (83) first presented evidence suggesting the increase in stearic acid noted in animals fed CPFA was due to the inhibition of the enzyme responsible for desaturating stearic acid to oleic acid. Johnson et al. (49) reported that sterculic and malvalic acids could inhibit the in vitro desaturation of ^{14}C-stearic acid to oleic acid in hen liver preparations.

One year later, Raju and Reiser (82) showed that CPFA would inhibit the conversion of ^{14}C-stearic acid to oleic acid in rat liver. They found that incorporation of ^{14}C-acetate into oleic acid was not inhibited in rats fed CPFA for seven days and proposed the existence of an alternate pathway of oleate synthesis. Allen et al. (4) confirmed earlier reports that loss of desaturase ability occurred in livers of

hens fed CPFA. They showed methyl sterculate to be a stronger inhibitor than methyl malvalate and claimed that kinetic data indicated the inhibition observed was irreversible. They demonstrated that possible metabolites of CPFA (dehydrosterculic acid and two isomers of 9 methyleneoctadec-10-enoic acid) did not inhibit desaturase activity at equivalent concentrations.

In 1967 Donaldson (27) reported that S. foetida oil increased liver stearate levels and inhibited conversion of labeled palmitate to palmitoleate in chicks. Later he observed that both chronic and acute dosage of CPFA resulted in inhibition of ^{14}C-palmitate incorporation into palmitoleate but the incorporation of ^{14}C-acetate into monoene fatty acids seemed to be effected only by an acute dose of CPFA. Donaldson said the alternative pathway of monoene synthesis suggested by Raju and Reiser (82) may be a repressed system which becomes active upon chronic CPFA dosing.

James et al. (48) studied sterculic acid as an inhibitor of unsaturated fatty acid biosynthesis in plants. They found that in the algae, Chlorella vulgaris, sterculic acid was a potent inhibitor of the desaturation of stearic acid but no inhibition of acetate incorporation into oleic acid was observed. These workers claim that a molar ratio of 2:1 inhibitor to precursor reduced stearate to oleate conversion to 25% of normal levels and a 20:1 ratio was necessary to cause any reduction in oleate to linoleate conversion in Chlorella vulgaris.

Glutathione added in concentrations equal to sterculic acid concentrations did not reduce inhibition. The authors claimed their results do not support the theory that sterculic acid is a general SH-enzyme inhibitor and they suggest an acyl transferase may be the enzyme blocked by sterculic acid.

Recently, Pearson et al. (78) published data showing that in chicken egg yolk, oleic acid synthesis was inhibited by dietary CPFA regardless of whether the precursor was stearate or acetate. This report was in agreement with data presented by Bickerstaffe et al. (11) who indicated that in goats there was no evidence for an alternate pathway of monoene synthesis.

Fogerty et al. (34) found that ring position in CPFA was important in determining whether or not inhibition will occur. They found that acids with the cyclopropene in the 8, 9; 9, 10 or 10, 11 position were effective inhibitors of desaturase activity in hen liver preparations. CPFA with the ring at the 11, 12 position was not inhibitory to the desaturase enzyme.

The inhibition of the desaturase system seems to occur in most experimental animals fed CPFA. The characteristic increase in the stearic to oleic fatty acid ratio has been reported in cows, rats and chickens, as mentioned, and in trout (86).

One of the earliest reports of experiments in which CPFA were fed to rats was a paper by Sheehan et al. (93) in 1965. He reported a

delay in sexual maturity in female rats fed S. foetida oil. Schneider et al. (90) found male weanling rats fed S. foetida oil at levels of 5% of the diet and above died with symptoms characteristic of B vitamin deficiency. Levels below 5% resulted in poor growth.

In an attempt to elucidate the cause of pre- and postnatal mortality of offspring from CPFA-fed rats, Miller et al. (64) studied the fetuses and newborn rat pups of CPFA-fed females grossly and histologically. They found 3% S. foetida oil prevented reproduction and levels of 1% caused pre- and postpartum death of the offspring. Degeneration of liver and kidney tissue was seen in the newborn pups. Hemorrhages into the lung alveoli appeared to be the cause of most postpartum deaths.

In 1971 Coleman and Friedman (24) studied fatty acids in tissue lipids of rats fed S. foetida oil for 34 weeks. They found dietary levels of 2% S. foetida oil caused increased levels of saturated fatty acids and decreased unsaturated fatty acid levels in heart, liver and adipose tissue. These tissues also contained Halphen-positive material (indicating the presence of intact CPFA). Increased fat was found in liver and heart tissue of experimental animals.

Nixon et al. (71) described effects in rats fed CPFA which were believed to be due to altered lipid metabolism and possible alteration of membranes. Erythrocyte hemolysis in 0.3 M glycerol was increased, glutathione-induced mitochondrial swelling was completely

inhibited and microsomal codine demethylase activity depressed in CPFA-fed rats. They confirmed earlier reports of large changes in saturated to unsaturated fatty acid ratios in rats fed CPFA. Increased liver weight to body weight was also noted.

In other studies of CPFA in mammals (11) it was shown that intravenous infusions of sterculic acid caused goats to produce milk with increased amounts of stearic acid. Sterculic acid had little effect on oxidation of acetate or on plasma lipid fatty acid composition.

Sinnhuber et al. (98, 99) reported that CPFA greatly enhanced the incidence and growth of aflatoxin-induced hepatoma in rainbow trout. A purified ration containing S. foetida oil (220 ppm CPFA) and 4 ppb aflatoxin B1 induced an 83% incidence of gross liver tumors in six months. Liver cells of fish receiving only B1 showed only slight damage. Lee et al. (55) found similar effects using Hibiscus syriacus oil which contained 2% sterculic acid and 19% malvalic acid. Two reports (36, 56) indicate that CPFA have little or no co-carcinogenic activity with aflatoxin when fed to rats.

It has also been shown that trout fed CPFA have increased saturated to unsaturated fatty acid ratios in adipose and liver lipids and morphological changes in liver cells including lipid infiltration and the formation of fiber-like structures. Recent work on trout fed CPFA suggests further effects include reduced liver protein, reduced activity of some dehydrogenase enzymes (102), and increased liver

lipid content (101). Intact CPFA have been detected in liver lipids, adipose lipid and in eggs of trout fed methyl sterculate (86).

Struthers (101) in 1973 reported on a study of rainbow trout fed methyl sterculate. She found increased liver lipid, reduced conversion of labeled oleic acid to carbon dioxide, and reduced P/O ratios after only a few days on the diet. (Reduced P/O ratios suggest uncoupling of ATP synthesis.) After two weeks, phospholipid turnover was reduced.

In 1959 it was reported that cottonseed oil, when fed to chickens, caused higher serum cholesterol levels and a greater degree of atherosclerosis than would be predicted by the oil's saturated fatty acid content (103). Goodnight and Kemmerer (38) suggested in 1966 that CPFA in the cottonseed oil were responsible for these effects. They demonstrated that CPFA fed as S. foetida oil could alter cholesterol metabolism in cockerels. Increased plasma cholesterol, aortic atherosclerosis, liver weight, bile volume and bile acid excretion were noted in cockerels given CPFA over a 20-week period. These authors saw no difference in sterol retention, weight gain or feed efficiency and hypothesized that CPFA cause depletion of liver cholesterol into the blood and bile. Their experiment demonstrated that CPFA could increase the severity of cholesterol-induced atherosclerosis in cockerels, but did not test the effect of CPFA on atherosclerosis induction in birds fed normal diets.

Further studies of the athrogenic potential of CPFA or their role in cholesterol metabolism have not been published. Nor are there any published reports of attempts to feed CPFA to mammals susceptible to experimental atherosclerosis, such as the rabbit.

Atherosclerosis

Nature of the Disease

Atherosclerosis is a disease of the large and medium-sized arteries and is characterized by a thickening of the inner portion of the artery wall in association with fatty deposits. Because atherosclerosis, or an associated thrombosis, can progressively or abruptly stop blood flow through the heart or brain, heart attack or stroke can result. A report of the National Heart and Lung Institute (68) indicates that atherosclerosis is the main cause of death in the United States.

In the study of atherosclerosis, plasma cholesterol, triglyceride and lipoprotein levels are often measured as indicators of general lipid metabolism and risk of atherosclerotic disease. A multitude of factors are involved in atherosclerosis but plasma cholesterol levels remain the single strongest indicator of individual or community risk of subsequent atherosclerosis and death from coronary heart disease (CHD) according to Shaper (92).

In 1973, Newall and Bliss (69) reported 60% of male patients with peripheral artery disease had hyperlipoproteinanemia; and most had significantly elevated triglyceride and cholesterol levels. These authors claim combined occurrence of elevated triglyceride and cholesterol levels may be of more significance clinically than either separately.

Theories of Induction and Promotion

Relatively little is known about the genesis of this disease. Current research is aimed at elucidating the sequence of pathological events at the cellular level which lead to atherosclerotic lesions and possibly explaining the role of cholesterol, triglycerides, and lipoproteins. One of the major cell types involved in atherosclerotic lesions is the smooth muscle cell. Ross and Glomset (87) concluded that arterial smooth muscle cells play a fundamental role in atherosclerosis. They theorize that local injury of the artery endothelium increases the concentration of plasma proteins in contact with smooth muscle cells, and in response, some of these cells migrate into the intima (the area bounded by endothelial cells and elastic lamina) and proliferate. The developing lesion may regress, the authors claim, unless repeated injury occurs. Then there is continued proliferation, lipid accumulation and eventual plaque formation. Ross and Glomset demonstrated that growth of medial smooth muscle cells in cell culture

could be stimulated by lipoproteins specifically low density lipoproteins (LDL) and high density lipoproteins (HDL). As early as 1950 Altshul (6) postulated that medial smooth muscle cells of the rabbit migrate into the intima under the influence of cholesterol feeding. Later, in 1966, Imai et al. (47) and Parker et al. (76) confirmed that theory.

In a 1973 paper, Paphadjopoulos et al. (75) reports evidence supporting the theory that increased cholesterol incorporation into plasma membranes of arterial intimal cells (induced by high levels of circulating LDL and/or endothelial injury) could alter the metabolic state of the cells by inhibiting critical membrane enzymes. These authors showed that cholesterol could inhibit the ability of phospholipids to activate a delipidated preparation of $(Na^+ - K^+)$ ATPase. Inhibition of the pump ATPase would result in loss of intracellular K^+ and reduced protein synthesis, inhibition of other enzymes and eventual osmotic lysis.

A recent paper by Kramsch and Hollander (54) suggests an interaction of plasma lipoproteins and arterial elastin in atherosclerotic lipid accumulation. A substantial portion of cholesterol accumulating in atherosclerotic plaques is in elastin (arterial elastin appears to be a lipid-protein complex) and their in vitro study suggests elastin protein may interact with plasma low density lipoproteins (LDL) or very low density lipoproteins (VLDL) and accumulate lipid, largely as cholesterol ester, from these lipoproteins. A precondition for the

lipid accumulation appeared to be an altered amino acid composition of the elastin protein, consisting of an increase in polar amino acids and reduced cross-linking.

Several risk factors have been described (92) which are involved in determining the incidence of CHD expected in a population with given plasma cholesterol and triglyceride levels. These factors, such as cigarette smoking, diabetes, hypertension, obesity and inactivity, increase the likelihood that a person or experimental animal will suffer from heart disease but the exact nature of their affect on the disease process has not been determined.

One of the most widely-used experimental animals in studies of atherosclerosis is the rabbit. It was discovered several decades ago (7) that atherosclerotic lesions could be induced in the rabbit by cholesterol feeding. Several authors have pointed out that there are differences in the pathogenesis of atherosclerosis in humans and rabbits (47, 76), but Imai et al. (47) claimed it was only a matter of degree. They stated that with appropriate diets rabbits were suitable for the study of atherosclerosis.

EXPERIMENTAL

Experimental Animals

Male New Zealand rabbits of approximately the same age and averaging 1800 grams in weight were used as test animals. They were housed in individual cages and given water and commercial rabbit pellets ad libitum until experimental diets were introduced.

Preliminary Feeding Trials

Preliminary experiments were designed to determine if a purified or a semi-purified ration could be developed for use as a vehicle in dosing Sterculia foetida oil. Composition of the first basal diet is given in Table 1. Table 2 lists fatty acid composition of lipid mixes used in diet formulation.

This first formulation had the consistency of a mealy powder and was grey in color. Consumption of the powdered diet was very low. Addition of 3% dehydrated alfalfa meal to the diet increased acceptance but the ration was proven unsatisfactory in the first preliminary feeding trial.

In this first trial, two powder diets were fed. Diet P1 contained 2% cholesterol and diet P2 contained 2% cholesterol and 0.7% S. foetida oil (see Table 1). Six animals were fed each diet. As

Table 1. Composition of diets used in preliminary study.

Component	Diet P1 (%)	Diet P2 (%)
Casein	20.0	20.0
Sucrose	23.8	23.8
Corn starch	28.0	28.0
Cellulose	14.8	14.8
Mineral mix[1]	4.0	4.0
Vitamin mix[2]	2.2	2.2
Cholesterol	2.0	2.0
Choline chloride	0.2	0.2
Corn oil	2.5	1.8
Hydrogenated vegetable oil	2.5	2.5
Sterculia foetida oil	0	0.7

[1] Jones-Foster Mineral Mix, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Vitamin Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio. Supplied the following per kg of diet (in mg)

Vitamin A conc.	99.0
Vitamin D. conc.	5.5
α-Tocopherol	110.0
Ascorbic acid	990.0
Inositol	110.0
Choline chloride	1650.0
Menadione	49.5
p-Amino benzoic acid	110.0
Niacin	99.0
Riboflavin	22.0
Pyridoxine hydrochloride	22.0
Thiamine hydrochloride	22.0
Calcium pentothenate	66.0
Biotin	0.44
Folic acid	1.98
Vitamin B-12	0.029

Table 2. Fatty acid composition of lipid mixes used for diet formulation.

Fatty acid	Diets A and C (% of total)	Diets B and C (% of total)	S. foetida oil[1] (% of total)
12:0	0.18	0.13	
14:0	0.18	0.35	0.19
16:0	12.60	13.79	18.42
16:1	0.74	0.24	0.51
18:0	7.40	8.30	2.10
18:1	38.79	32.85	8.40
18:2	38.48	35.83	14.19
18:3	1.63	3.01	
18 Δ[2]		0.70	6.63
19 Δ		4.80	48.39
Total saturates	20.40%	22.57%	

[1] From Reohm (85)

[2] 18 carbon CPFA

mentioned, the rabbits ate poorly and some of those receiving diet P2 stopped eating entirely after three weeks. Animals which refused the experimental diets for three days were given normal rabbit pellets until the end of the trial. This preliminary trial was terminated at eight weeks. Plasma cholesterol and triglyceride levels were measured three times during the trial. Results were not conclusive due to poor acceptance of the CPFA-containing ration.

Main Feeding Trial

The diet formulation finally adopted for the main experiment was similar to the first basal diet described except that 3% dehydrated alfalfa meal, 2% ground barley and 5% molasses were added. After all ingredients were combined, the diet was pelleted using a California Pellet Mill. The basal diet proved to be acceptable to the animals when S. foetida oil was added at the level of 0.5% and gave normal weight gains in young rabbits.

Four diets were used in this study. Composition of experimental diets is given in Table 3. Diet A (basal diet) contained 20% casein and 5% lipid, 2.5% corn oil and 2.5% hydrogenated vegetable oil (Crisco). Diet B was identical except that 0.5% of the corn oil was replaced with S. foetida oil. The Sterculia foetida oil used in this experiment contained 48% sterculic acid and 6% malvalic acid. Diet C differed from the control only in that it contained 0.5% cholesterol, replacing

Table 3. Composition of diets used in main feeding trials.

Component	Diet A (%)	Diet B (%)	Diet C (%)	Diet D (%)
Casein	20.0	20.0	20.0	20.0
Sucrose	16.45	16.45	16.45	16.45
Corn starch	29.5	29.5	29.5	29.5
Cellulose	12.3	12.3	11.8	11.8
Molasses	5.0	5.0	5.0	5.0
Alfalfa meal	3.0	3.0	3.0	3.0
Ground barley	2.0	2.0	2.0	2.0
α-Tocopherol[1]	0.15	0.15	0.15	0.15
Mineral mix[2]	4.0	4.0	4.0	4.0
Vitamin mix[3]	2.2	2.2	2.2	2.2
Cholesterol	-	-	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2
D-L methionine	0.2	0.2	0.2	0.2
Corn oil	2.5	2.0	2.5	2.0
Hydrogenated vegetable oil	2.5	2.5	2.5	2.5
S. foetida oil	-	0.5	-	0.5

[1] α-Tocopherol acetate, 1.5 g provides 375 I. U. per kg.

[2] Jones-Foster Mineral Mix, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Vitamin Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

an equal amount of cellulose in the basic formulation. The fourth diet, diet D, contained both 0.5% S. foetida oil and 0.5% cholesterol. All diets were isocaloric at 3.4 calories per gram and saturated fatty acids made up approximately 21% of total lipid.

Ten animals received each of the four pelleted rations described for a period of five weeks. Rabbits receiving the control diet and those receiving diet B were fed 80 g/day based on preliminary feeding studies which indicated experimental animals would consume 80 to 85 g/day of diet containing 0.5% S. foetida oil when fed ad libitum. Animals receiving diets C and D were fed identical amounts based on the average amount group D animals ate in a 24-hour period. Animals receiving diets C and D were fed 60 g/day for the first three weeks, then 50 g/day through the end of the trial. Feed consumption and weight gains were recorded throughout the feeding trial. The animals will be referred to as group A, B, C, or D according to the diet they received.

Two-ml blood samples were taken from each animal initially and after three weeks on the experimental diets. Blood samples were also taken at five weeks when the animals were sacrificed. Blood samples were drawn from the marginal ear vein using a 20 gauge, one-inch needle. Veins were distended by rubbing a small amount of xylene on the back of the ear with cotton.

Freshly drawn blood was transferred to centrifuge tubes

containing 0.1 ml heparin solution (1000 units/ml). As soon as possible, all samples were centrifuged for 20 minutes at top speed using an I. E. C. clinical centrifuge. Plasma was transferred to labeled vials which were flushed with nitrogen, closed and frozen until analysis could be carried out.

In all cases, animals were starved but given free access to water for at least 12 hours before blood samples were taken.

After five weeks on the test diets animals were fasted overnight and sacrificed by cervical fracture. The jugular vein was immediately severed, blood samples taken and internal organs removed for gross examination. The weights of liver, spleen, kidney, lungs and heart were recorded for all animals and slices of liver, heart and aorta tissue collected for histological examination. The thoracic and abdominal aorta to the femoral branch was removed, split longitudinally and fixed in 10% formalin to facilitate lipid staining. The remaining liver was frozen for later proximate analysis.

Analytical Methods

Total Cholesterol

Total cholesterol in plasma was determined according to the method of Rudel and Morris (88). The method they describe is a simple, rapid spectrophotometric determination using o-phthalaldehyde as a color reagent. Plasma samples were saponified,

nonsaponifiable lipids were extracted with hexane and cholesterol content of an aliquot of the hexane layer determined. Details can be found in Appendix A.

Free Cholesterol

Levels of nonesterified cholesterol in plasma were determined using the procedure of Crawford (25). This procedure makes use of the digitonin precipitation of free cholesterol. Appendix B outlines the method in detail.

Plasma Triglycerides

Total triglyceride levels in plasma were determined using a Calbiochem Triglyceride Stat-Pack (Calbiochem, LaJolla, Calif.). The reaction sequence carried out in this enzymatic assay was as follows:

$$\text{Triglycerides} \xleftrightarrow[\text{hydrolysis}]{\text{enzymatic}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xleftrightarrow[\text{kinase}]{\text{Glycerol}} \alpha\text{-Glycerol phosphate} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \xleftrightarrow[\text{kinase}]{\text{Pyruvate}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xleftrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^{+}$$

Essentially, the level of glycerol in the sample is measured by the reduction in absorbance at 340 nm as NADH is oxidized to NAD^+ in a series of coupled reactions. Details of this method and calculation of results are given in Appendix C.

Liver Cholesterol Levels

Liver cholesterol levels were determined by extracting the total liver lipids using a modified Folch extraction (see Appendix D) and the method of Rudel and Morris for measuring total cholesterol in a portion of the total extract. Liver protein, ash and moisture contents were determined by proximate analysis.

Histological Examination

Slices of heart and liver tissue were placed in Bouin's solution immediately after the organs were excised and weighed. Tissue was held for 48 hours in Bouin's solution before washing, imbedding and sectioning. Tissue sections (4 μ) were stained with hematoxylin and eosin.

Aorta grading

Aorta sections were removed from the animals, trimmed of external tissue, and placed in 10% formalin. The thoracic and abdominal portions of the aorta were split longitudinally and stained

following the procedure of Holman et al. (44) and graded on a zero to four scale as described by Adams (2).

Specimens were washed in 70% ethanol, then immersed in staining solution for 15 minutes. Staining trays which held six specimens were used. Agitation from time to time during staining was essential. Aortas were then de-stained in 80% ethanol for 20 minutes and washed in running water for one hour. Immediately following staining, the arteries were graded for atherosclerotic lesions. Following visual grading, all specimens were labeled and color photographs were taken.

The scoring system used to grade the severity of atherosclerosis in this study was a subjective visual grading system which has been used by several authors (2, 38). Grade one indicated mainly fatty streaks; grade two, elevated intimal thickening; grade three, expanded thickened areas with apparent fibrous plaques; and grade four, ulcerated, complicated plaques with hemorrhage and calcium deposits. Normal arteries were assigned a score of zero.

Fatty Acid Analysis

Fatty acid composition of lipid mixes for diet formulation and of tissue lipids was determined using gas-liquid chromatography.

The instrument used was an Aerograph model 600-B gas chromatograph with a flame ionization detector. The recorder was a

Barber-Coleman with one millivolt full scale sensitivity. Column parameters were as follows:

Column	Aluminum
length	9 ft
diameter	0.085 inches I. D.
Solid support	Anakrom 110-120 mesh
Liquid phase	10% DEGS
Carrier gas	Nitrogen
Flow rate	20 ml/minute
Column temp.	191°C

Methyl esters of sample fatty acids were injected and percent composition of each fatty acid calculated by triangulation. Normal sample size was 0.15 µl.

Total liver lipids were extracted from 5 g of liver tissue using a modification of the method of Folch et al. as mentioned previously. After a portion of the total extract was taken for cholesterol analysis, the remaining lipid was transferred to a labeled vial which was flushed with nitrogen and frozen until fatty acid analysis could be carried out. Methyl esters were prepared using boron trifluoride and methanol (see Appendix E).

Erythrocyte ghosts were prepared from blood taken when the animals were killed after five weeks on diet. Twelve to 15 ml of blood were centrifuged to remove the red cells from the plasma. The erythrocytes were then lysed by freezing in an equal volume of distilled water. Following thawing, the cells were washed twice in

0. 9% saline solution and the resulting ghosts were prepared for extraction. Lipid was extracted using the modified Folch procedure described previously and methyl esters were prepared using boron trifluoride-methanol. For all lipid samples, fatty acid analysis was completed within 48 hours after lipid extraction.

CPFA Analysis

CPFA content of adipose and serum lipids was measured using the Halphen method described by Hammonds et al. (43) (see Appendix F). Liver lipids were analyzed for CPFA using the same procedure but high background levels prevented useful measurements. Roehm (85) reported similar problems in using the Halphen method on trout liver lipids.

Rabbit liver cholesterol esters were isolated and analyzed for CPFA content. Column and thin layer chromatography were utilized in preparing the cholesterol ester fraction and details are given in Appendix G. Adipose and serum lipids were analyzed for CPFA directly as extracted with no further cleanup or preparation.

RESULTS

Semi-purified Diet Performance

The diet developed for this feeding study was found to be very acceptable and may be useful as a basal diet in other types of experiments. Average daily gain for animals receiving the control diet was 17.6 g/day and the feed conversion ratio was 5.2 to 1 (g of feed per g weight gain). It was found that feed efficiency and average weight gains for the CPFA-fed rabbits were slightly below those for control animals at the end of the second week of the trial. By the fifth week no difference in feed conversion efficiency or weight gain was evident. Liver weight to body weight ratios were not altered in animals fed CPFA. These results are summarized in Table 4. When groups C and D were compared, they were nearly equal in feed conversion, weight gain and liver to body weight ratio. However, groups C and D were approximately 50% less efficient than groups A and B in feed conversion (see Figure 1).

Adamson and Fisher (3) recently described a similar chemically defined diet used to determine amino acid requirements for New Zealand rabbits. They found an unusually high requirement for arginine (in comparison with other young animals) and suggested an arginine level of 1% of the diet for maximum growth. The diet used in these experiments supplies approximately 0.7% of the diet as arginine.

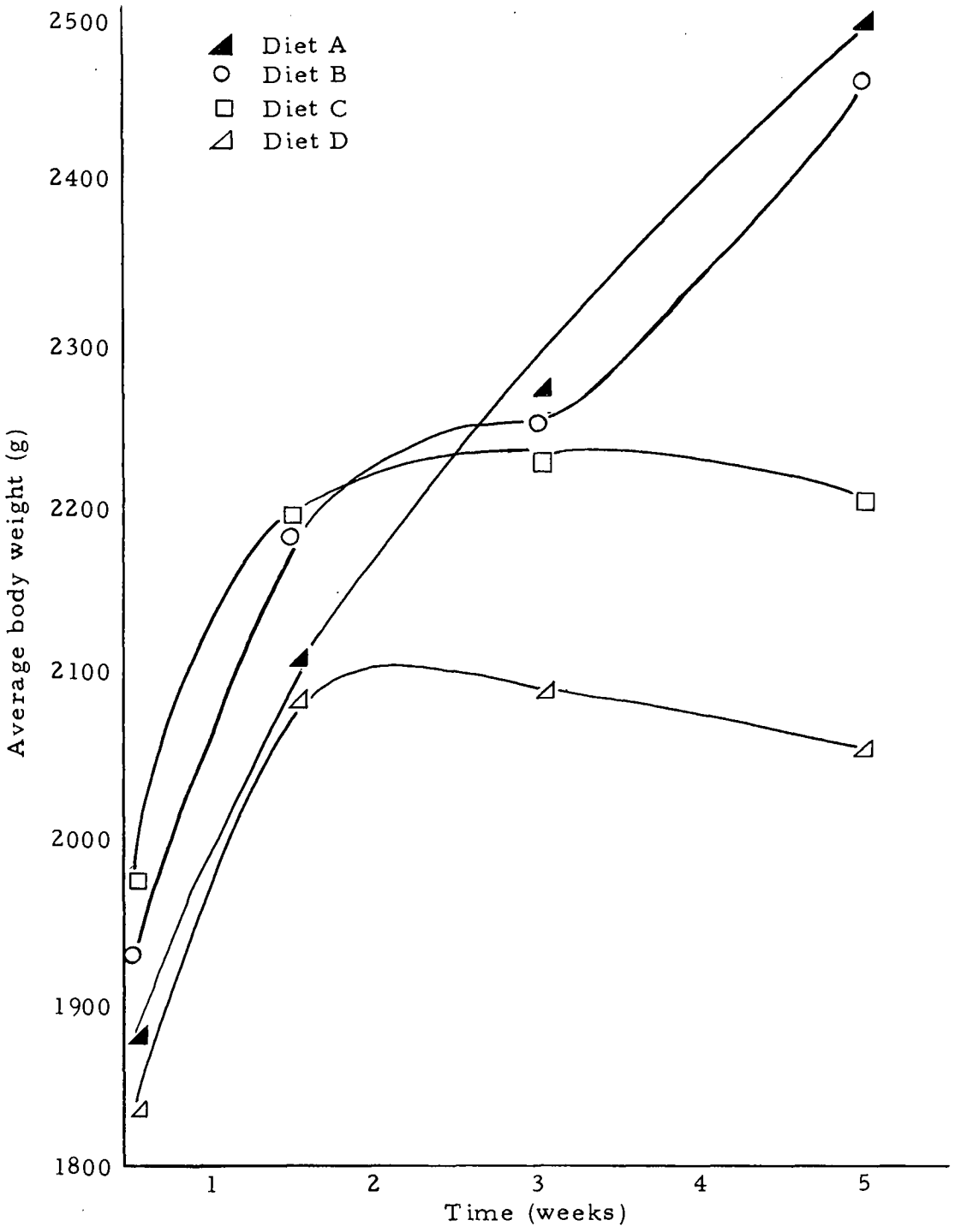


Figure 1. Effect of CPFA on growth rate.

Table 4. Effect of CPFA on liver weight as percent of body weight and feed conversion.

Group	Number of animals	Liver weight as % of body weight	g Feed/ g gain
A	8	3.7 ± 0.70[1]	5.24
B	8	3.9 ± 0.96	4.05
C	8	3.2 ± 0.46	9.04
D	10	3.3 ± 0.51	10.73

[1]Mean ± standard deviation

Effects of CPFA Feeding

Plasma Cholesterol Levels

When mean cholesterol levels of animals fed CPFA were compared with those of control animals, large differences were seen at three and five weeks (see Table 5). Two animals receiving CPFA had plasma cholesterol levels ten times normal levels after only three weeks on the experimental diets. Due to the large standard deviation found in cholesterol levels of experimental animals, and the relatively small number of experimental animals, differences between groups A and B were not statistically significant. Total cholesterol levels in animals fed CPFA (groups B and D) increased continually throughout the trial and were higher than cholesterol levels in animals fed similar diets without CPFA (groups A and C) at three and five weeks. Mean cholesterol levels in group A increased for the first three weeks

Table 5. Effect of CPFA on plasma cholesterol levels.

Group	No. animals	Diet	Initial Cholesterol		Three weeks Cholesterol		Five weeks Cholesterol	
			Total (mg/100 ml)	Free (%)	Total (mg/100 ml)	Free (%)	Total (mg/100 ml)	Free (%)
A	8	Basal	85.3 ± 26.8[1]	33.2	187.7 ± 85.5	42.1	148.3 ± 59.0	37.1
B	8	Basal + SF oil	87.3 ± 29.5	30.0	404.6 ± 294.0	58.9	460.6 ± 453.0	45.6
C	8	Basal + cholesterol	83.9 ± 16.8	29.7	1,170 ± 467.0	44.5	1,442 ± 600.0	37.6
D	10	Basal + SF oil & cholesterol	83.1 ± 28.2	42.8	1,231 ± 503.0	44.7	1,719 ± 615.0	48.1

[1]Mean ± standard deviation

then decreased at five weeks. These data are graphically represented in Figure 2.

At both three and five weeks the percentage of nonesterified cholesterol (free cholesterol) in the plasma of group B animals was higher than in the plasma of control animals. Group D animals had higher free cholesterol levels than group C at five weeks. When groups A and C were compared, cholesterol-fed animals had higher plasma and liver cholesterol levels, but the percentage of free cholesterol in plasma of both groups was nearly identical. They averaged 37.3 and 37.0% free cholesterol, respectively, over the entire trial. Camejo et al. (16) reported 39% free cholesterol in control rabbit plasma. Group B and D animals averaged 45.6% and 48.1% free cholesterol after five weeks on experimental diets. Increased levels of free cholesterol in plasma have been found in cases of familial lecithin-cholesterol acyl transferase (LCAT) deficiency and in cholesterol-fed guinea pigs (67).

Plasma Triglyceride Levels

Post-absorptive plasma triglyceride levels varied widely between individual animals in each treatment and showed no direct relationship to plasma cholesterol levels (Table 6). The CPFA-containing diets produced plasma triglyceride levels higher than

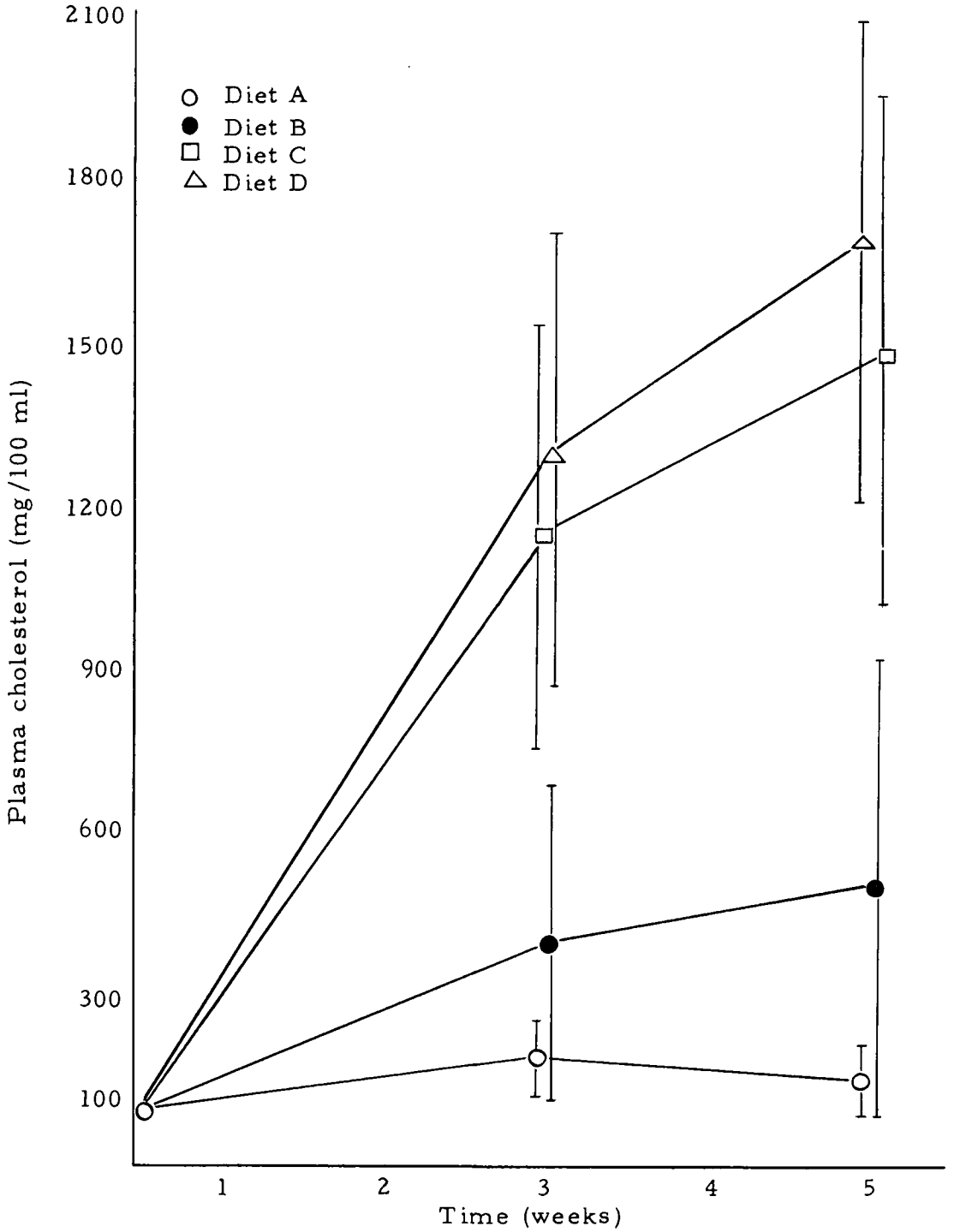


Figure 2. Effect of CPFA on group mean plasma cholesterol levels.

similar diets without CPFA. The extreme animal-to-animal variation limited statistical analysis.

Table 6. Effect of CPFA on plasma triglycerides.

Group	Diet	Plasma triglycerides (mg/100 ml)		
		Initial levels	Three weeks	Five weeks
A	Basal	48.6 ± 35.0[1]	24.8 ± 7.1	42.5 ± 37.1
B	Basal + SF oil	33.1 ± 11.8	58.0 ± 61.2	64.4 ± 30.3
C	Basal + cholesterol	30.7 ± 15.3	20.3 ± 14.5	--[2]
D	Basal + SF oil and cholesterol	30.4 ± 13.7	96.9 ± 39.2	--[2]

[1]Mean ± standard deviation

[2]Not determined

Liver Cholesterol Levels

Liver cholesterol levels expressed as mg/g wet weight and mg/g of protein are summarized in Table 7. Statistically significant differences in liver cholesterol content were not seen but an obvious trend was established. Control livers contained an average of 22.2 mg of cholesterol per gram of protein (4.5 mg/g wet weight). This value is in agreement with published values for normal rabbit liver (41). In animals receiving CPFA the mean liver cholesterol level was 44.2 mg/g protein but the standard deviation was large (S.D. = 34.6). Similar patterns were seen when liver cholesterol levels of

Table 7. Effect of CPFA on liver cholesterol levels.

Group	Diet	Cholesterol	
		mg/g Wet weight[1]	mg/g Protein
A	Basal	4.5 ± 1.02	22.2 ± 4.99
B	Basal + SF oil	7.7 ± 6.05	44.2 ± 34.60
C	Basal + cholesterol	18.3 ± 4.57	98.6 ± 24.72
D	Basal + SF oil and cholesterol	20.9 ± 8.52	114.8 ± 46.82

[1]Mean ± standard deviation

animals fed cholesterol were compared with those of animals receiving cholesterol and CPFA. The level of cholesterol accumulated in liver tissue correlated well with plasma cholesterol levels.

Liver samples from two animals from each group were subjected to complete proximate analysis. Table 8 lists the percent dry matter, crude protein, crude fat and ash for each sample. These data show a dramatic increase in total liver lipid of group B animals with a corresponding decrease in total liver protein. Struthers (101) observed that trout livers increased in lipid content within a few days after introduction of a diet containing methyl sterculate.

Atherosclerosis Induction

Table 9 summarizes the results obtained when aortic atherosclerosis was graded and the four groups compared. Rabbits

Table 8. Effect of CPFA on liver composition.

Animal no.	Diet	Liver composition (% of dry weight)			
		Total solids	Crude protein	Crude fat	Ash
A2	Basal	31.5	60.9	1.9	4.9
A3		31.6	68.1	9.0	5.3
B2	Basal + SF oil	32.0	48.0	46.4	4.3
B3		34.9	56.4	24.6	4.6
C3	Basal + cholesterol	33.9	51.5	13.4	4.3
C4		33.4	58.6	19.0	4.5
D1	Basal + SF oil & cholesterol	33.4	57.1	23.1	4.7
D3		29.7	58.6	10.2	4.9

Table 9. Effect of CPFA on extent of atherosclerosis.

Group	Diet	Number of animals	Mean aorta scores[1]
A	Basal	8	0
B	Basal + SF oil	8	0.50 ± 0.46[2]
C	Basal + cholesterol	8	1.31 ± 1.0
D	Basal + SF oil and cholesterol	10	2.42 ± 1.3

[1]Graded on a 0-4 scale as described by Holman (44) and Adams (2).

[2]Mean ± standard deviation

receiving the control diet for five weeks showed no signs of plaque formation, fatty streaks or any other degeneration of the intimal surface. Five of eight animals in group B developed fatty streaks (accumulated foam cells under the intact endothelial surface) and small plaques in the abdominal aorta and aortic arch. The extent of atherosclerotic disease correlated well with increases in plasma cholesterol levels noted in the CPFA-fed rabbits.

Animals in groups C and D had much more severe atherosclerosis as reflected by the mean aortic grades of 1.31 and 2.42 respectively. Animals receiving cholesterol plus CPFA tended to have more extensive lesions; however, individual variations were large. One animal in group D had a normal appearing aorta (score 0), nearly normal plasma cholesterol levels, normal liver cell structure, and

appeared to be immune to the effects of both dietary cholesterol and CPFA. All other animals in group D were found to have an aortic grade of at least 1.5 indicating the presence of some plaques elevated above the intimal surface. Figure 3 shows individual atherosclerotic scores and Figures 4 through 7 are photographs of typical aortas from each group.

Alteration of Liver Composition and Morphology

Histological examination showed that liver cells from animals fed diets A and C were normal. Parenchymal cells of group C animals contained inclusion bodies, probably accumulated lipid. Liver tissue from group B animals showed degeneration of parenchymal cells in most livers. In some dying cells the subcellular organelles were organized into what appeared to be fibers. These fiber-like structures were found only in cells from rabbits consuming CPFA. Liver cells from animals of group D were abnormal and showed many signs of toxicity including disintegrated cell walls, pyknotic nuclei, binucleate cells and abnormal, folded bile duct walls. Many fiber-containing cells were found in this group. Photographs of typical liver cells from animals on all four diets are shown in Figure 8. Kidney tissue from CPFA-fed rabbits was abnormal also. Numerous blood-filled vacuoles were seen and some were surrounded by necrotic cells.

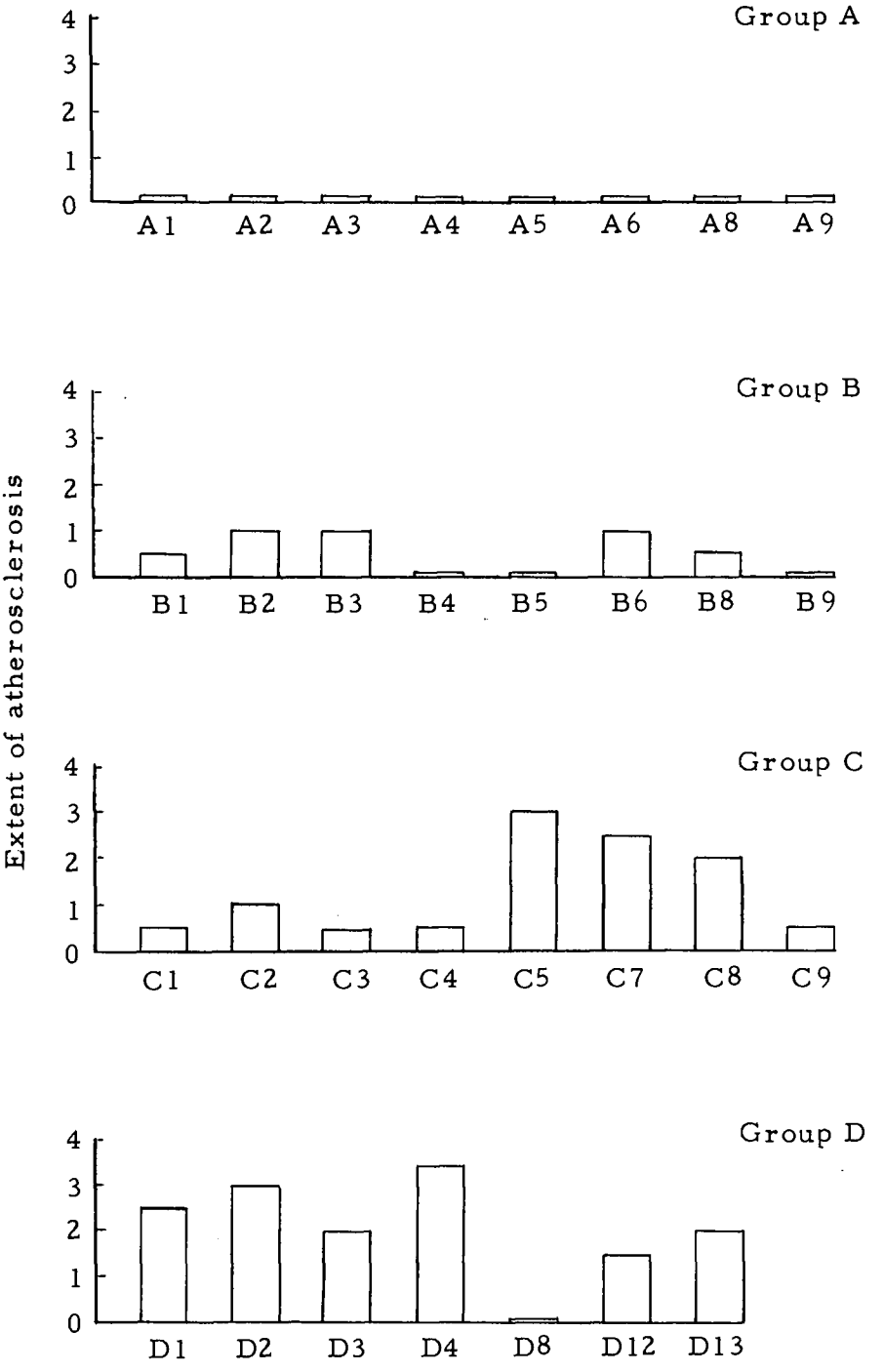


Figure 3. Individual atherosclerotic scores.

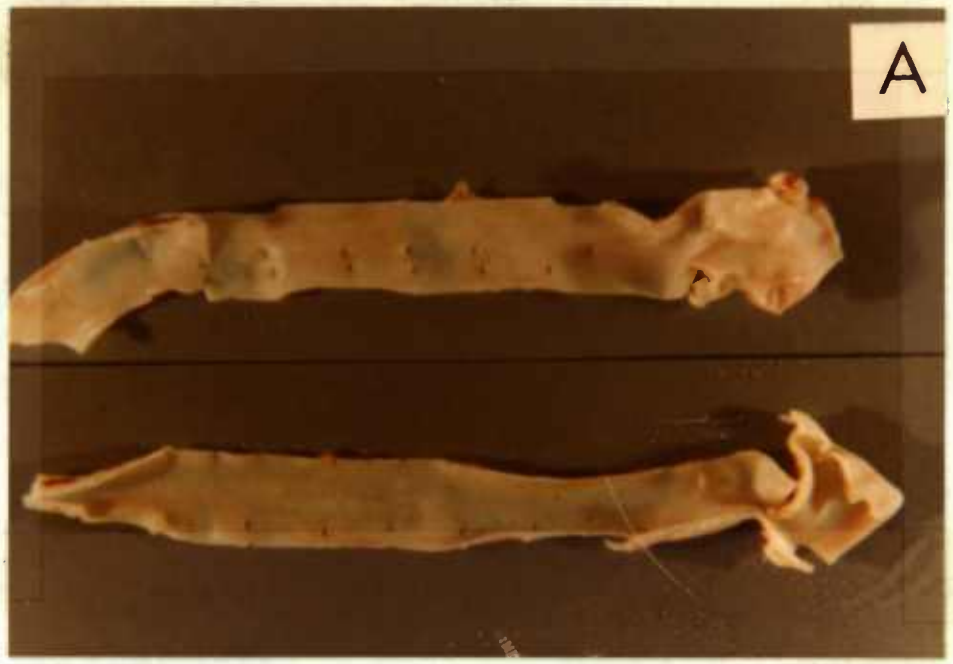


Figure 4. Typical aortas from control animals stained with Sudan IV.

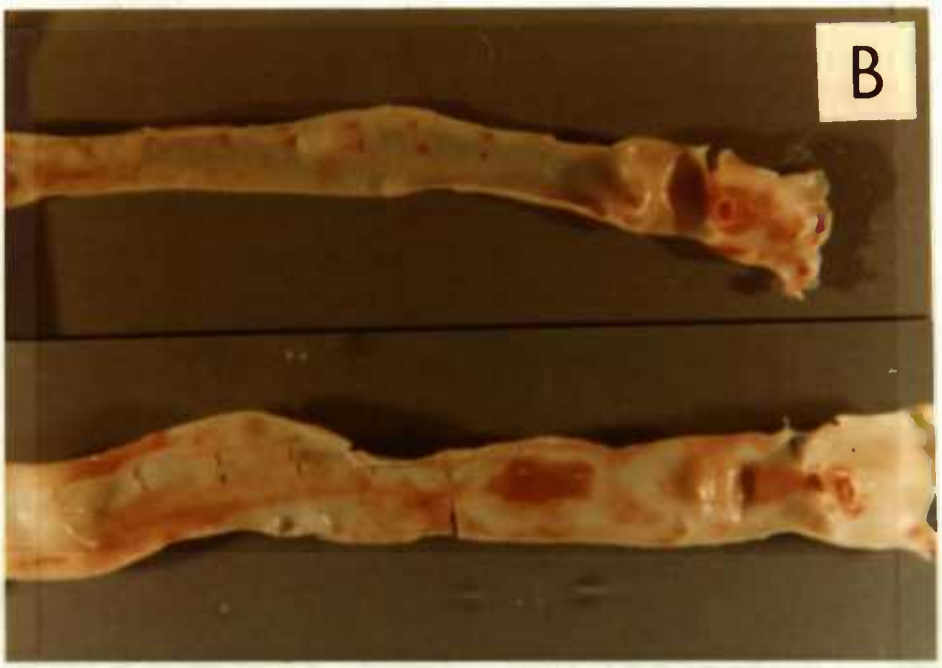


Figure 5. Typical aortas from group B animals stained with Sudan IV.

Figure 6. Typical aortas from group C animals stained with Sudan IV.

Figure 7. Typical aortas from group D animals stained with Sudan IV.

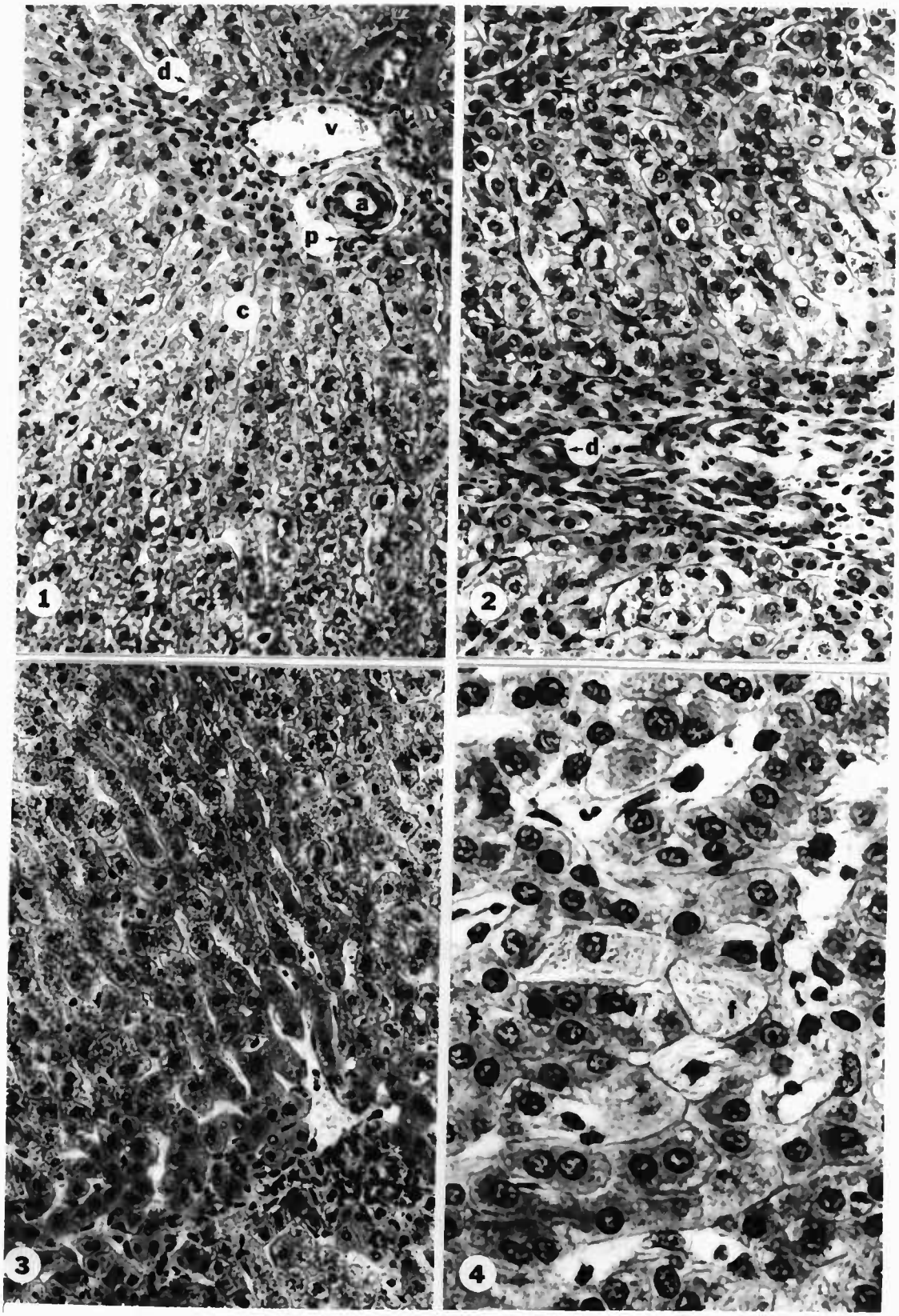
Figure 8. Liver tissue sections from groups A, B, C and D.

1. Control rabbit liver. Shown are an artery (a), a vein (v), at least two bile ductules (d), a portal branch of connective tissue (p), and normal chords of parenchymal cells (c). H. & E. stain, X250.

2. Liver of a group B animal. This picture covers an area which contains a fork of the portal tissue with several bile ductules (d). This portal tissue is abnormally massive. The chords of parenchymal cells have lost their individuality and the entire area shown is a mass of enlarged, bizarre cells with abnormal nuclei. Some of the nuclei are vacuolated. H. & E. stain, X250.

3. Liver of a group C animal. No abnormalities evident. H. & E. stain, X250.

4. Liver of a group D animal. Note several dying parenchymal cells with fibers (f). Cells are irregular in size and shape. N. & E. stain, X560.


d
v
a
p
c
1
d
2
3
f
4

CPFA Accumulation in Lipids

Roehm (85) found that trout accumulated dietary CPFA in intermuscular, depot and liver lipids. Rabbits deposited dietary cyclopropenoids in adipose lipids at levels of 1.1 to 3.45% of total lipid. Halphen-reactive materials were also detected in plasma lipids at the level of approximately 0.75%. Liver cholesterol ester fatty acids contained no detectable CPFA. Table 10 lists the CPFA content of the lipids tested.

Changes in Fatty Acid Composition

The fatty acid composition of liver lipids and erythrocyte ghost lipids is given in Table 11. As has been reported in most species consuming CPFA, the ratio of 16:0 to 16:1 and 18:0 to 18:1 fatty acids was increased in rabbit lipids. When the fatty acid composition of liver lipids from animals fed both cholesterol and CPFA were compared, it was seen that cholesterol accentuated the increase in the ratio of saturated to unsaturated fatty acids. In contrast, cholesterol feeding alone decreased the ratio below levels seen in control animals.

Erythrocyte Ghost Lipids

Red blood cells (erythrocytes) develop in bone marrow and, when mature, contain no nuclear material. When these cells are suspended in a hypotonic solution, they swell and rupture. Virtually all internal constituents are soluble in saline solutions and washing

Table 10. CPFA content of adipose and plasma lipids.

Animal no.	CPFA content (%)	
	Adipose lipid	Plasma lipid
A2	0	
A8	0	
B6	2.03	
B11	3.45	0.61
B3	2.13	
B8	2.15	
B9	1.83	
B10		0.91
D12	2.08	
D13	1.10	
D14	1.85	
D7	1.75	
D8	1.86	
Pooled plasma		
Group B		0.50
Group D		0.76

Table 11. Fatty acid composition of tissue lipids.

Fatty acids	Liver lipids[1]				RBC ghost lipids[2]	
	A	B	C	D	A	B
14:0	0.40	1.51	0.54	0.53		
14:1	0.46	0.48	0.28	0.27		
16:0	18.10	19.56	21.20	24.64	24.80	21.40
16:1	2.23	2.17	3.20	1.45	2.40	1.70
17:0	1.03	1.24	0.76	0.73		
18:0	16.70	20.50	14.80	23.10	14.20	18.70
18:1	21.60	17.45	28.90	17.74	19.50	14.90
18:2	31.30	29.03	25.80	20.80	29.20	32.90
18:3	1.57	2.69	1.50	2.39		
20:4	6.47	5.17	2.90	7.92	6.80	7.10
16:0/16:1	8.12	9.02	6.74	17.00	10.30	12.60
18:0/18:1	0.77	1.18	0.51	1.31	0.73	1.25

[1] Figures shown are average values for two animals for groups A, C and D. Values for group B are averages from three animals.

[2] Figures shown are averages obtained from two group A animals and three group B animals.

of lysed cells gives a residue referred to as "ghosts" which serve as a crude preparation of red cell membrane. Red cell ghost lipids exhibit a fatty acid composition similar to that in liver lipids of CPFA-fed rabbits. The ratio of saturated to unsaturated fatty acids increased and 18:2 content increased slightly in ghost lipids from CPFA-fed rabbits. Red cell ghosts were not prepared from animals receiving diets C or D.

A recent study (65) sets the mean life span of human red blood cells at 126 days but in conditions such as sickle cell anemia, the life span can be reduced to 15 days. Erythrocytes used in this study were from rabbits receiving experimental diets for five weeks.

DISCUSSION

Rabbits as Experimental Animals

Rabbits were chosen as experimental animals because they have been shown to be quite susceptible to changes in plasma cholesterol levels and experimental atherosclerosis can easily be induced. It was also felt the identification of a mammal more susceptible to the effects of CPFA than the rat might be useful in studying the role of CPFA in altered lipid metabolism.

Most recent work on the physiological effects and metabolism of CPFA has been done with fish, rats and poultry. Rats, the only mammal studied extensively, are not extremely sensitive to CPFA and fairly high levels must be fed to produce physiological changes (71).

Rabbits, in contrast, appear to be quite sensitive to CPFA. Ingestion of 200 mg CPFA per day induced morphological changes in liver cells and led to the development of aortic atherosclerosis in five weeks. A few animals fed this level of CPFA plus 0.5% cholesterol reduced feed consumption after three weeks on the experimental diet and appeared ill. Two of these animals were sacrificed early (after approximately four weeks). A third animal died shortly afterwards. The two rabbits examined showed signs of extensive liver damage including liver cell degeneration, bile duct proliferation and the appearance of bile in the plasma.

An important observation in this study was that while control animals showed no signs of atherosclerosis, over 60% of the animals receiving CPFA developed atherosclerosis to some extent. Comparison of animals fed diet C and D showed that CPFA also increased the severity of cholesterol-induced atherosclerosis developed in five weeks.

The extreme increases in plasma cholesterol levels of most rabbits in group B indicated CPFA had some affect on cholesterol balance in these animals. The fact that increases in liver cholesterol levels show a similar pattern to those of plasma cholesterol levels lends weight to the suggestion that increases in total cholesterol in CPFA-fed animals may be significant. The cause of the increased levels was not determined.

When studied individually, animals within groups B and D which had the largest increases in plasma cholesterol levels were found to also have the most extensive atherosclerosis. However, induction of atherosclerosis in CPFA-fed rabbits cannot be accounted for solely by increased plasma cholesterol levels. Two animals in group A had plasma cholesterol levels above the levels noted in group B animals but the Group A animals showed no signs of atherosclerosis.

Liver cholesterol levels corresponded well with plasma cholesterol levels and extent of arterial degeneration in the CPFA-fed rabbits. Animals with the most elevated cholesterol levels were the

most inefficient in feed conversion and showed the most severe histopathological changes (see Tables 12 and 13). Plasma triglycerides were not greatly elevated and showed no direct relationship to plasma cholesterol levels or extent of atherosclerosis.

Plasma cholesterol levels in the control animals rose slightly. This was thought to be due to the large difference in alfalfa content of the test diets and the commercial pellets the animals received prior to introduction of the experimental diets. Alfalfa has been shown to act as a hypocholesterolemic agent in rabbits (10). Cheeke (18) reported that alfalfa contains several saponins (compounds which have been shown to reduce plasma cholesterol levels in chickens [40]). This may account for the cholesterol-lowering ability of alfalfa.

Possible Causes of Increased Cholesterol Levels

Increased plasma and cholesterol levels in group B animals could indicate increased absorption, increased synthesis or decreased excretion of cholesterol. Since these animals were fed a diet which contained no added cholesterol and no animal fats, and rabbits normally absorb 77% of dietary cholesterol (37), increased absorption seems unlikely as an explanation of the increased cholesterol levels. Goodnight et al. (38) found no change in excretion of sterols when cockerels were fed CPFA. If this can be assumed to be the case in the rabbit, increased synthesis can be suggested as the cause of the

Table 12. Relationships of cholesterol levels with other parameters within groups A and B.

Animal no.	Plasma cholesterol (mg/100 ml)	Liver cholesterol (mg/g prot.)	Atheroma score	Feed conversion[1]	Histology
A9	67.4	20.5	0	6.9	N[2]
A2	87.4	17.1	0	12.0	N
A1	129.9	16.4	0	3.0	N
A4	137.7	22.2	0	2.6	N
A6	143.4	20.5	0	3.1	N
A3	176.9	24.3	0	7.4	N
A8	195.1	24.3	0	3.7	N
A5	251.4	32.2	0	3.2	N
B9	180.6	22.7	0	3.6	N
B8	181.6	30.5	0.5	2.6	Fibers, nuc. irr.
B1	203.3	22.7	0.5	3.2	Inclusion bodies
B4	235.0	57.9	0	2.9	Inclusion bodies
B5	303.7	29.7	0	2.7	Fibers
B6	343.9	28.7	1.0	6.0	Fibers
B2	754.3	36.1	1.0	7.3	Fibers
B3	1482.6	125.2	1.0	No gain	Fibers

[1] Grams of feed per gram of weight gain.

[2] Liver cells appear normal.

Table 13. Relationships of cholesterol levels with other parameters within groups C and D.

Animal no.	Plasma cholesterol (mg/100 ml)	Liver cholesterol (mg/g prot.)	Atheroma score	Feed conversion[1]	Histology
C9	774	64.5	0.5	8.4	N[2]
C4	817	122.3	0.5	12.8	N
C7	1295	111.8	2.5	5.3	Sl. degeneration
C3	1293	70.4	0.5	11.8	N
C8	1318	125.1	2.0	8.7	N
C5	1375	74.1	3.0	7.9	Sl. degeneration
C2	2200	109.9	1.0	5.2	N
C1	2466	111.2	0.5	12.2	N
D8	254	30.0	0	5.1	N
D11	1371	--	0.5	18.0	N
D10	1444	--	1.0	6.7	Fibers
D1	1473	163.6	2.5	10.0	Fibers
D7	1523	122.5	3.5	2.8	Fibers
D3	1787	150.7	2.0	No gain	Fibers
D2	2184	128.7	3.0	No gain	Fibers
D4	2559	134.1	3.5	24.8	Fibers

[1] Grams of feed per gram of weight gain.

[2] Liver cells appear normal.

observed increases. Increased cholesterol synthesis while plasma and tissue cholesterol levels are high would require that feedback inhibition of cholesterol synthesis be reduced or eliminated. Preliminary evidence from this laboratory indicates CPFA-fed mice do incorporate three times as much labeled acetate into cholesterol as controls (58).

Recent evidence has suggested that in the rat and perhaps other animals (61, 104) the liver is not the main source of endogenous cholesterol. The intestine has been suggested as another important synthesis site. HMG CoA reductase (β-hydroxy β-methyl glutaryl CoA reductase, E. C. 1. 1. 1. 34) has been shown to be the rate-limiting enzyme in hepatic and intestinal cholesterol synthesis (14). Control mechanisms for this enzyme are not clear. It appears that enzymatic activity is controlled by alteration of the rate of synthesis of enzyme protein (13). Entry of cholesterol or cholesterol esters, facilitated by the bile salts, into intestinal crypt cells is important in regulation of HMG CoA reductase in rat intestine (94). Recent studies with human fibroblasts grown in cell culture show serum very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) inhibit HMG CoA reductase synthesis strongly, while high density lipoproteins (HDL) do not suppress enzyme synthesis or activity (14). The authors suggest cholesterol in combination with specific apoproteins may be the important agents in control of cholesterol synthesis, particularly extrahepatic synthesis.

According to Nixon et al. (71) most of the observed effects of CPFA in rats can be attributed to altered lipid metabolism and membrane function. Alteration of membrane function was thought to be due to increased saturation of membrane lipids. Altered sterol content can also affect activity of membrane-associated enzymes (21).

In a current theory of atherosclerosis induction, Papahadjopoulous et al. (75) suggest that the increased incorporation of cholesterol into plasma membranes of arterial intimal cells could alter the metabolic state of the cells, inhibit enzyme action, and eventually lead to cell death. Accumulation of necrotic cells and intra- and extracellular lipid would lead to plaque formation. In rabbits fed CPFA, it is possible that degree of saturation and sterol content both may be altered in important membrane structures. This may partially explain a loss of control over cholesterol synthesis and the induction of atherosclerotic lesions in CPFA-fed rabbits.

Altered Lipoprotein Patterns

The possibility of altered lipoprotein patterns being secreted in animals fed CPFA is also interesting. As mentioned, VLDL and LDL have been shown to be active in control of cholesterol synthesis in fibroblasts. It has also been suggested recently that lipoproteins are important in the early stages of atherosclerosis induction. Ross and Glomset (87) theorize that local injury or any situation which increases

the concentration of plasma LDL in the vicinity of medial smooth muscle cells can cause these cells to migrate to the intima and proliferate.

Comparison of Experimental Animals

Rats fed 2% S. foetida oil as the only lipid source in a six-month trial showed reduced feed conversion, increased liver weights and increased saturated to unsaturated fatty acid ratios (71). The reactions of rainbow trout to dietary CPFA are similar, but are more nearly the same as those of the rabbit. Fibers apparently identical to those reported in fish liver cells (101) were found in liver cells of most rabbits fed CPFA. These unusual structures have not been found in liver cells from rats or mice fed cyclopropenoids according to Nixon (70). Proximate analysis of livers from two animals of each group in this study indicate that triglyceride as well as cholesterol accumulates in liver cells of CPFA-fed animals. Fatty livers also develop in trout fed CPFA (86, 101). Other unpublished work from this laboratory suggests there is some increase in serum cholesterol levels of trout fed S. foetida oil (80), similar to the rabbit.

The exact nature of the fatty liver common to fish and rabbits fed CPFA has not been studied. Carbon tetrachloride, ethionine, orotic acid and puromycin all cause fatty livers, directly or

indirectly, by impaired lipoprotein synthesis or impaired release of lipoproteins from liver cells (59). D-galactosamine (66) produces histological changes, triglyceride accumulation, and other changes in rat liver cells similar to those seen when CPFA are ingested and the suggested mechanism for these D-galactosamine induced changes is inhibition of lipoprotein apoprotein synthesis.

Ethanol feeding also can produce a fatty liver. Gordon (39) recently studiedthe metabolism of liver cells from rats with ethanol-induced fatty livers. She found an accumulation of CoA esters of long-chained fatty acids in liver cells, a decreased flux of substrate through the TCA cycle, a shift to a more reduced state in mitochondria and reduced ATP synthesis. These results correspond very closely to the findings of Struthers (101) in CPFA-fed trout. Gordon attributed the accumulation of long-chained fatty acid CoA esters to the fact that oxidation of ethanol was occurring in preference to β- oxidation of fatty acids. As long-chained CoA esters accumulated, they could inhibit adenyl transferase and reduce ADP transport across the mitochondrial membrane, causing the observed changes in rat livers.

Co-carcinogenic Effect

The co-carcinogenic effect of CPFA is still unexplained but the suggestion that it may eliminate or alter control of cholesterol synthesis is interesting. Malignant liver cells appear to lose the ability

to control cholesterol synthesis (13). This has been shown in mice and humans (100). Moreover, in the case of 2-acetylaminofluorene (AAF) (46), and other hepatocarcinogens (45), this defect in feedback control occurs months before hepatomas develop. In rainbow trout, CPFA acts as a co-carcinogen with both aflatoxin (98) and AAF (55). Holeman et al. (46) believes elimination of cholesterol synthesis control in liver cells may be one of the important early changes in liver cancer. Possibly it is also important in co-carcinogenesis.

SUMMARY

In summary, CPFA have been shown to act as a hepatotoxin in rabbits much as they do in trout. CPFA induce atherosclerosis in rabbits and alter cholesterol metabolism. The similarity of the hepatotoxic reactions caused by CPFA in trout and rabbits raises the question as to whether these fatty acids might not act as co-carcinogens in rabbits. There was evidence of increased saturation of tissue lipids fatty acids and accumulation of CPFA in adipose lipids of CPFA-fed animals. The rabbit shows promise as an experimental model for further study of CPFA metabolism.

Suggestions for Further Study

The similarity of the reactions of trout and rabbits to dietary CPFA suggests that a study of the co-carcinogenic potential of CPFA in rabbits may be warranted. The desired carcinogens could easily be added to diets similar to the ones prepared for this study.

Because of the suspected importance of lipoproteins in the atherosclerotic process and their proven importance in lipid metabolism, a complete study of the effect of CPFA on production, synthesis and composition of lipoproteins in the rabbit would be useful.

It has been shown that cholesterol feeding in rabbits leads to the biosynthesis of VLDL with apoproteins very similar to those of

control LDL (16). CPFA feeding, which alters plasma cholesterol levels markedly in rabbits, most likely causes some changes in lipoproteins. A study of these changes may shed some light on the athrogenic effect of CPFA and on the triglyceride accumulation in liver cells associated with CPFA feeding.

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APPENDICES

APPENDIX A

TOTAL CHOLESTEROL DETERMINATION

(Rudel and Morris [88])

Reagents:

1) cholesterol standard solution: 1 mg/ml in ethanol

2) o-phthalaldehyde working solution: 50 mg/100 ml in glacial acetic acid prepared fresh daily.
(o-phthalaldehyde was purchased from K and K Laboratories, Inc., Hollywood, California)

Procedure:

In a typical assay, 0.1 ml of plasma or serum, 0.3 ml of 33% KOH (w/v), and 3 ml of 95% ethanol are placed in a glass-stoppered tube and mixed thoroughly. The tube is then stoppered and placed in a 60 ^{o}C heating block or water bath for 15 minutes. After the mixture has cooled, 10 ml of hexane are forcefully added to the tube to mix with the lower layer. Three ml of distilled water are added, and the tube is capped and shaken for 1 minute to ensure complete mixing. A blank, a standard, and a sample of pooled plasma are saponified and extracted at the same time. Appropriate aliquots (usually 1 ml) of the hexane layer are pipetted in duplicate into spectronic tubes, and the solvent is evaporated under nitrogen. Two ml of the o-phthalaldehyde reagent are added to each tube, and the solution is thoroughly mixed to dissolve all of the sample. About 10 minutes after the addition of the o-phthalaldehyde reagent, 1 ml of concentrated sulfuric acid is carefully added by allowing it to run down the inside of

the tube; the solutions are immediately mixed with a vortex mixer. Absorbance is read at 550 nm between 10 and 90 minutes after the addition of the concentrated sulfuric acid.

A modification of this method (using thin-layer chromatography) can be used to measure both free and esterified cholesterol. Lower limits of detection are 4 to 5 μg.

APPENDIX B

FREE CHOLESTEROL DETERMINATION

(Crawford [25])

Reagents:

1) methanol-ethyl ether 3:1

2) digitonin solution; 1 g dissolved in 50 ml ethanol, dilute to 100 ml with distilled water

3) ferric chloride stock solution; 2.5 g/100 ml glacial acetic acid

4) ferric chloride working solution; 1 ml stock/50 ml glacial acetic acid

Procedure:

Fifteen ml of methanol-ether (3:1) are placed in 25 ml volumetric flasks, one for each sample and standards. Exactly 0.5 ml of plasma or standard solution is added to each flask and they are held at room temperature with swirling for 30 minutes. Flask contents are diluted to the mark with methanol-ether (3:1) and mixed by inversion ten times. Samples are filtered and 2 ml of filtrate are transferred to a centrifuge tube which is then placed in a 65^{o}C water bath until tube volume is reduced to 1 ml. One ml of digitonin solution is added to precipitate free cholesterol. After standing 10 minutes, tubes are centrifuged at 3000 rpm for 10 minutes. The precipitate is washed with acetone, centrifuged and solvent removed. Three ml of working ferric chloride solution are added and the tubes heated to boiling for exactly 5 minutes. When cool, 2 ml concentrated sulfuric acid are

added and tube contents are mixed on a vortex mixer. Absorbance at 560 nm is determined after 30 minutes. Results are expressed as mg free cholesterol per 100 ml plasma.

APPENDIX C

PLASMA TRIGLYCERIDE DETERMINATION AND CALCULATIONS

Total triglyceride levels in plasma were determined using a Calbiochem Triglyceride Stat-Pack obtained from Calbiochem, LaJolla, California.

In this assay, a substrate solution containing buffers, lipase enzymes, pyruvate kinase, lactate dehydrogenase, ATP, PEP and NADH is prepared by adding ice cold distilled water to a vial containing the proper amounts of enzyme and co-factors. To 3 ml of this solution (warmed to 30°C in a cuvet) 50 µl of plasma are added. The mouth of the cuvet is covered with parafilm and the contents mixed by gentle inversion. The cuvet is incubated for exactly 10 minutes at 30°C and the initial absorbance (A_0) read at 340 nm. Absorbance at 340 nm measures the amount of NADH in the solution.

Next, 50 µl of a glycerol kinase solution are added to each cuvet and they are mixed as before and returned to a water bath or heating block held at 30°C. Incubation time is 10 minutes. Glycerol kinase is prepared by adding cold distilled water to a specific amount of lypholyzed enzyme prepackaged in small evacuated vials. Following the second incubation, final absorbance (A_{10}) is read at 340 nm. Essentially, the incubation of plasma with substrate solution hydrolyzes the triglycerides present to glycerol and free fatty acids.

Addition of glycerol kinase starts a series of reactions shown below:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \alpha\text{GP} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow[\text{kinase}]{\text{Pyruvate}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

With the conversion of pyruvate to lactate, NADH is concurrently oxidized to NAD^+, a compound which does not absorb at 340 nm.

To determine blank values, samples are allowed to incubate for another 10 minute period at 30°C. The final reading at 340 nm is called A_{20}. Then $A_{10} - A_{20}$ = blank value (A_b).

Calculations

Triglyceride concentration is expressed in mg/100 ml of plasma. The following formula is used:

$$\text{T.G., mg\%} = \Delta A \times 883 \times \text{Dilution factor}$$

A dilution factor is used if plasma must be diluted to bring initial absorbance below 1.0. The change in absorbance is found by multiplying A_0 by .98 to account for dilution by glycerol kinase solution, and subtracting the final absorbance (A_{10}) and the blank absorbance (A_{20}). The factor 883 is derived as follows: the millimolar extinction coefficient of NADH (the absorbance of a solution containing 1 μM per ml

at 340 nm in a 1 cm light path) is 6.22. The conversion of 1 μM in the 3.1 ml of reaction mixture corresponds to an absorbance change of 6.22/3.1 = 2.006.

In the assay system described, this is the equivalent of 1 μM of triglycerides in the 3.1 ml reaction mixture. Assuming an average triglyceride molecular weight of 885.4, the concentration of triglyceride in the 50 μl sample is $\Delta A \times 885.4/2.006$. The concentration in 100 ml is:

$$\Delta A \times \frac{885.4}{2.006} \times \frac{2000}{1000} = \Delta A \times 883$$

APPENDIX D

LIPID EXTRACTION

Liver tissue of other lipid-containing material was extracted using a modification of the method of Folch et al. (35). The solvent was choloroform-methanol 2:1 (v/v). Tissue is blended in 20 volumes of solvent with a Tissumizer model STD homogenizer (Tekmar Company, Cincinnati, Ohio). The homogenate is filtered and washed with additional solvent. To the filtrate, 0.2 volumes of distilled water are added. The resulting milky solution is held overnight or for 12 hours at refrigerator temperatures until a two-phase system forms. The upper phase (methanol and water) is aspirated off. The chloroform layer is dried over sodium sulfate and evaporated to dryness on a rotary evaporator. Lipid samples to be stored were redissolved in diethyl ether and transferred to vials. The ether is evaporated at low temperature, the vials flushed with N_2 and held at -32°C.

APPENDIX E

METHYLATION WITH BORON TRIFLUORIDE IN METHANOL

Samples of 25 to 40 mg of lipid are transferred to a clean, dry test tube. One ml of benzene is added and the contents mixed. Two ml of dry methanol are added and the solution is mixed again. One ml of boron trifluoride is added, the tube is capped and heated in boiling water for 20 minutes. Tubes are removed and allowed to cool. Two or 3 ml of saturated sodium sulfate solution are added (sodium sulfate in water), the solution shaken, then an equal volume of ethyl ether is added. The tube is shaken again and the ether layer is removed to another tube. The ether extract is washed with an equal volume of distilled water and removed to an evaporating flask and evaporated using a rotary evaporator. The resulting methyl esters may be directly injected on a GLC column for analysis.

APPENDIX F

HALPHEN METHOD OF CPFA DETERMINATION

(Hammonds et al. [43])

Reagents:

1) n-butanol, redistilled reagent grade

2) sulfur solution, 1% precipitated sulfur in carbon disulfide

3) morpholine solution, 4% in n-butanol

Apparatus:

1) 15 x 415 Kimex screw-topped test tubes, Teflon-lined caps

2) oil bath, $110^{\circ}C \pm 1.0^{\circ}C$

Procedure:

A weighed sample (usually 1 to 20 mg) of lipid is placed in a test tube. Lipid samples of high cyclopropenoid content are usually diluted to a known volume in n-butanol and aliquots taken for analysis. Next, 0.1 ml of 4% morpholine in n-butanol is added, and total volume made up to 5.0 ml with n-butanol. One ml of 1% sulfur solution is added to each tube, the tube capped tightly, shaken, and placed in the oil bath for exactly 110 minutes. Sulfur addition should take place under subdued lighting and tubes should be heated in the dark. A standard cyclopropene solution must be run with each group of samples. After heating for 110 minutes, the tubes are cooled to room temperature in cold water. Nineteen ml of n-butanol are added to bring the total

volume of the tubes to 25 ml. Absorbance is measured at 495 nm immediately after dilution.

If a standard solution containing 0.25 mg/ml CPFA is used, 0.4 ml of this solution gives 100 μg/tube, a convenient reference point,

APPENDIX G

ISOLATION OF CHOLESTEROL ESTERS FROM LIVER LIPID

In an attempt to isolate a pure cholesterol ester fraction from liver lipids of CPFA-fed animals, a silica gel G column was prepared according to the method of Crinder.[1]

Silica gel G, adjusted to 10% moisture content, was suspended in hexane and poured into a glass column 1.9 x 30 cm. A small glass wool plug was placed in the bottom of the column, then sufficient silica gel-hexane slurry was slowly added to give a column 5 inches in height. Solvent was simultaneously drained from the column and the silica packed under slight pressure. The top surface of the silica gel was covered with a small amount of sand. Samples were dissolved in 5 or 10 ml of petroleum ether and added to the column at the rate of 10 or 15 mg of sample per gram of silica gel. The lipid sample was eluted with the following solvent systems:

Solvent	Fraction to be Eluted
50 ml petroleum ether	Hydrocarbons
50 ml 6% diethyl ether in petroleum ether	Cholesterol esters
150 ml 10% ethyl acetate in petroleum ether	Triglycerides and cholesterol
50 ml diethyl ether	Free fatty acids
100 ml methanol-acetic acid-water (8:1:1)	Phospholipids

[1]Reference: Crinder, Q. E. et al. J. Lipid Res. 5:479-480. 1964.

Elution was carried out under slight nitrogen pressure and a flow rate of 1 to 2 ml/minute was used; 5 to 10 ml fractions were collected manually. Tubes which were supposed to contain only cholesterol esters were checked for purity by thin layer chromatography (TLC).

Several attempts were made to elute a pure cholesterol ester fraction but some contamination with hydrocarbons or triglycerides usually occurred. All cholesterol ester-containing fractions were combined, reduced to small volume on a rotary evaporator and spotted on preparatory silica gel G plates. Bands corresponding to pure cholesterol oleate were scraped from the plate and the cholesterol esters extracted from the silica gel with hexane. The solvent was evaporated with a rotary evaporator and the cholesterol esters were transferred to tared, screw-topped test tubes in preparation for CPFA analysis.

APPENDIX H

AVERAGE GROUP WEIGHTS AT 1-1/2, 3 AND 5 WEEKS

(in grams)

Weeks	Group A	Group B	Group C	Group D
0	1869	1933	1971	1820
1-1/2	2103	2182	2185	2074
3	2250	2267	2228	2089
5	2504	2460	2208	2043