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Title: [9, 10-METHYLENE-^{14}C]STERCULIC ACID METABOLISM IN THE RAT: URINARY METABOLITES, LIVER TISSUE DISTRIBUTION, AND INDUCED ω-HYDROXYLASE ACTIVITY

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Corn oil (CO) and Sterculic foetida oil (SFO) fed rats were injected with [9, 10-methylene-^{14}C]sterculic acid. Less than 1% of the label was expired as carbon dioxide. The majority of the label was excreted in the urine as short-chain dicarboxylic acids with an intact cyclopropane ring. The major metabolites for both CO and SFO fed rats were cis-3, 4-methylene adipic acid and cis-3, 4-methylene suberic acid. Sterculic acid must undergo β- and ω-oxidation to form these urinary metabolites. α-oxidation played a minor role in the formation of cis- and trans-3, 4-methylene pimelic acid. Rats on the SFO diet could metabolize sterculic acid faster than rats on the CO diet. However, both CO and SFO fed rats produced the same urinary metabolites.

CO fed rats incorporated more label from sterculic acid into protein and acid soluble liver fractions than SFO fed rats. Less than 0.01% of the label from either group was found in liver lipid sterol or glycerol fractions.

There was a tendency for SFO fed rats to metabolize n-[1-^{14}C] octadecane faster than CO fed rats. This suggests that sterculic acid may induce ω-hydroxylation of n-octadecane.

[9, 10-methylene-^{14}C]Sterculic Acid Metabolism in the Rat: Urinary Metabolites, Liver Tissue Distribution, and Induced ω-hydroxylase Activity

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Thomas Anthony Eisele

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Head of Department of Food Science and Technology

Dean of Graduate School

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ABBREVIATIONS

α-	alpha
β-	beta
CPFA	cyclopropene fatty acids
cpm	counts per minute
CO	corn oil
GC	gas chromatograph
GLC	gas-liquid chromatography
IG	intragastric
IP	intraperitoneal
IR	infrared
M	molecular weight
MS	mass spectrum
NMR	nuclear magnetic resonance
P	parent (molecular weight)
SFO	Sterculia foetida oil
ω	omega

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INTRODUCTION

The main source of cyclopropenoids in the United States is the seed oil of the cotton plant (Gossypuim hirsutum); while in some countries, cyclopropenoids are supplied by kapok oil. Cyclopropenoids (CPFA) are unique naturally occurring fatty acids containing a highly strained and reactive unsaturated three membered ring in the center of an 18 carbon (malvalic) or 19 carbon (sterculic) chain.

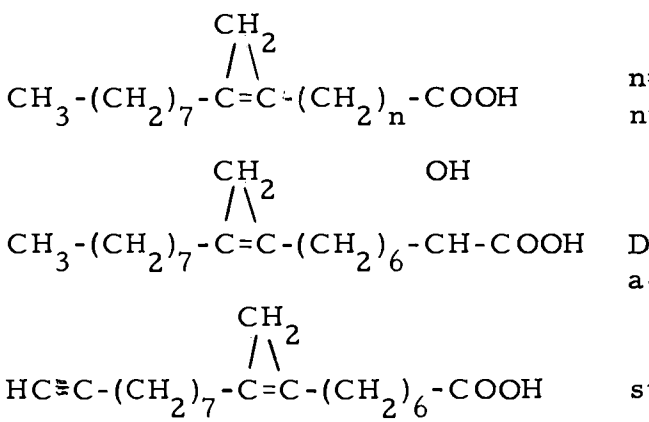
A review by Phelps et al. (62) reported that dietary CPFA impaired reproduction in rats and chickens, delayed sexual maturity in females of these species, and caused pink discoloration in avian egg whites during storage. Other recent work with CPFA fed rats (46, 47) showed fatty infiltration and degeneration of the liver along with renal tubule degeneration; and altered lipid metabolism, and membrane and mitochondrial function (50). Also, Sinnhuber and coworkers (84) discovered that CPFA were powerful co-carcinogens when fed together with aflatoxin B_1. Furthermore, Lee et al. (38) found a higher incidence of tumors in rats receiving a crude mixture of aflatoxins B_1 and G_1 with cyclopropene than in those fed the mycotoxins alone.

There is strong evidence that microsomal activation is required for carcinogenicity of aromatic hydrocarbons. Brodie (6) and Gillette (20) suggested that the various oxidative pathways in microsomes are closely related and may all be assumed to be hydroxylation reactions involving the direct substitution of a hydroxyl group for a hydrogen. Preliminary studies by Yoss (97) has shown that the formation of the major urinary metabolite of sterculic acid in rats required microsomal ω-oxidation. It would, therefore, be important to know all the actual urinary metabolites formed from sterculic acid, and the extent the formation of these metabolites may have on the ω-hydroxylase system in the rat. Thus, the purpose of this investigation was: 1) to identify the rat urinary metabolites and the distribution of [^{14}C] in the liver tissue from [9, 10-methylene-^{14}C]sterculic acid, and 2) to determine the effect of sterculic acid metabolism on the induction of the n-[1-^{14}C]octadecane ω-hydroxylation system.

LITERATURE REVIEW

Cyclopropenoid Fatty Acids: Structure and Occurrence

In 1952, Nunn (53) isolated the cyclopropene fatty acid, sterculic acid, from Sterculia foetida oil (SFO). Shortly thereafter, McFarlane et al. (4) isolated a second cyclopropene fatty acid, malvalic acid, and followed by Craven and Jeffery (12) with D-2-hydroxy-sterculic acid, and Morris and Hall (48) with sterculynic acid.



n=6 malvalic acid
n=7 sterculic acid

D-2-hydroxysterculic acid

sterculynic acid

Cyclopropene acids were found principally in seed lipids of the order Malvales. They exist largely as triglycerides in both the neutral and phospholipid components (27). Gossypium hirsulum (cotton), Eridendron anfractuosum (kapok), and Sterculia foetida (java olive) seed oils are the common sources of cyclopropene. Cotton seed oil is used extensively in the U.S. as an important source

of oil in many food products, and kapok oil is used chiefly in oriental countries. Cotton seed oil contains 1.1-1.6% malvalic and 0.4-0.8% sterculic acid (8), kapok (20) 12-14% CPFA, and Sterculia foetida oil (29) 4-10% malvalic and 45-54% sterculic acid. A review by Christie (8) lists the cyclopropene fatty acid content of many seed oils.

Reactions and Analysis

Cyclopropenes are highly reactive materials due to the strain of the unsaturated ring. They oxidize (24) with conventional reagents and add hydrogen as do most olefins only more easily and with milder catalyst (98). Hydrogen halids in glacial acetic acid react slowly but quantitatively with cyclopropenes (7), and this has been developed into a routine analytical technique for determining total cyclopropene content of seed oils. Mercaptans also add readily across the cyclic double bond to give two thiol esters (31). Silver nitrate in methanol (32) reacts with cyclopropenes to form a keto and alkyoxy derivative which can be easily separated by gas chromatography. The silver nitrate method (76) along with the thio adducts has been used extensively for establishing CPFA standards.

Coleman (10) has reviewed many methods for the assay of cyclopropenes. He concluded the Halphen (22) reaction was the quickest and the simplest of the "wet" methods. A more recent method developed by Pawlowski (61) involves NMR which measures

the ratio of the cyclopropene area hydrogens to the terminal ω-methyl hydrogens. It is a quick, simple, nondestructive, direct method that is very accurate and suitable for samples greater than 1.0% CPFA. Other spectroscopic methods such as IR (3) which measures the in-plane wagging vibration of the methylene group at 1009 cm^{-1} is less accurate and requires a dual cell set-up. Mass spectrometry coupled with gas chromatography of cyclopropenes (59) and their silver nitrate in methanol derivatives seems to be the most sensitive method. The molecular weight of the CPFA and the position of the ring can be easily determined from the mass fragmentation pattern (15).

Biological Effects

Cotton seed oil incorporated into the diet of laying hens was observed by Sherwood (80) to cause egg discoloration on storage, with the whites turning pink and the yolks turning a salmon-orange color. Lorenz et al.(40) found that the fat from the pink eggs and the cotton seed oil both gave a positive Halphen test and suggested that the substance in both were one and the same. In 1952 Nunn (53) established the structure of sterculic acid, the fatty acid responsible for the positive Halphen test, and found sterculic acid in very small quantities in the glycerides of cotton seed oil.

The biological activity of sterculic acid has been shown to

reside in the unsaturated ring. When the ring was destroyed by mild hydrogenation or gaseous hydrogen chloride (79) both cotton seed oil and SFO no longer gave a positive Halphen test nor did they cause pink egg discoloration. Furthermore, Nordby et al. (52) showed the acid or ester group was not a requirement for activity.

Effects other than pink egg discoloration were noted by Schneider et al. (74, 75, 77). They observed that feeding SFO to hens caused embryo mortality, delay of sexual maturity in pullets, and poor egg production and enlarged gall bladders and livers. A complete review on this subject and other biological effects was written by Phelps et al. (62).

A more recent finding by Johnson et al. (26) showed that sterculic acid inhibited the in vitro desaturation of [14^{C}]stearic acid to oleic acid in the hen. Allen and coworkers (1) confirmed these reports and showed sterculic acid to be a stronger stearic fatty acyl desaturase inhibitor than malvalic acid. Shortly thereafter, the same desaturase effect was observed by Reiser and Raju (67) in the rat and by Lehman (35) in the mouse.

Nixon et al. (50) in a recent report showed that dietary CPFA caused the partial loss of membrane-associated functions in the rat. There was a noted increase in the rate of erythrocyte hemolysis, a decrease in microsomal codeine demethylase activity, and a complete inhibition of glutathione-induced mitochondrial swelling. It was

suggested that the change in saturation to unsaturation fatty acid ratio by CPFA altered the physical properties of the membrane and thus influenced the associated membrane functions. Other workers (11, 21, 66) also reported CPFA induced alterations in rat tissue lipids with a noted increase in lipid saturation. Reduced growth rates, poor feed conversion, and elevated liver/body weight ratios were common in female and male rats.

Rainbow trout, Salmo gairdneri, have proven to be very susceptible to CPFA with dietary levels below 200 ppm cyclopropene producing some of the same effects as in mammals (68, 69). Malevski et al. (42) noted a decrease in protein synthesis in trout fed 0.5 mg CPFA/kg body wt/day while Struthers (88) found increased liver lipids, reduced P/O ratios, and reduced conversion of [^{14}C]oleic acid to [$^{14}CO_2$] in only a few days. Taylor et al. (89) and again Malevski (42) noticed a decrease in normal lipid synthesis from [^{14}C]acetate in fish fed CPFA. Histological abnormalities including fatty infiltration, the formation of "fibers" in liver parenchymal cells, and bile duct proliferation were common (37, 69, 84, 86, 87).

Goodnight and Kemmerer (21) suggested in 1966 that CPFA fed to chickens were also responsible for their high serum cholesterol levels. They demonstrated that CPFA could indeed alter cholesterol metabolism in cockerels by increasing serum

cholesterol, bile volume, bile acid excretion, and aortic atherosclerosis. Ferguson (16) noted higher plasma cholesterol levels and higher incidence of aortic atherosclerosis using New Zealand rabbits fed CPFA when compared to a control group. Nixon (49) observed that mice fed CPFA had a tendency to have higher serum cholesterol levels.

Cocarcinogenic and Carcinogenic Activity

Cyclopropene fatty acids fed as a component of Sterculia foetida oil was reported by Sinnhuber et al. (84) to have cocarcinogenic activity together with aflatoxin B_1 in rainbow trout. This was confirmed by Lee et al. (37), not only with aflatoxin B_1 but also with 2-acetyl-aminofluorene. Lee showed that levels as low as 20 ppm of methyl sterculate in the diet promoted the growth of hepatoma in rainbow trout.

Nixon et al. (51) reported that CPFA fed with aflatoxin B_1 to Wistar rats showed a tendency to increase the hepatoma incidence. At low levels of aflatoxin B_1, hepatoma incidence increased 11%, while at higher aflatoxin B_1 levels, the CPFA effect was not apparent. Friedman and Mohr (17) could find no interaction between CPFA and aflatoxin B_1 in rats. Conversely, Lee et al. (38) fed Long-Evans rats 220 ppm CPFA with a mixture of aflatoxin B_1 and G_1 over an 18-month period and reported an increased tumor incidence

from 59% to 70%. The results were not significant due to the low numbers of animals used in the experiment. Thus, cocarcinogenic activity of CPFA in species other than rainbow trout has not been adequately demonstrated.

Recently, Sinnhuber and coworkers (83) reported that CPFA also were carcinogens in rainbow trout. Levels as low as 45 ppm CPFA in the diet produced liver cancer in 11 out of 40 fish while 405 ppm CPFA in the diet produced even higher incidence of 10 out of 30 fish.

Cytoplasmic Alterations

Scarpelli et al. (73) reported that hepatocytes from rainbow trout and rats fed 200 ppm CPFA for four weeks showed induced cytoplasmic alterations. These alterations, cleft-like striations, parallel arrays, and whorled membrane profiles of rough-surfaced endoplasmic reticulum, became progressively more marked with continued feeding. A follow-up paper by the same author (72) showed CPFA at 500 ppm in the diet for two weeks of male Sprauge-Dawley rats significantly increased mitotic activity in the pancreas as well as in liver helatocytes. The significant point made in Scarpelli's paper was that CPFA were acting as a mitogen. Cell division usually occurs only in the presence of adjacent cell death

and active growth (young animals). CPFA was stimulating cell division of the rat pancreas without regards to death or active growth.

Metabolism of Cyclopropenes, Cyclopropanes, and Related Compounds

Wood and Reiser (96) studied the metabolism of cis- and trans-9, 10-methylene octadecanoic acid in weanling rat adipose tissue. The main metabolites were cis- and trans-3, 4-methylene dodecanoic acid. The products were apparently the result of the inability of the β-oxidation enzyme system to continue down the fatty acid chain. This was supported by the fact that large amounts of the material accumulated in the adipose tissue.

Chung (9) carried this one step further by using the [methylene-^{14}C]cis-9, 10-methylene hexadecanoic acid and cis-9, 10-methylene octadecanoic acids incubated with rat liver mitochondria. He showed that the cyclopropane long chain fatty acids were converted to short chain cyclopropane fatty acids, and that the [methylene-^{14}C] labelled ring was not converted to [$^{14}CO_2$].

In a review on lipids (13), Deuel reported that in branched chain fatty acid metabolism in mammals, whenever there is a hinderence of β-oxidation, that ω-oxidation plays a much larger role in metabolizing these fatty acids. Hence, a dicarboxylic acid of shorter chain length is formed then eliminated through the urine as a conjugate or a free dicarboxylic acid.

Altenburger and coworkers (2) intravenously administered [methylene-^{14}C]sterculic acid to fasting hens. They showed that limited amounts of $^{14}CO_2$ formed over a 24-hour period, and that more than 50% of the label was found in the fecal material (feces and urine). This indicated that the compound was being metabolized to a certain extent and then excreted in the fecal material with an intact methylene group, much the same as a branched fatty acid would be. The same observations were made by Berry (5) along with the fact that methyl sterculate was absorbed through the gut at a slower rate then methyl oleate.

Yoss (97) did a comparative study on the metabolism of ^{14}C-sterculic acid in the rat and rainbow trout. He found that the rat could excrete approximately 48% of the administered dose in 16 hours while the fish required five days. The distribution of the label in subcellular fractions in both species showed that radioactivity peaked first in the microsomes and then in the mitochondria. He suggested that CPFA metabolism by mitochondria may first require an initiation step by the microsomes. The major urinary metabolite formed in the rat was cis-3, 4-methylene adipic acid. Formation of this metabolite requires β- and ω-oxidation plus reduction of the cyclopropene ring to a cyclopropane ring. No metabolites were identified in the rainbow trout.

In summary, CPFA in rats, trout, chickens, and rabbits alter

lipid metabolism, modify certain enzymic activities, and are hepatotoxic. CPFA are potential cocarcinogens in mammals and also carcinogens in rainbow trout. Humans may consume CPFA in small quantities along with other carcinogenic nitrosamines and mycotoxins. Therefore, it is of great importance that the metabolism of CPFA be studied in mammalian systems to help evaluate the potential health hazard.

It is the intent of this investigation that the results will aid in the understanding of the mode of action of CPFA on mammalian systems, especially in regards to its action as a cocarcinogen.

EXPERIMENTAL

Experimental Animals

Eight weanling Wistar strain male rats (30 days old) from a closed colony maintained by the Department of Agricultural Chemistry at Oregon State University were housed in plastic box cages containing ground corn cobs. The animals were fed water ad libitum and a solid agar semipurified diet. Food consumption and individual weight was taken on a weekly basis as an index of overall growth and health.

Diet

The composition of the semipurified diet (33) is shown in Appendix I. The total diet consisted of 92.8% premix, 2.2% vitamin mix, and 5.0% corn oil mixed 1:1 (w/v) with 3.0% agar (DIFCO) dissolved in distilled water at approximately 80°C. The hot mix was solidified at two to five degree Centigrade, cut into small blocks, and stored in plastic bags at -30°C until used.

After two weeks on 5.0% corn oil diet, four of the eight rats were transferred to a 4.0% corn oil plus 1.0% Sterculia foetida oil diet on a dry weight basis. The diets were labelled CO for corn oil control and SFO for cyclopropene experimental. The SFO diet

contained 0.5% cyclopropene (malvalic and sterculic acid) or 10% of the lipid on a dry weight basis.

Sources of Labelled Compounds

Sterculic acid, labelled on the 9,10-methylene bridge of the cyclopropene ring, was synthesized by Pawlowski (60). The Halphen test (Appendix III) and NMR (61) indicated 99.0 ± 0.2% and 103.0 ± 2.0% cyclopropene respectively. The free acid was prepared by saponification with alcoholic KOH (Appendix II). Pure methyl sterculate, 110 μCi/mmole, was diluted to 4.0 μCi/0.41 grams corn oil. n-[1-^{14}C]-octadecane was purchased from Amersham/Searle. 21.2 mCi/mmole was diluted to 10 μCi/0.4 grams cold n-octadecane.

The mode of administration to the rats was intragastric (IG) for sterculic acid and intraperitoneal (IP) for n-octadecane. Immediately after injection, animals were placed in metabolism chambers (Figure 1). Air was pulled through several KOH traps to collect $^{14}CO_2$, and ten ml. samples were taken every hour and counted in scintillation fluor (Appendix V). Urine was allowed to flow into glass tubes packed in ice. After the allotted time, animals were decapitated and the livers were removed and frozen on dry ice, then stored in a freezer until fractionated.

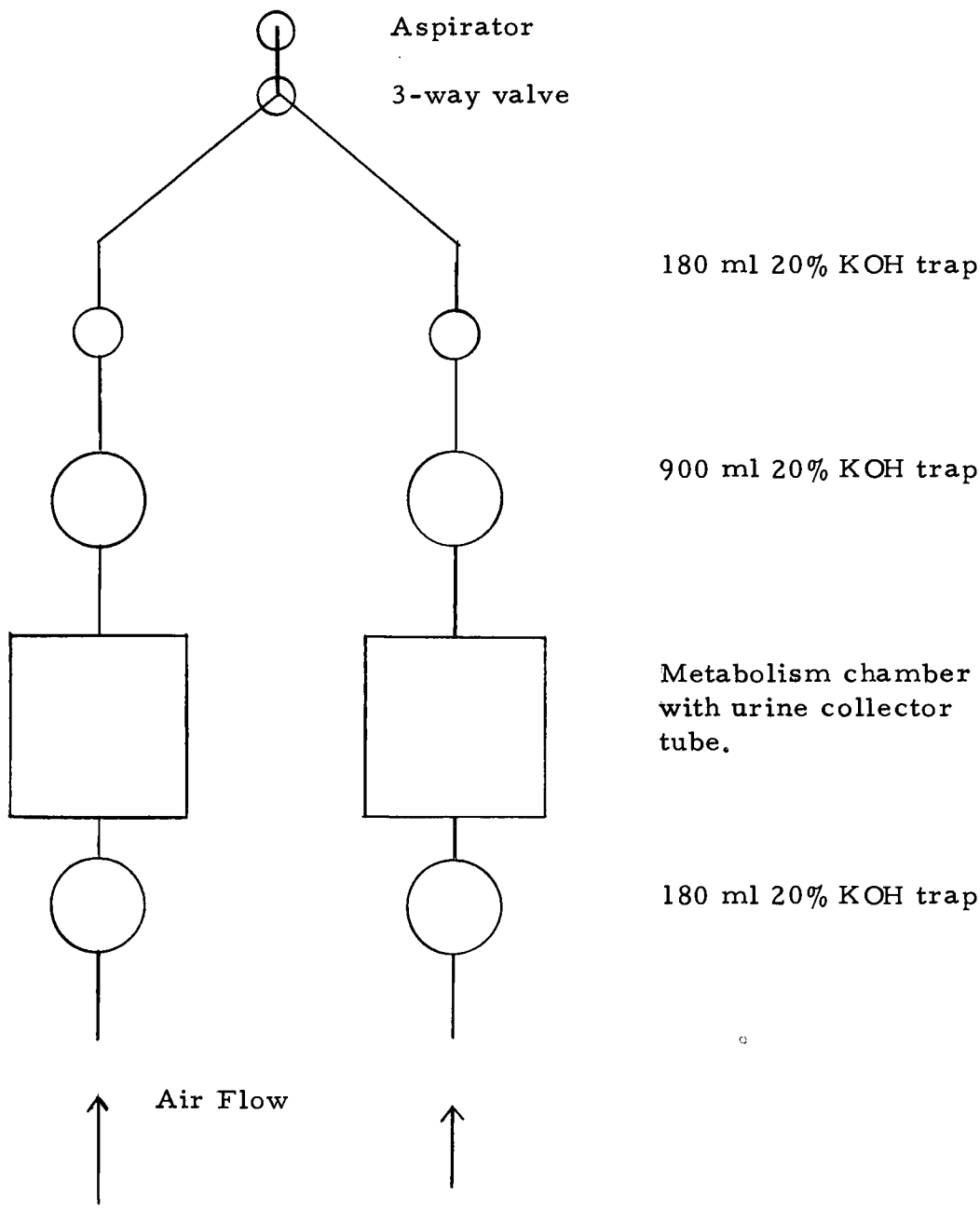


Figure 1. Schematic of apparatus for trapping CO_2.

Method of Administering Labelled Compounds

Rats numbered 1 through 4 were given [9, 10-methylene-^{14}C] sterculic acid by IG. This route was considered the "natural" way animals consume food oils. Animals were given food up to the time of injection of label, but only water was provided in the metabolism chamber. The experiment was terminated at the end of ten hours for the CO and SFO fed rats. Yoss (97) determined that the rat on CO diet excreted the maximum amount of label from ^{14}C-sterculic acid between eight and 16 hours. Hence, ten hours seemed an appropriate time.

Rats numbered 5 through 8 were given n-[1-^{14}C]octadecane by IP. The experiment was terminated at the end of eight hours for CO and SFO rats.

Table 1 shows the dose in μCi of label given to each animal. Each experiment was run with one CO and one SFO fed rat, then repeated a second time to correct for biological variability.

Urine Extraction

An equal volume of distilled water was added to the urine. pH was adjusted to 1.0-2.0 with HCl, and extracted with four volumes of ethyl ether (64). The combined extract was centrifuged at 2000 rpm to break up emulsions, if formed. The combined ethyl

ether extract was washed with one volume distilled water and the solvent evaporated off on a vacuum rotary evaporator. The free acids were esterified with diazomethane (Appendix IV).

The label in the rat urine could be extracted only under acid conditions. The maximum extractable [^{14}C] was 88 to 90% for both CO and SFO fed rats. Extracting the urine with additional ethyl ether did not improve the yield. Therefore, about 10% of the label was probably excreted as the glucuronide (30) or some other conjugate.

Gas Chromatography

Preparative GLC was carried out on a Varian Model 1400 flame ionization unit. The column, 14 ft by 0.085 in I.D. aluminum tubing packed with 10% SP-222-PS 01-1885 110/120 mesh (Supelco, Inc., Bellefonte, PA), was temperature programmed at 1°C/min for 120° to 180°C and then held isothermally at 180°C until the end of the run. The injector temperature was 200°C and the detector 220°C. Nitrogen at a flow rate of 20 ml/min was used as the carrier gas. The detector was equipped with a 5:1 split for trapping purposes: 1 part detector and 5 parts trap.

The same GLC conditions were used in combination with the mass spectrometer.

Spectroscopy Equipment

IR spectra were obtained on a Beckman Model IR-18A using a beam condensor. A micro salt cell with path length of 0.1 mm or a salt plate was used to hold the sample. Spectra were run in carbon techtrachloride or carbon disulfide.

NMR spectra were run on a Varian HA-100 with a time averaging computer using carbon tetrachloride as a solvent and benzene as a lock signal.

Mass spectra were obtained with a Finnigan Model 1015C Quadrupole mass spectrometer interfaced to a Varian Model 1400 gas chromatograph oven with a Gholke glass jet molecular separator. The ion source pressure was 10^{-6} mm Hg; ionizing current, 400 μA; ionizing potential, 70 ev; and manifold temperature 150^{o}C.

Isolation of Metabolites From Urine by Gas Chromatography Trapping

Glass capillary tubes packed in dry ice (size slightly larger than internal diameter of splitter to prevent back pressure) were attached to the splitter of the GC with teflon tubing. A heat gun was used to keep the sample from condensing near the splitter-capillary interface and to drive the condensed material to the cold section of the capillary tubing. All peaks and the base line area between the peaks were trapped separately. The capillary tubes were sealed

at the ends with microflame and stored in the freezer until needed.

To determine which peaks were labelled, the capillary tubes were washed with 1.0 ml of appropriate scintillation solution (Appendix V) into vials to be counted for [^{14}C]. Radioactive peaks and the more abundant non-radioactive peaks were trapped several times to obtain sufficient amounts of each compound for identification.

Liver Tissue Fractionation

Livers were fractionated according to the method of Shibko and coworkers (81) into three fractions (Appendix VI): glycogen fraction (polar water soluble compounds), lipid fraction, and protein fraction. These were counted in the appropriate scintillation solution. The lipids in turn were fractionated into three more fractions (Appendix VII): steroid fraction (hexane non-polar soluble), free fatty acid fraction (ethyl ether soluble), and glycerol fraction (water-alcohol soluble). These were also counted for activity.

RESULTS AND DISCUSSION

Animal Health and Growth

Animals were fed the SFO diet for at least two months to enable them to develop a sufficiently active pathway for metabolizing CPFA. Weekly body weights and feed consumption showed CO and SFO fed rats to be healthy. Table 1 indicates the perspective body weight, liver weight, days on diet, and the dose of labelled compound given to each animal.

Identification of [9, 10-methylene-^{14}C]sterculic Acid Metabolites in SFO Fed Rats

A typical gas chromatograph of methylated urine extract from a SFO fed rat given [9, 10-methylene-^{14}C]sterculic acid is illustrated in Figure 2. Peaks labelled with the letters A through J contain radioactivity. The scale on the left of the GC indicates the dpm's representative of each peak. Since peak C contains the largest amount of material and label, it was identified first.

Peak C

The IR (Figure 3) of the largest peak C (Figure 2) shows an intense carbonyl absorption at 1740 cm^{-1} indicative of an aliphatic ester (82); strong 2860 cm^{-1} due to carbon-hydrogen methyl

Table 1. Body weight, liver weight, days fed diet, and [^{14}C] dose given to corn oil and SFO fed rats.

Animal #	Diet	Body Weight (gm)	Liver Weight (gm)	Days on Diet	Dose μCi	^{14}C-compound
1	CO	279	6.52	89	3.08	sterculic acid
2	SFO	284	8.31	77	4.36	sterculic acid
3	CO	352	7.34	107	3.35	sterculic acid
4	SFO	353	7.40	94	4.62	sterculic acid
5	CO	411	10.02	134	4.18	n-octadecane
6	SFO	407	10.10	117	3.55	n-octadecane
7	CO	396	10.31	155	4.54	n-octadecane
8	SFO	351	9.50	138	4.92	n-octadecane

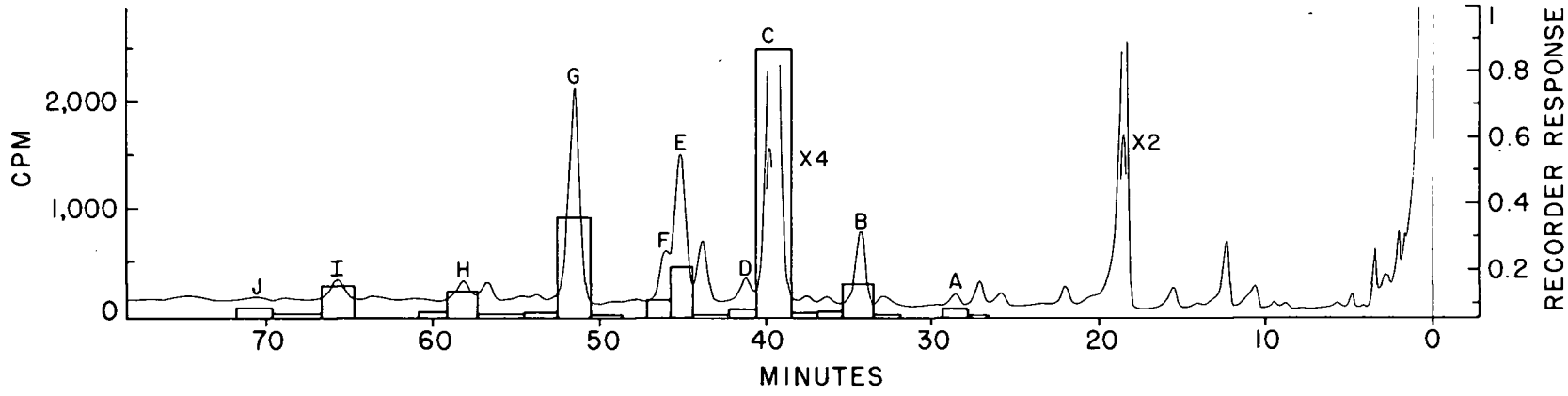


Figure 2. Gas chromatograph of methylated urine extract of a SFO fed rat injected with [9, 10-methylene-^{14}C]sterculic acid.

stretching; strong 2930 cm^{-1} of methylene carbon-hydrogen stretching; moderate 1465 cm^{-1} of carbon-hydrogen methylene bending; moderate 1440 cm^{-1} of methylene cyclopropane bending; a moderate to weak 1260 cm^{-1}, 1195 cm^{-1}, and 1175 cm^{-1} for carbon-oxygen stretching indicative of a methyl ester; and a medium 1020 cm^{-1} absorption from skeletal vibration of the cyclopropane ring (9, 96).

The MS (Figure 4) of peak C (Figure 2) shows fragments characteristic of methyl esters of dibasic acids (Table 2). P-32 (m/e 154), P-59(m/e 127), P-64(m/e 122), and P-73(m/e 113) are present. The series m/e 53, 67, 81 and 95 corresponding to the empirical formulae $[C_nH_{2n-5}O]^+$ and the series m/e 71, 85, 99, 113, and 127 corresponding to $[C_nH_{2n-3}O_2]^+$ are also present. Ryhage and Stenhagen (71) observed in the mass spectra of methylated dibasic acids that the most intense peak in the high mass range was m/e(P-31) or m/e(p-32), that the m/e(p-64) was also very intense, and that the parent ion was very small or nonexistent. Based on these observations, the molecular weight of compound C would be m/e(154 + 32) = 186. The parent peak is not present in the MS of compound C (Figure 4).

The NMR (Figure 3) of C indicates a short chain cyclopropane dibasic ester. The large singlet at 6.40τ (14) indicates six methoxyl protons of a methyl ester and the doublet at 7.74τ is assigned to four methylene protons adjacent to a carbonyl group. Therefore, there

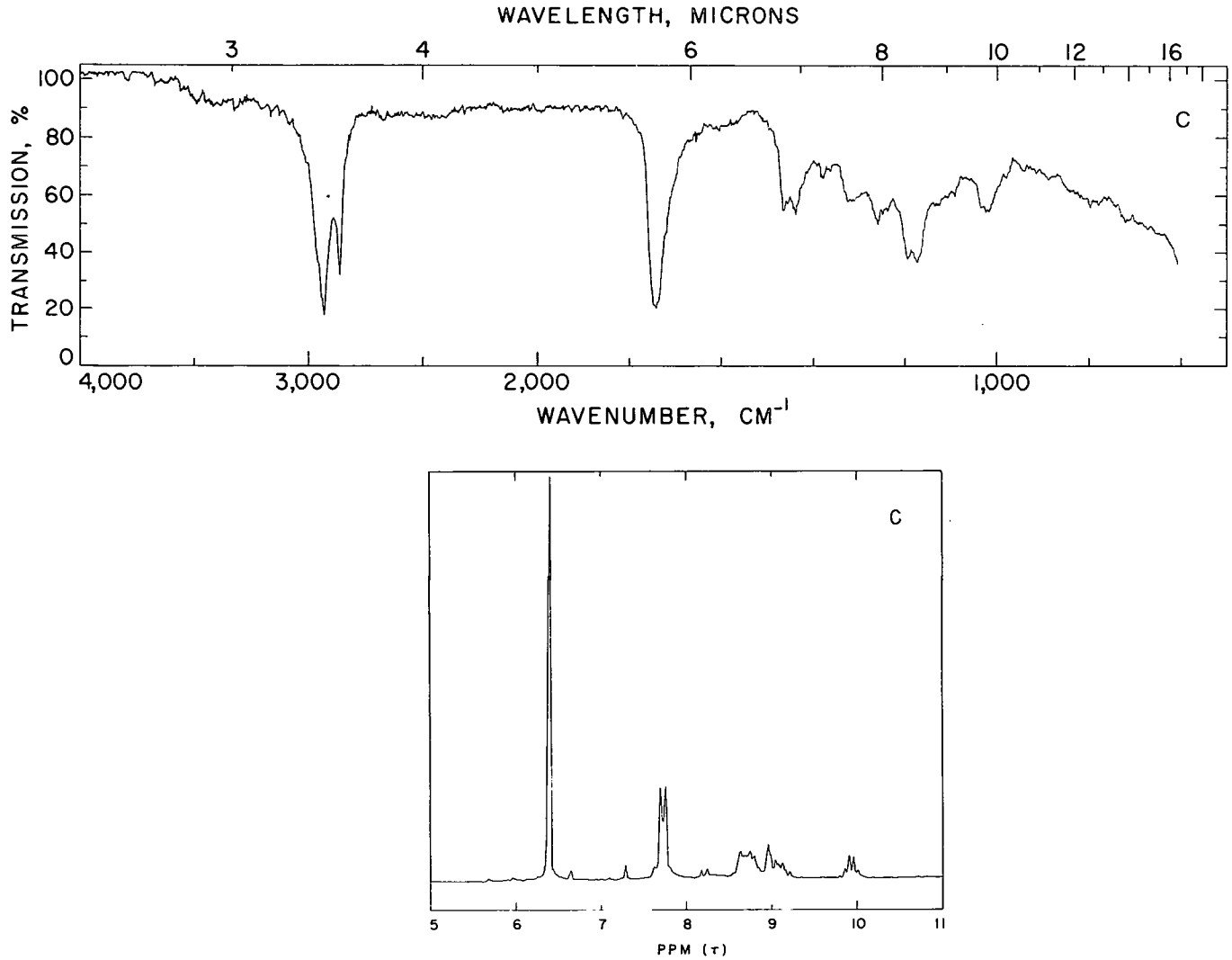


Figure 3. IR (thin film) and NMR of compound C.

Table 2. Mass fragments characteristic of methyl esters of dibasic acids (71).

m/e	Fragment lost	Remarks
P-31	$-OCH_3$	Characteristic of all methyl esters of dibasic acids.
P-32	$-OCH_3 + H$	
P-60	$-COOCH_3 + H$	This peak is prominent only for short chain esters.
P-63	$(-OCH_3)_2 + H$	Prominent for all esters except methyl adipate.
P-64	$(-OCH_3)_2 + 2H$	
P-73	$-CH_2-COOCH_3$	Characteristic of all esters.
P-91	$-COOCH_3 + -OCH_3 + H$	P-92 is higher than P-91 from methyl azelate upwards.
P-92	$-COOCH_3 + -OCH_3 + 2H$	
P-105	$-CH_2-COOCH_3 + -OCH_3 + H$	Low for methyl adipate, high for higher esters.
P-106	$-CH_2-COOCH_3 + -OCH_3 + 2H$	Marked only for very long chain esters.

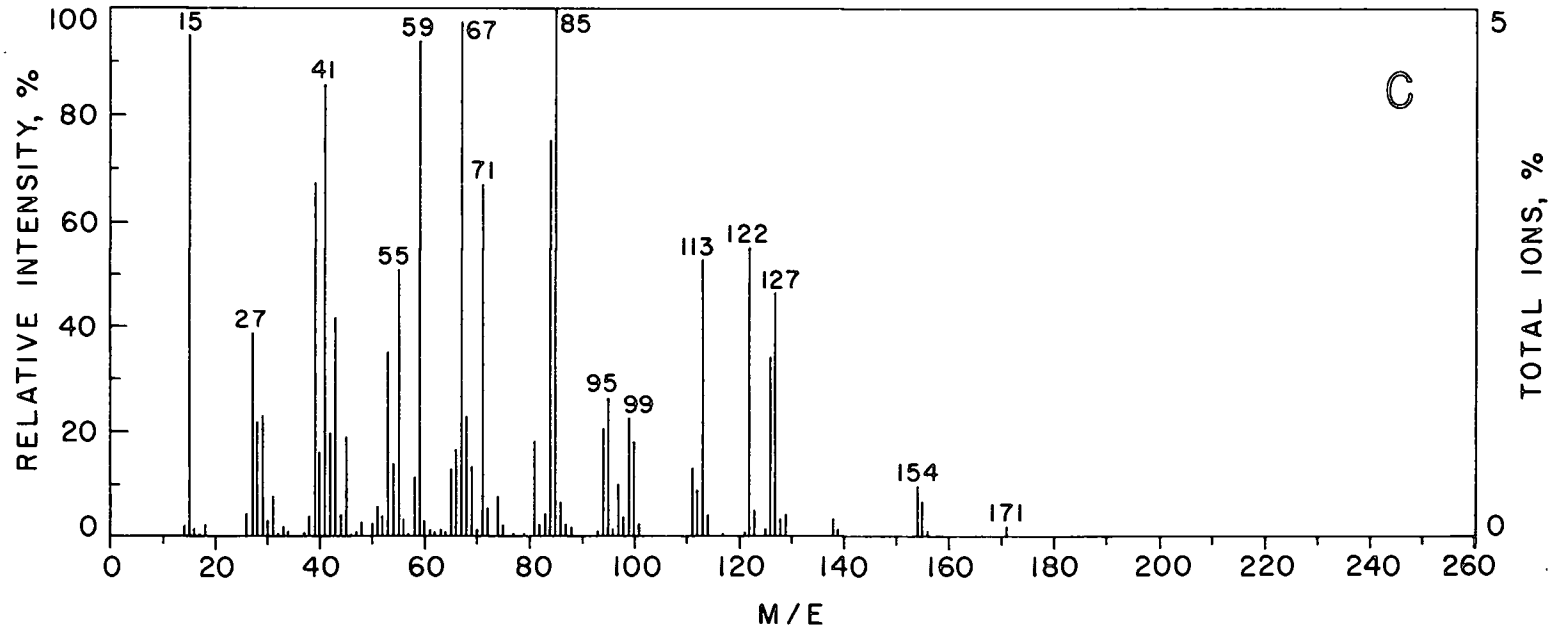
are two methoxyl groups and two methylene groups. A proton upfield at 9.94 - and another at 9.08τ indicate a methylene group of a cyclopropane (25, 39) of the cis-configuration. The two methine protons of the ring absorb at 8.72τ. Absorptions at 6.65τ 7.29τ 8.18τ, 8.25τ, and 8.97τ are from solvent contamination. The above data suggests a compound with the structure shown in Figure 4.

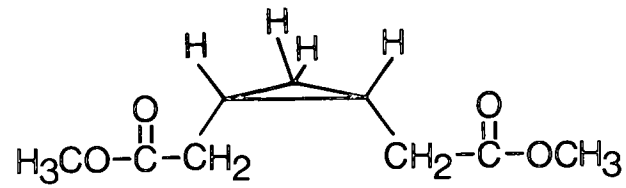
Peak B

Peak B (Figure 2) exhibits the same IR (Figure 5) and MS (Figure 6) as compound C (Figures 3 and 4). Also, the NMR (Figure 5) shows the same methoxyl (6.40τ) and methylene (7.74τ) protons. The difference between the two compounds is the downfield shift of the methylene cyclopropane protons (9.49τ) in the NMR. Wood and Reiser (96) assigned the trans configuration to this absorption. Furthermore, compound B has a lower retention time on a GC column (Figure 2), 1.55 versus 1.80 for compound C, a characteristic expected of a trans isomer on the GC column used. The remaining NMR absorptions are due to solvent contamination. (This sample was scanned 208 times and averaged on a computer). Therefore, the data suggests a structure for compound B shown in Figure 6.

Peak G

The IR (Figure 7) of peak G exhibits the same absorption





M=186

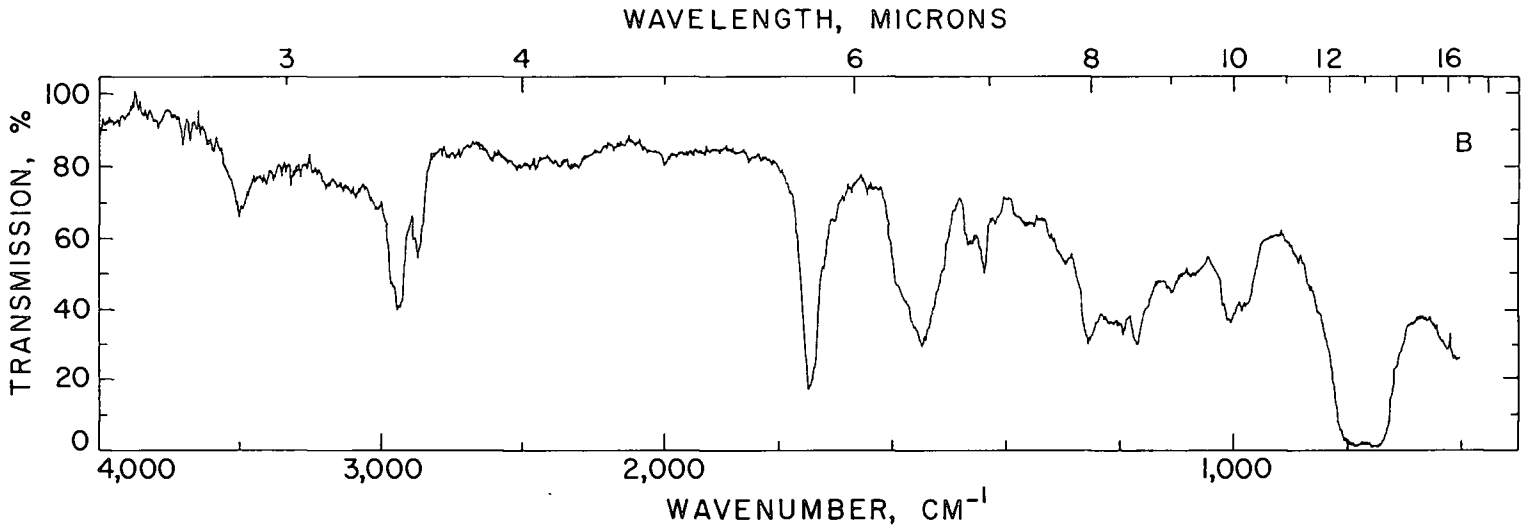
cis-methyl-3,4-methylene adipate

Figure 4. MS, molecular weight, and formula of compound C.

pattern as compounds B (Figure 5) and C (Figure 3), only less intense. Again, there is a carbonyl stretch at 1740 cm^{-1}; carbon-hydrogen methyl stretch at 2860 cm^{-1}; carbon-hydrogen methylene stretch at 2930 cm^{-1}; carbon-oxygen stretch from the methyl ester at 1260 cm^{-1}, 1195 cm^{-1}, and 1175 cm^{-1}; and a weak cyclopropane ring skeletal vibration at 1020 cm^{-1}.

The MS (Figure 8) fragments characteristic of dibasic methylated acids (Table 2): P-32 (m/e 182), P-60 (m/e 154), and P-64 (m/e 150) and the series $[C_nH_{2n-5}O]^+$ and $[C_nH_{2n-3}O_2]^+$ are present. The parent peak is absent, but corresponds to m/e 214. This is 28 mass units or two methylene groups larger than compound B (Figure 6). Also, the m/e 74 representative of the McLafferty (45) rearrangement of typical methyl esters is moderately intense (40%). This would suggest that at least three saturated carbons exist adjacent to one of the ester ends.

The NMR (Figure 7) shows a singlet for two methoxyl groups at 6.39τ and what appears to be a doublet superimposed on a triplet at 7.8τ. The doublet indicates that there is one methylene group between the cyclopropene ring and the ester. The other methylene protons, equal to four, appear in a complex pattern at 8.2τ. A porton at 10.02τ and one at 9.16τ indicate a methylene group of a cyclopropane with cis configuration. The above data suggests a structure for G shown in Figure 8.



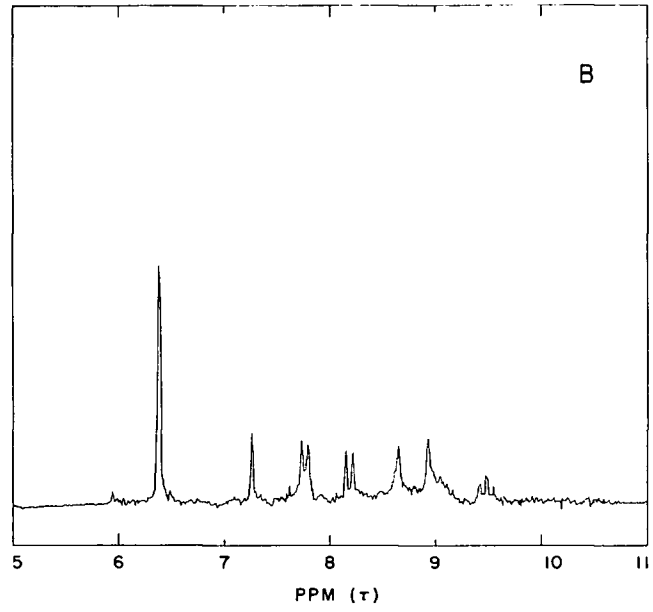
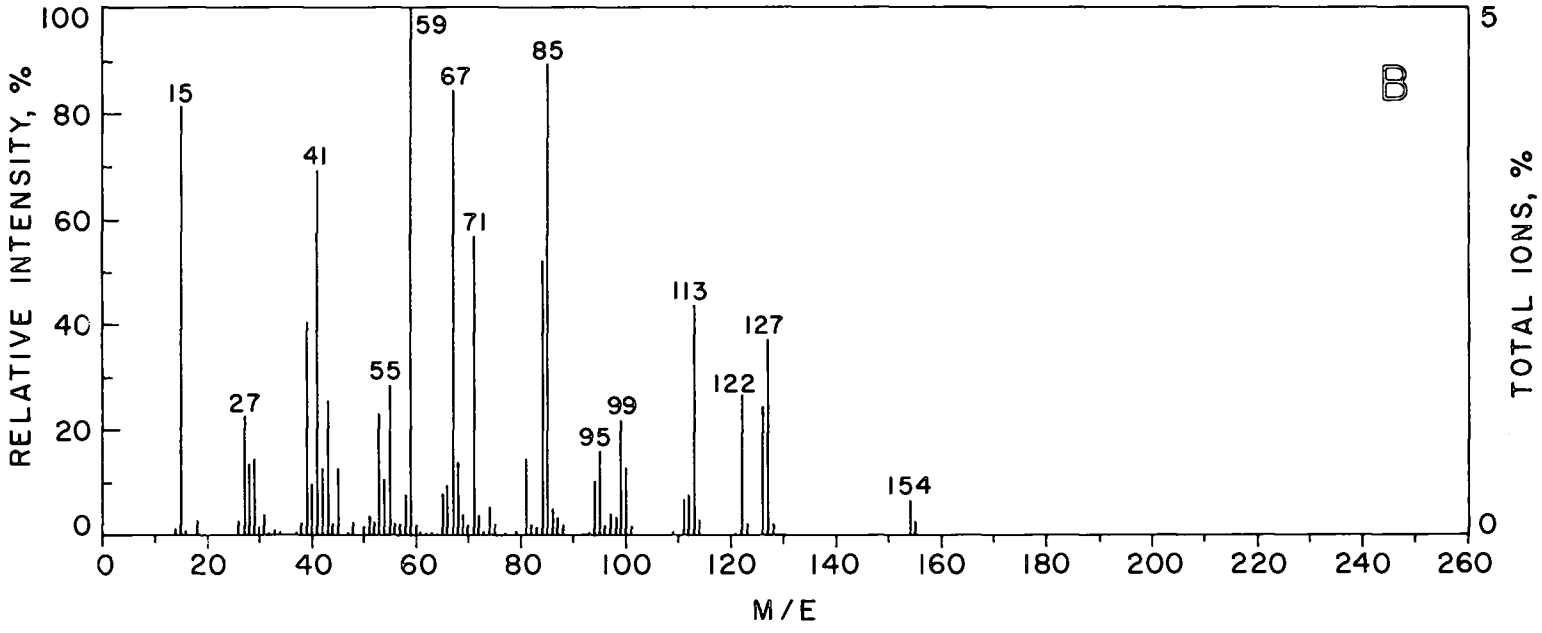
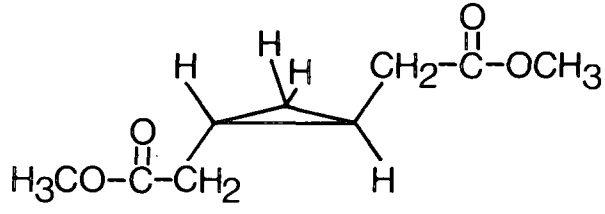


Figure 5. IR (solvent-CCl_4) and NMR of compound B.





M=186

trans-methy-3,4-methylene adipate

Figure 6. MS, molecular weight, and formula of compound B.

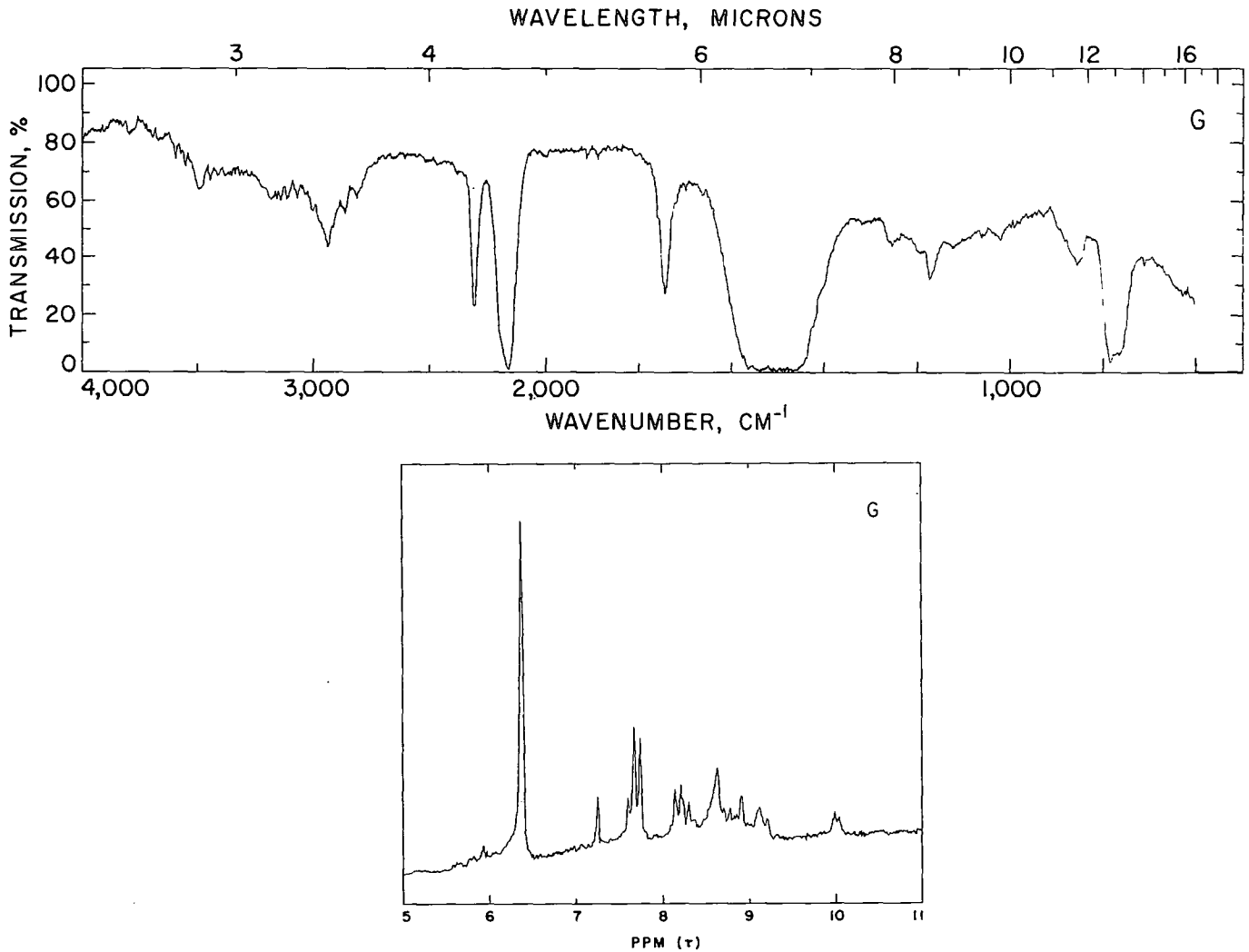
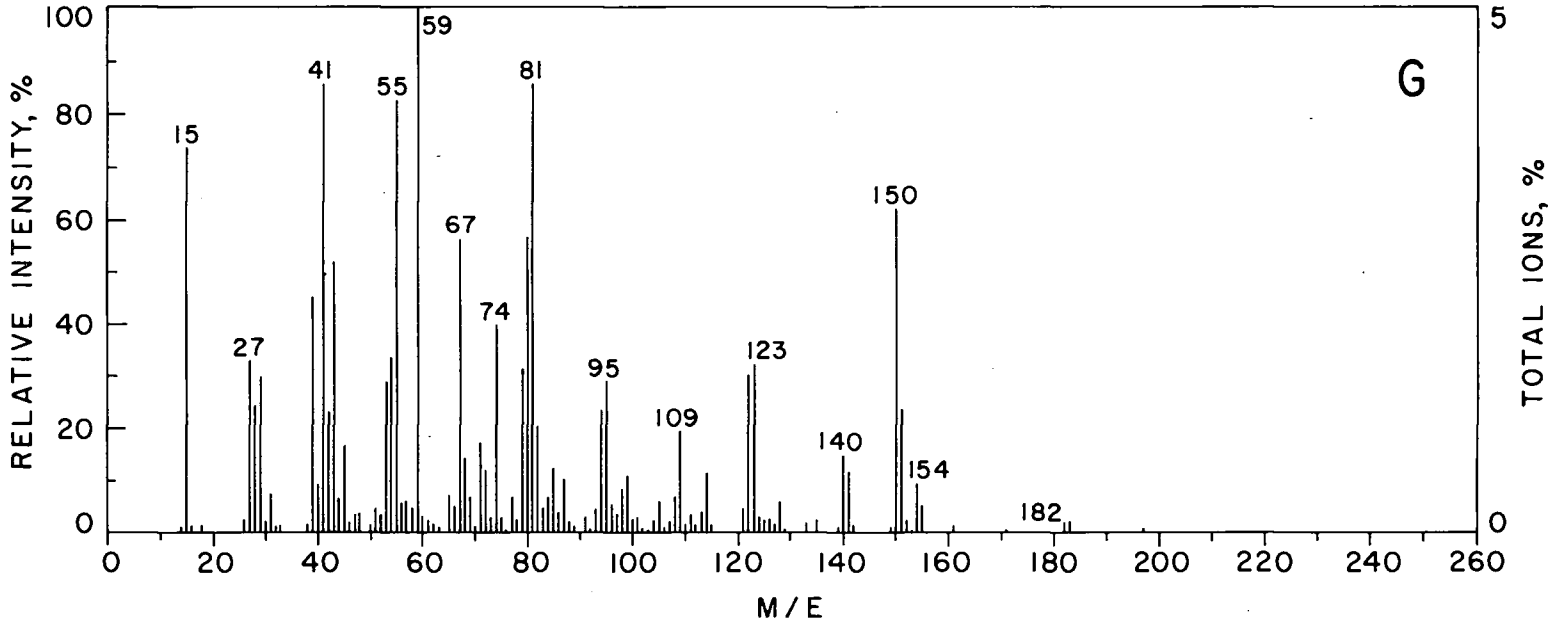
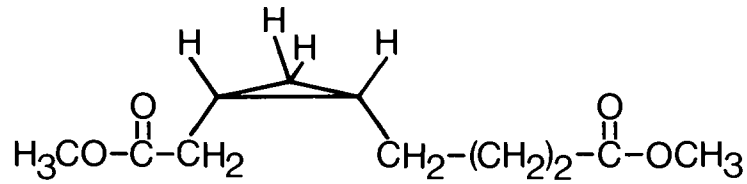


Figure 7. IR (solvent-CS_2) and NMR of compound G.





M=214

cis-methyl-3,4-methylene suberate

Figure 8. MS, molecular weight, and formula of compound G.

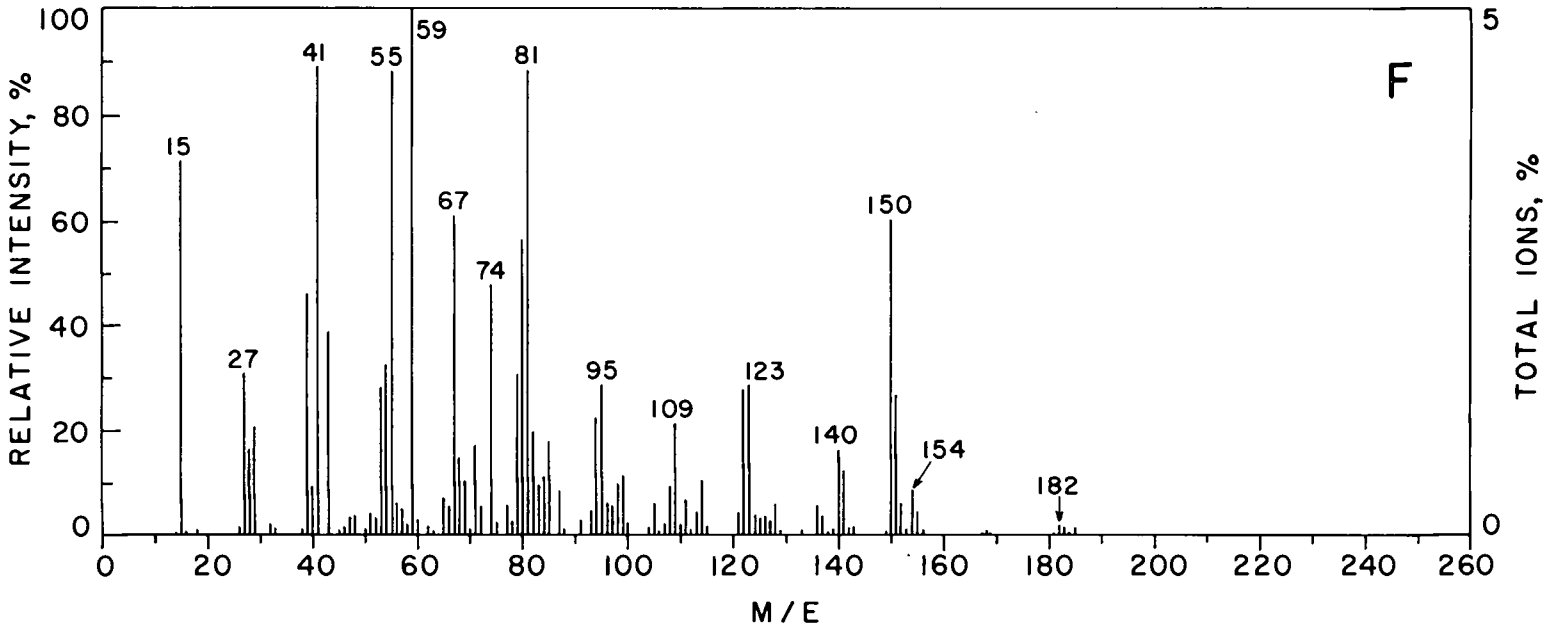
Peak F

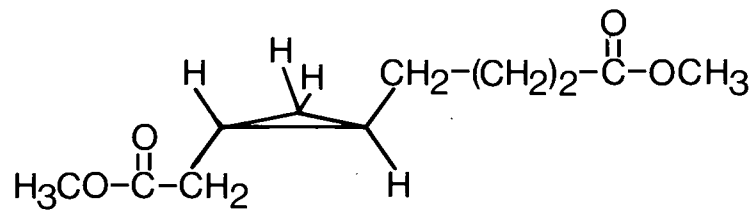
Peak F (Figure 2) has an MS (Figure 9) identical to compound G (Figure 8). There was an insufficient amount of sample for an NMR, and the IR was very weak. The IR did indicate a carbonyl stretch at 1740 cm^{-1} and the carbon-oxygen stretch at 1260 cm^{-1}, 1195 cm^{-1}, and 1175 cm^{-1} for a methyl ester. Compound F has a lower retention time on the GC (Figure 2) than compound G, 2.09 versus 2.35. Since both have the same molecular weight and MS pattern, compound F is probably the trans isomer of compound G (Figure 8). Thus, a probable structure for F is shown in Figure 9.

Peaks E and D

An NMR could not be obtained for peak E. The IR (Figure 10) absorptions were very weak showing only carbonyl stretch at 1740 cm^{-1}, carbon-oxygen stretch of methyl esters at 1260 cm^{-1}, 1195 cm^{-1}, and 1175 cm^{-1}, and also a possible very weak 1020 cm^{-1} for cyclopropane skeletal vibration.

Compound E shows the typical MS (Figure 11) fragmentation pattern of methylated dibasic acids: P-31 (m/e 169), P-60 (m/e 140), P-64 (m/e 136), and P-92 (m/e 108) and the series $[C_nH_{2n-5}O]^+$ and $[C_nH_{2n-3}O_2]^+$. There is very little m/e 74 (14%) from the McLafferty rearrangement, implying that there are less than three





M=214

trans-methyl-3,4-methylene suberate

Figure 9. MS, molecular weight, and formula of compound F.

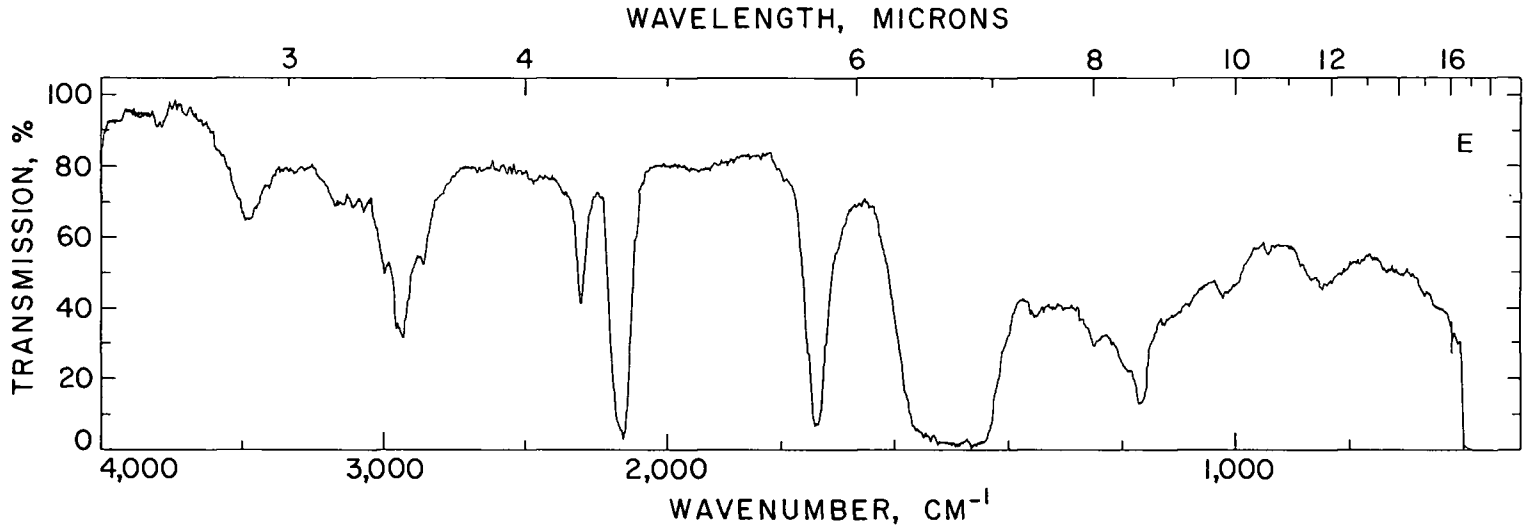


Figure 10. IR (solvent-CS_2) of compound E.

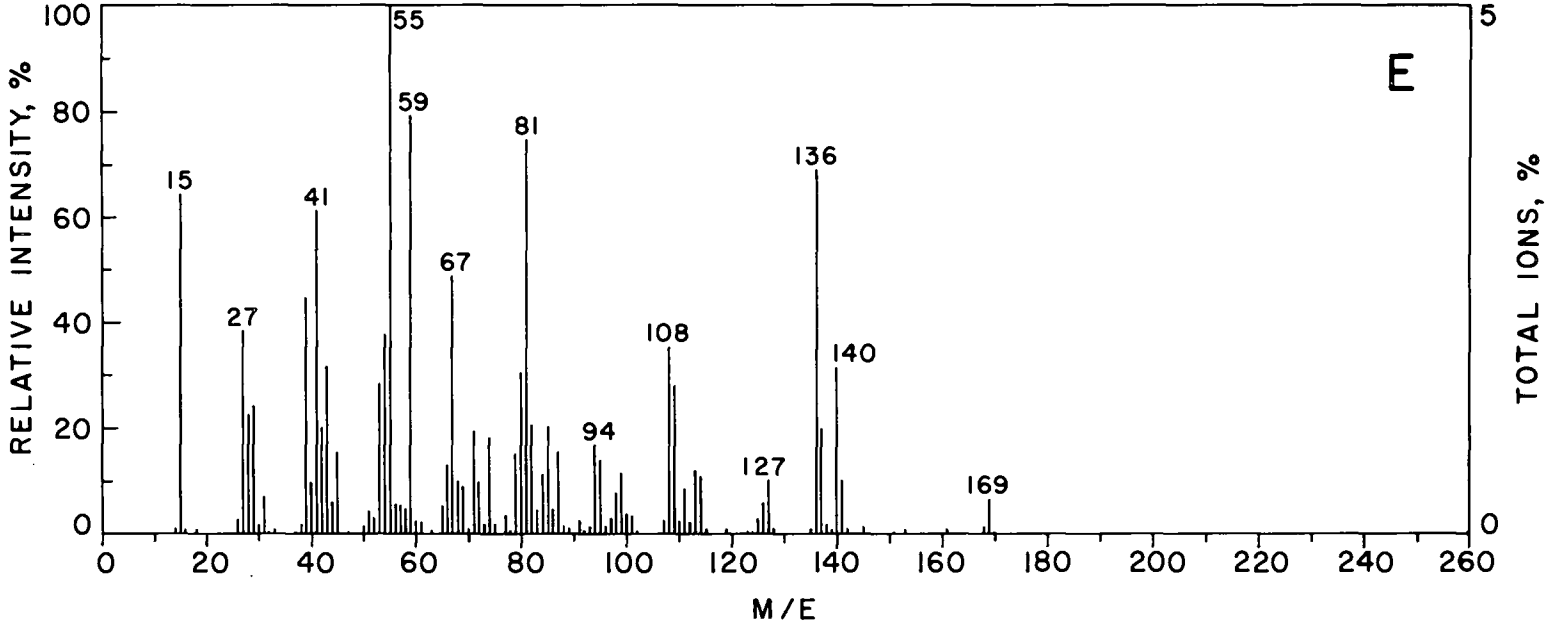
saturated carbons between the ring and the ester group. The parent at m/e 200 is absent. This is 14 mass units or one methylene group larger than compound C (Figure 4).

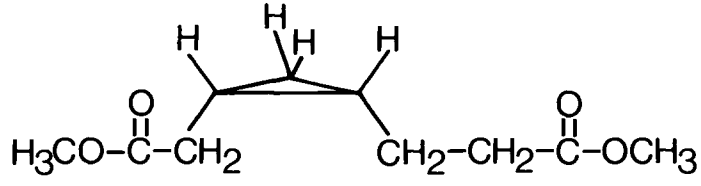
Compound D (Figure 2) has an MS (Figure 12) identical to compound E (Figure 11). Infrared or NMR spectra could not be obtained. Compound D has a lower retention time, 1.86 versus 2.04, than compound E. Since both have the same molecular weight, the data weakly suggests a structure for E as shown in Figure 11 and for D as shown in Figure 12.

Peak H

An NMR could not be obtained for compounds H or I. The IR's were very weak due to insufficient material and indicated only carbonyl stretch at 1740 cm^{-1}.

The MS (Figure 13) of compound H (Figure 2) shows the typical fragments of dibasic acid methyl esters: P-32 (m/e 196), P-60 (m/e 168), P-64 (m/e 164), and P-92 (m/e 136). The parent peak is absent, but would correspond to a molecular weight of m/e 228. This is 14 mass units or one methylene group larger than compound G (Figure 8). The same series $[C_nH_{2n-5}O]^+$, m/e 53, 67, 81, and 95, and $[C_nH_{2n-3}O_2]^+$, m/e 71, 85, 99, 113, and 127 are present. Also, m/e 74 (88%) is very intense indicating a carbon length of three or more from the ester. Since the MS of H is very similar to

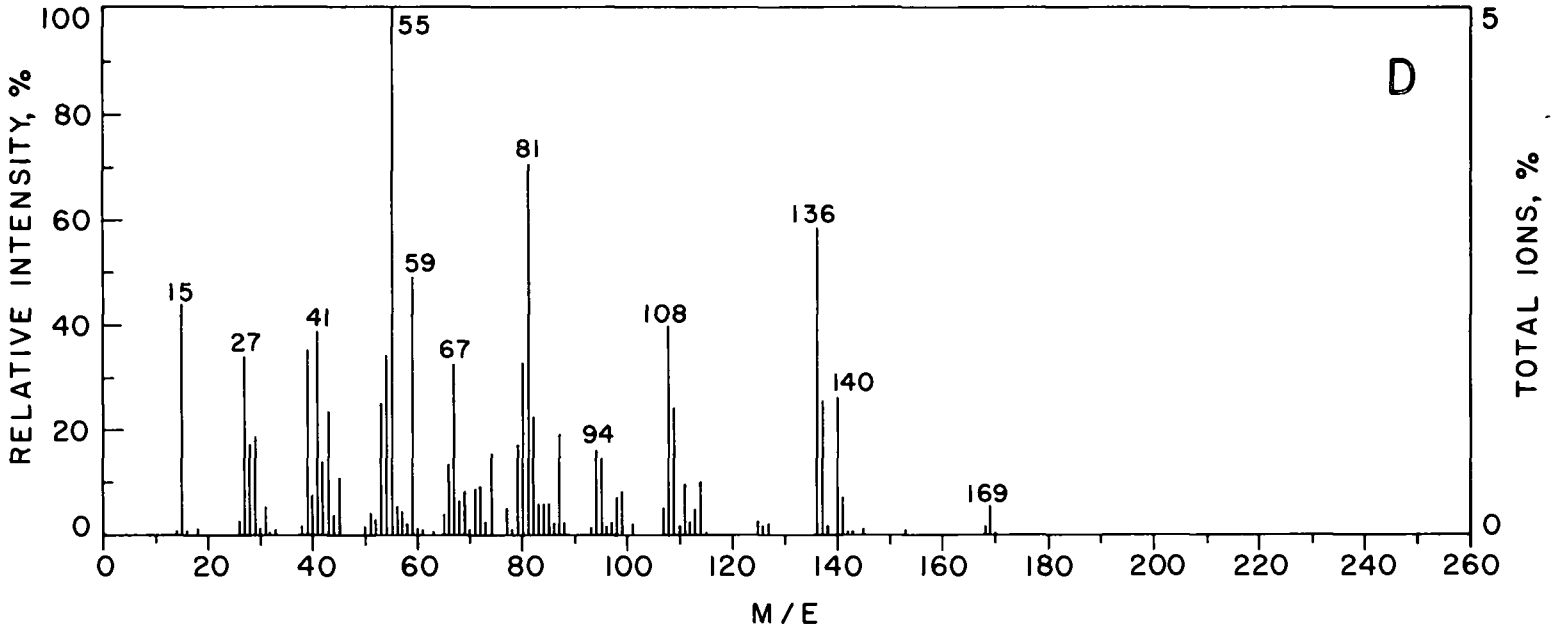


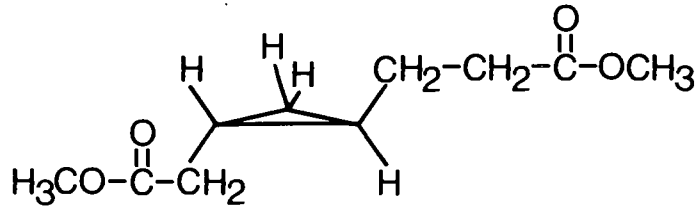


M=200

cis-methyl-3,4-methylene pimelate

Figure 11. MS, molecular weight, and formula of compound E.

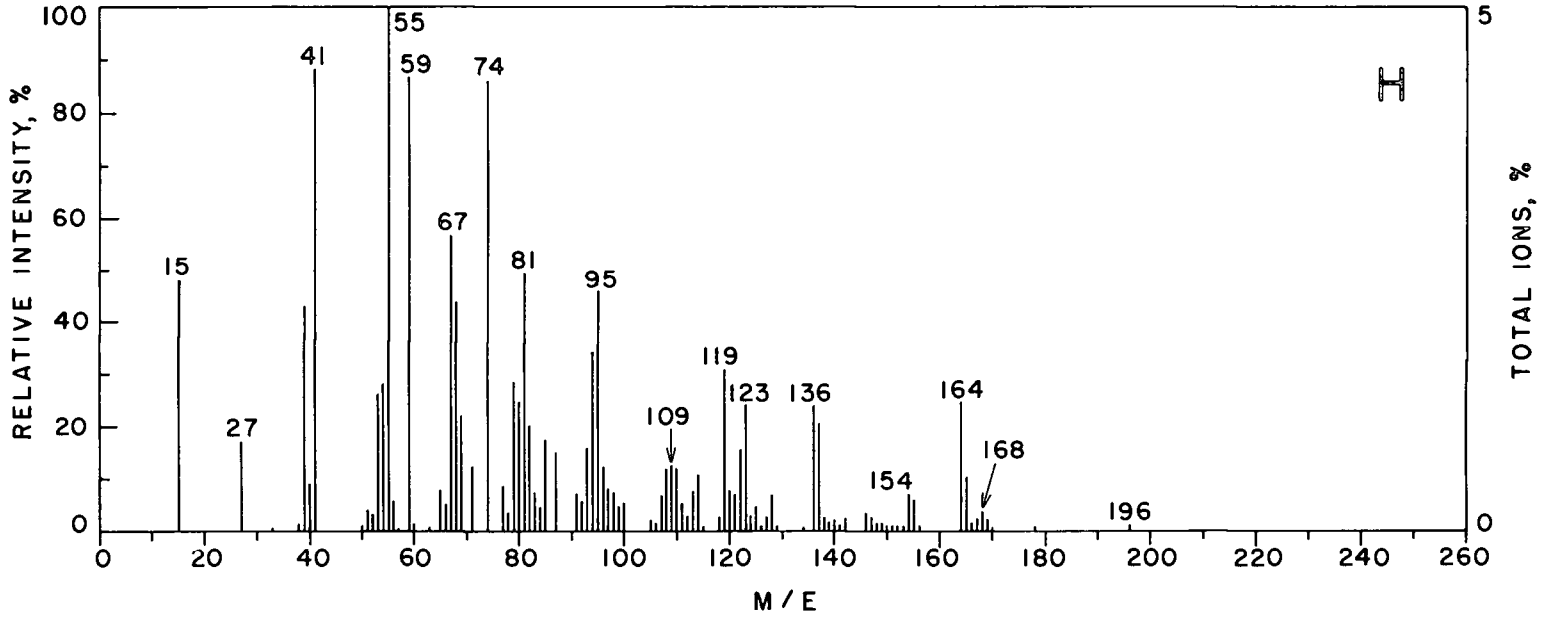


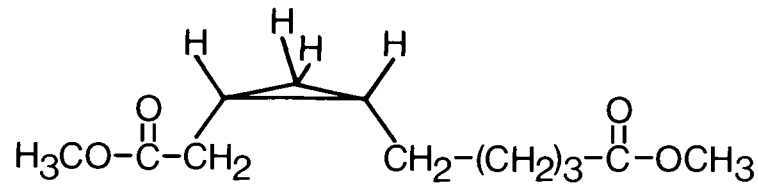


M=200

trans-methyl-3,4-methylene pimelate

Figure 12. MS, molecular weight, and formula of compound D.





M=228

cis-methyl-3,4-methylene azelate

Figure 13. MS, molecular weight, and formula of compound H.

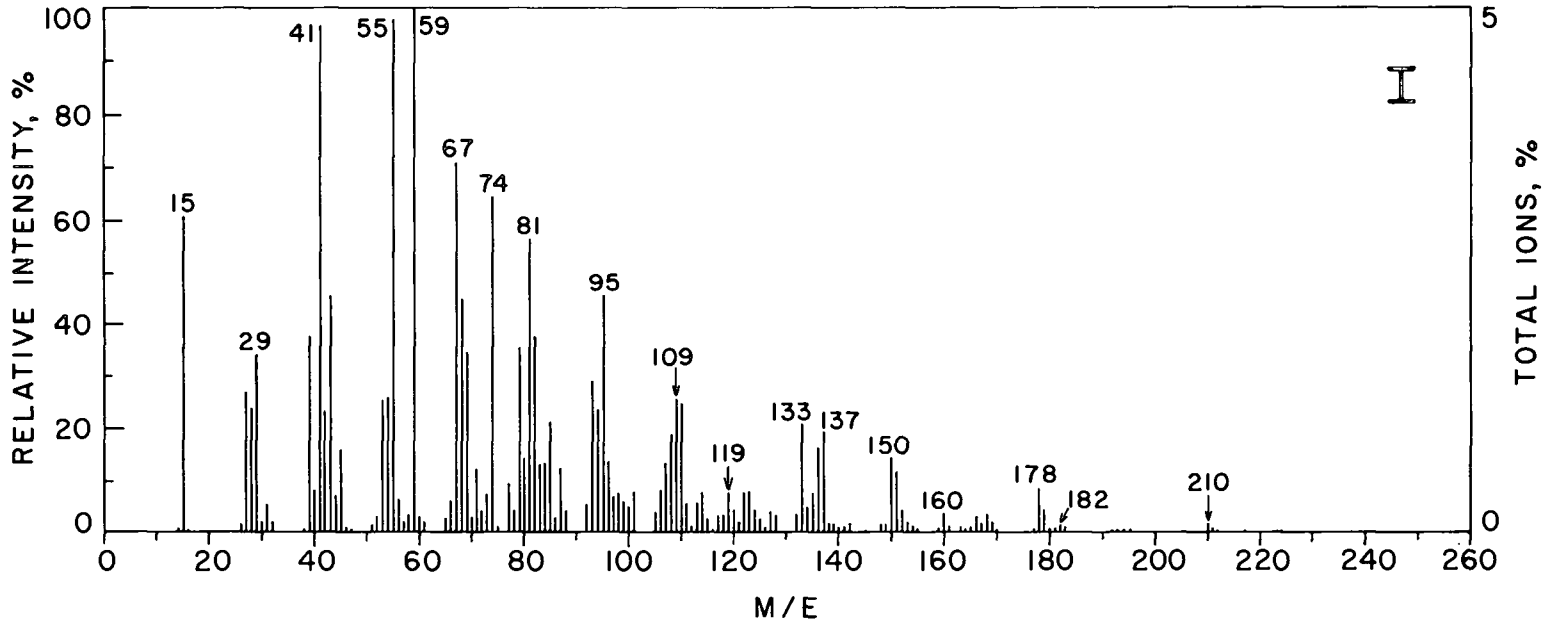
that of G, the structure of H is probably like G. Also, considering that the amount of cis isomers are prevalent over the trans isomers, a probable structure for compound H is shown in Figure 13.

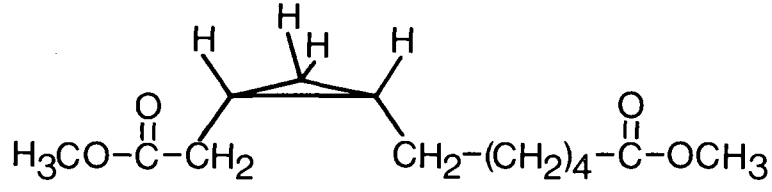
Peak I

Peak I (Figure 2) has an MS (Figure 14) fragmentation pattern similar to G (Figure 8) and H (Figure 13). The mass spectra of the cyclopropane urinary metabolites all appear to have the same basic fragments. The typical fragments of methylated dibasic esters are present: P-32 (m/e 210), P-60 (m/e 182), P-64 (m/e 178), and P-92 (m/e 150), along with the two series $[C_nH_{2n-5}O]^+$ and $[C_nH_{2n-3}O_2]^+$. The parent peak is absent, but would correspond to m/e 242. This is 14 mass units or one methylene group larger than compound H. Again, based on the same considerations given compound H, a probable structure of I is shown in Figure 14.

Peak A

The MS (Figure 15) of peak A (Figure 2) shows a parent of m/e 172. This is 14 mass units less than compound C (Figure 4), cis-methyl-3, 4-methylene adipate. The fragmentation pattern is different from the other compounds of this series. The IR and NMR could not be obtained, thus no attempt was made to assign a structure to A.

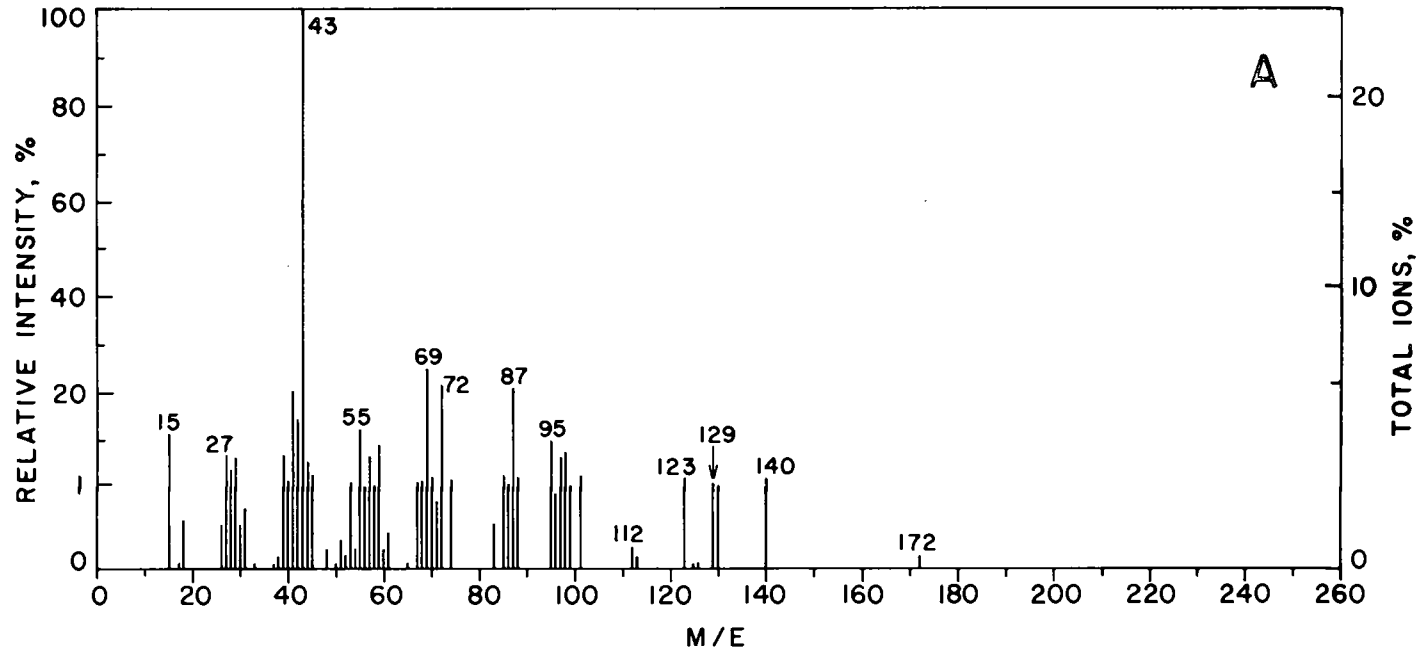




M=242

cis-methyl-3,4-methylene sebacate

Figure 14. MS, molecular weight, and formula of compound I.



M = 172

unknown

Figure 15. MS and molecular weight of compound A.

Peak J

A closer look at the GC (Figure 2) indicates another possible labelled metabolite numbered J. No spectral data was obtained on this compound due to insufficient amounts. However, being the last compound to come off the column, it is probably an analog of the methylated dibasic cyclopropane esters.

In summary, eight metabolites were tentatively identified out of ten labelled peaks shown on the GC (Figure 2). They are listed in Table 3 giving the molecular weight and name of each compound.

Identification of [9, 10-methylene-^{14}C]sterculic Acid Urinary Metabolites in Corn Oil Fed Rats

The purpose of this section was to test if corn oil fed rats given sterculic acid (IG) produced the same urinary metabolites as SFO fed rats. A typical gas chromatograph of methylated CO fed rat urine is illustrated in Figure 16. Peaks that contained label [^{14}C] were analyzed by MS and found to have identical mass spectra as the corresponding peaks of SFO fed rats. Table 4 shows the relative retention times of each peak from both groups of animals, and the total percentage of activity for each labelled peak. Peak E in the CO fed rat could not be detected due to its close similarity to peak F. The dashed line in the GC (Figure 16) shows where it would be present.

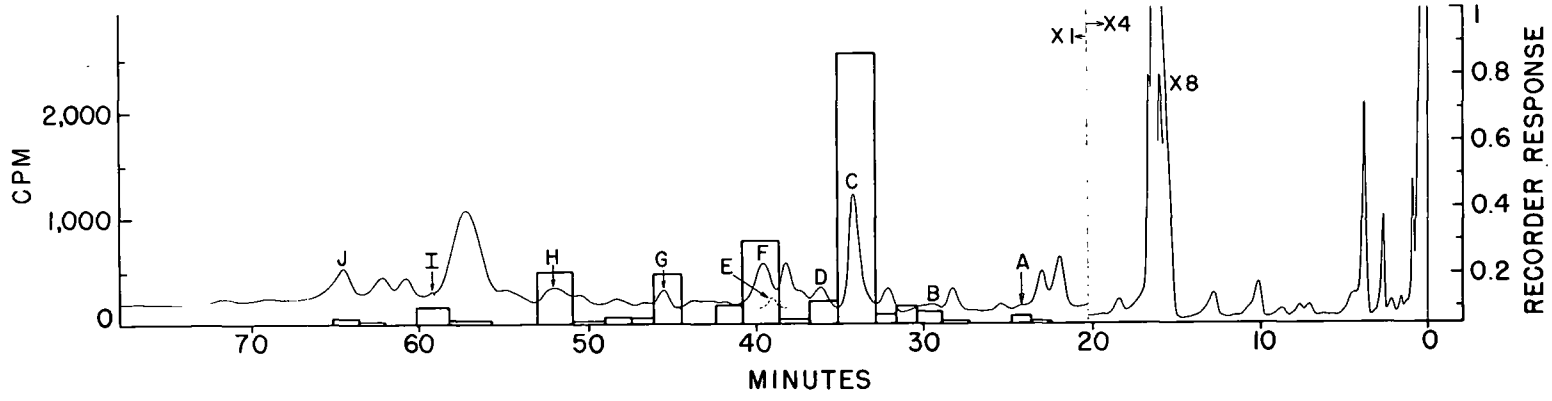


Figure 16. Gas chromatograph of methylated urine extract of CO fed rat injected with [9, 10-methylene-^{14}C] sterculic acid.

Table 3. Urinary metabolites of [9, 10-methylene-^{14}C]sterculic acid from SFO fed rats.

Peak	Molecular Weight	Name (Methyl Ester)
A	172	Unknown
B	186	Trans-methyl-3, 4-methylene adipate
C	186	Cis-methyl-3, 4-methylene adipate
D	200	Trans-methyl-3, 4-methylene pimelate
E	200	Cis-methyl-3, 4-methylene pimelate
F	214	Trans-methyl-3, 4-methylene suberate
G	214	Cis-methyl-3, 4-methylene suberate
H	228	Cis-methyl-3, 4-methylene azelate
I	242	Cis-methyl-3, 4-methylene sebacate
J		Unknown

Table 4. Retention time and percent label from [9, 10-methylene-^{14}C]sterculic acid urinary metabolites of CO and SFO fed rats.

	SFO Fed Rat		CO Fed Rat	
Compound #	R_t [a]	%CPM	R_t [a]	%CPM
A	1.30	1.42	1.31	1.61
B	1.55	7.37	1.59	5.83
C	1.80	47.51	1.85	46.79
D	1.86	1.42	1.91	3.37
E	2.04	8.75	2.09	[b]
F	2.09	2.50	2.15	2.32
G	2.35	18.06	2.44	9.57
H	2.64	5.89	2.81	11.88
I	2.98	5.36	3.20	3.35
J	3.21	1.73	3.53	1.11

[a] Retention time was calculated using methyl phenyl acetate as a reference, peak 6 (Figure 17).

[b] Compound E CPM's are summed with compound F.

The data indicates that CO fed rats can metabolize CPFA the same as SFO fed rats even though they had not been acclimated to the CPFA diet. The main metabolites for both groups were cis-methyl-3, 4-methylene adipate, compound C (46-47%), and cis-methyl-3, 4-methylene suberate, compound G (9-18%). These are the same two metabolites that Yoss (97) identified in his corn oil fed rats given labelled sterculic acid.

The GC elution pattern of Figure 16 differs from that of Figure 2 because the relative amount of label and metabolites in the CO fed rat urine (1-40%) was lower than the amount of label and material in the SFO fed rat urine (46-57%) (Table 6).

Identification of Urinary Metabolites in SFO Fed Rats

The purpose of this section was to determine if SFO fed rats excreted the same urinary metabolites as SFO fed rats injected (IG) with labelled sterculic acid.

Figure 17 shows a typical GC of methylated rat urine from a SFO fed rat. The lettered peaks A through J correspond to the sterculic acid metabolites. Compound D and E, cis and trans methyl-3, 4-methylene pimelate, could not be detected by MS. The chopped line indicates where they would be on the chromatograph.

The numbered peaks 1 through 11 were also identified by

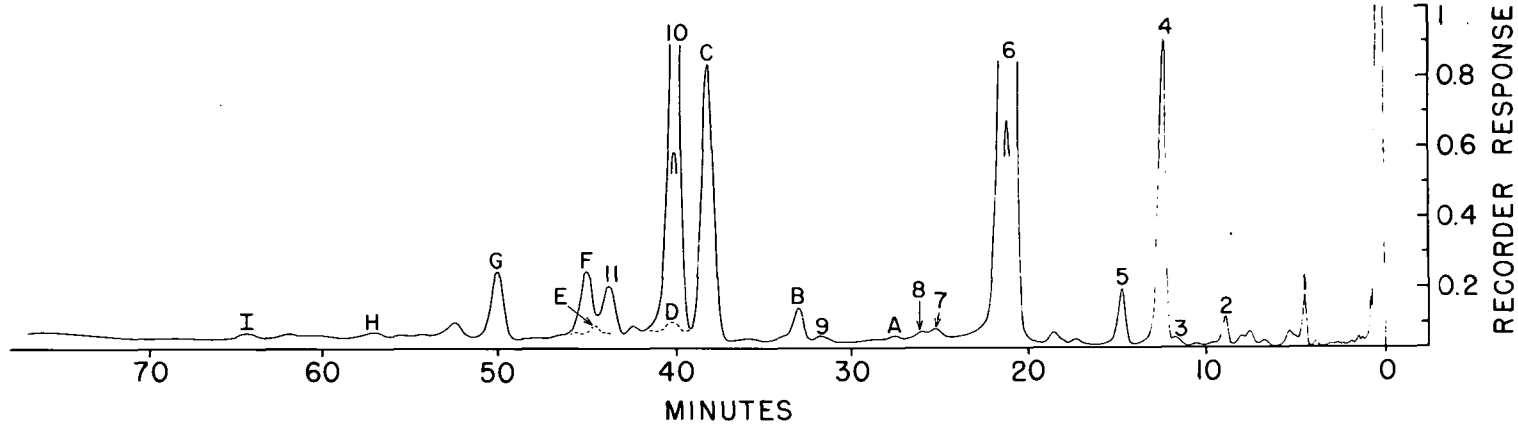


Figure 17. Gas chromatograph of methylated urine extract of SFO fed rat.

combinated MS, IR, and GC retention time (Table 5). Compound 6, methyl phenyl acetate which was the largest amount, is formed by the normal metabolism of pehnylalanine to phenylpyruvic by by transamination and further decarboxylation to phenyl acetic acid (56). The other large peak 10, p-cresol, is formed in the gut by bacterial action on tyrosine, followed by absorption in the intestional tract, and then excretion through the urine (57). The other lesser compounds are found in various amounts in the urine of most mammals (43, 58, 64).

Label [^{14}C] in Liver Fractions, Liver Lipid Fractions and Carbon Dioxide from [9, 10-methylene-^{14}C] sterculic Acid

The primary purpose of fractionating the liver and collecting carbon dioxide was to investigate the possibility of [9, 10-methylene-^{14}C]sterculic acid being incorporated into other products. Table 6 shows the distribution of label in the various fractions expressed as a percent of the administered labelled dose.

The most obvious observation is that the majority of the label was located in the urine over the ten hour period. The labelled carbon dioxide accounted for about 1.0% or less of the activity. Hence, very little of the ring was metabolized. In the liver, the majority of the label is in the lipid fraction with most of this in the fatty acid material. A closer look at the liver fractions shows a

Table 5. Other urinary metabolites in SFO fed rats.

Peak #	Compound Name	Identification Method			Source of Reference
		GC (R_t)[a]	MS[b]	IR[c]	Compound
1	Methyl lactate	0.21	X		Eastman Org. Chem.
2	Dimethyl oxalate	0.42	X		MCB
3	Dimethyl malonate	0.55	X		Sigma
4	Methyl benzoate	0.58	X	X	MCB
5	Dimethyl succinate	0.70	X		Eastman
6	Methyl phenyl acetate	1.00	X	X	Aldrich
7	Dimethyl-2-methyl glutarate?	1.19	X		
8	Dimethyl adipate	1.23	X		Sigma
9	Dimethyl pimelate	1.50	X		Sigma
10	P-cresol	1.89	X	X	Aldrich
11	Dimethyl octadecen (?) ioate	2.02	X		

[a] Methyl phenyl acetate was used as a reference standard.

[b] MS were compared to known compound (92).

[c] IR were compared to known compounds listed in source.

Table 6. Percent of administered [^{14}C] dose in urine, carbon dioxide, liver fractions, and liver lipid fractions of CO and SFO fed rats injected with [9, 10-methylene-^{14}C]sterculic acid.

		Activity Expressed as Percent of Administered Dose								
					Liver Fractions			Liver Lipid Fractions		
Rat #	Diet	Liver	Urine	CO_2	Protein	Lipid	Acid Sol.	Steroid	FA	Glycerol
1	CO	5.04	0.87	0.34	0.34	3.78	0.93	a/	3.78	a/
2	SFO	4.40	56.98	0.44	0.12	3.97	0.30	a/	3.97	a/
3	CO	9.50	39.47	1.05	0.80	8.00	0.70	a/	8.00	a/
4	SFO	11.14	46.55	0.65	0.35	10.36	0.43	a/	10.36	a/

a/ The amount of activity in these fractions was less than 0.01% of dose.

significant amount of label in the protein fraction for CO and SFO fed rats. Kircher (31) suggested that sulfhydryl groups of proteins may react with cyclopropenes, thus causing the physiological effects observed from feeding CPFA. The procedure used in the fractionation of the liver should remove all lipid from the protein. Consequently, this adds more evidence that Raju and Reiser (65) may be correct in implying that CPFA binds irreversibly to enzymatic sulfhydryl groups.

In summary, a large percentage of sterculic acid is being metabolized to dicarboxylic acids and excreted in the urine. Corn oil fed rats can metabolize CPFA almost as effectively as SFO fed rats with the difference being only the time required.

Oxidative Mechanisms in [9, 10-methylene-^{14}C]sterculic Acid Metabolism

The metabolites indicate a combination of α, β, and ω-oxidative mechanisms taking place. β-oxidation occurs in the mitochondria (34) and α- and ω-oxidation takes place in the microsomes (54, 55). Normal straight chain fatty acids undergo β-oxidation, however, it has been shown that in certain cases, straight chain as well as branched chain (13) fatty acids do undergo ω-oxidation.

Bergstrom et al. (4) studied the metabolism of

[1-^{14}C]-2,2-dimethylstearic acid in the rat. They found very little labelled carbon dioxide and recovered 90% of the label in the urine as [1-^{14}C]-2,2-dimethyladipic acid. In another study on α-substituted alkyl myristates and stearates in dogs, Wetzel (95) observed that when the side chain was greater than an ethyl group, large amounts of the corresponding α-substituted adipic acid was excreted in the urine. The metabolism of [9,10-methylene-^{14}C]sterculic acid produces cis and trans-3,4-methylene adipic and cis and trans-3,4-methylene suberic acid as the main products. Therefore, sterculic acid metabolism seems to be a special case of branched chain fatty acid metabolism with the 9,10-methylene of the ring acting as a branch.

Another laternate pathway, α-oxidation, was demonstrated by Stokke et al. (85) to be prominent in certain types of branched chain fatty acid metabolism. After the ingestion of 3,6-dimethyl[8-^{14}C] octanoic acid by man, labelled carbon dioxide was found in the expiratory air, and 2,5-dimethylheptanioic acid was demonstrated to be present in the urine. He suggested that this pathway would account for about 1-2% of all fatty acid oxidation. The total amount of cis- and trans-3,4-methylene pimelic acid, the products of α, β, and ω-oxidation, isolated in the urine from CO and SFO fed rats injected with labelled sterculic acid, was approximately 4% and 10% respectively (Table 4). However, the SFO fed rat that was not given

label (Figure 17), did not form these two products indicative of α-oxidation. Thus α-oxidation seems to occur only when an animal is given a large dose of oil at one time. During moderate, regular exposure to SFO and/or CO, this pathway was less evident.

Proposed Metabolic Pathway for Sterculic Acid

Yoss (97) observed in a time distribution study of [9, 10-methylene-^{14}C]sterculic acid in IG injected rats, that initially, 0-1 hour, the 12,000 Xg mitochondrial fraction contained more label than the 105,000 Xg microsomal fraction. From two to four hours the activity was higher in the microsomes. After four hours the microsomal counts dropped below the mitochondrial and remained there for 26 hour study period. Based on the above observations, the urinary metabolites identified, and the fact the ring in hydrogenated, the following pathway (Figure 18) is proposed for sterculic acid.

Sterculic acid is transported to the mitochondria where it is β-oxidized to within two carbons of the ring. Further transport to the microsomes leads to reduction of the cyclopropene ring to a cyclopropane ring plus ω-oxidation of the methyl group to an acid. It is probably during reduction that the two isomers, cis and trans, are formed. Evidence is lacking as to the order that reduction and ω-oxidation take place and also why the cis is formed in preference

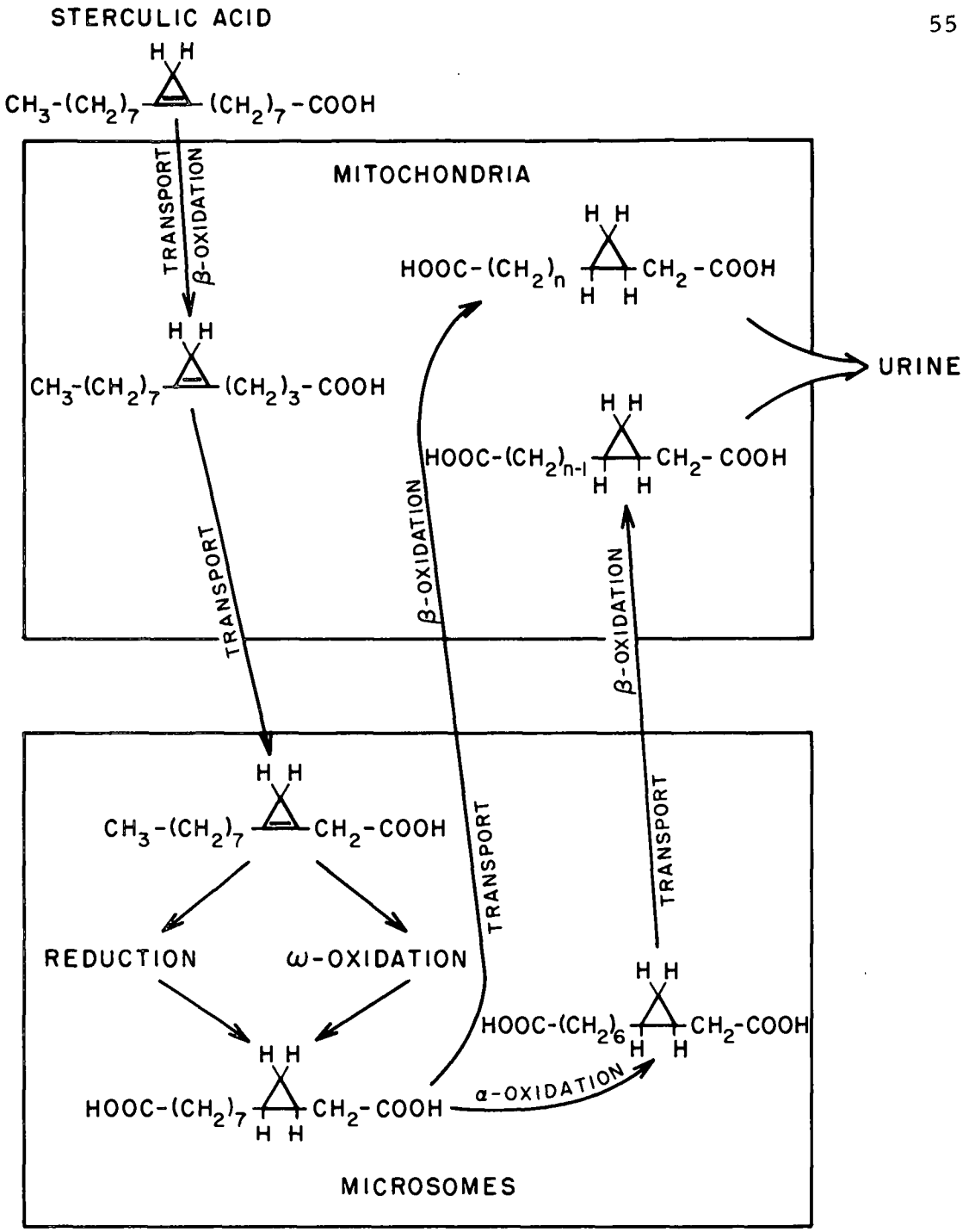


Figure 18. Proposed pathway for Sterculic Acid Metabolism in the rat.

to the trans. Some of the dicarboxylic acids are then transported back to the mitochondria and further β-oxidized to the corresponding urinary metabolites. Whether this is the preferred pathway has yet to be extablished, but to form the dicarboxylic acids and to reduce the ring, sterculic acid must be transported through both oxidative systems.

Label [^{14}C] in Liver Fractions, Liver Lipid Fractions, and Carbon Dioxide from n-[1-^{14}C]octadecane in Rats

In the previous sections, it has been shown that sterculic acid undergoes ω-oxidation as part of its metabolism. The purpose of this experiment was to determine whether CPFA induced ω-hydroxylation and effected the metabolism of n-[1-^{14}C]octadecane. Even though the hydrocarbon is a completely different compound than a fatty acid, Gillette (19) visualized all hydroxylation reactions as being catalyzed by simple microsomal metabolizing systems that use a mixed function oxidase mechanism consisting of NADPH, oxygen, cytochrome c reductase, cytochrome P-450, and cytochrome P-450 reductase. The amount of labelled carbon dioxide would be proportional to the amount of oxidation taking place and this would be an index of the amount of ω-hydroxylation. Table 7 compares the label distribution in liver fractions, liver lipid fractions and carbon dioxide from CO and SFO fed rats over an eight hour period.

Table 7. The percent of administered [^{14}C] dose in urine, carbon dioxide, liver fractions, and liver lipid fractions of CO and SFO fed rats injected with n-[^{14}C]octadecane.

		Activity Expressed as Percent of Administered Dose								
					Liver Fractions			Liver Lipid Fractions		
Rat #	Diet	Liver	Urine	CO_2	Protein	Lipid	Acid Sol.	Steroid	FA	Glycerol
5	CO	0.44	--	a/	0.02	0.40	0.02	0.27	0.09	0.04
6	SFO	0.44	0.09	a/	0.03	0.39	0.02	0.20	0.12	0.07
7	CO	0.56	--	a/	0.02	0.48	0.04	0.35	0.07	0.06
8	SFO	2.14	0.02	a/	0.08	2.00	0.06	1.83	0.09	0.08

a/ The amount of activity in these fractions was less than 0.01% of the dose.

Animals on both diets metabolized n-octadecane very slowly as evident by the lack of label in the carbon dioxide. There was very little difference between either group. However, the SFO fed rats indicated a tendency to metabolize the compound faster as observed by the amount of label present in the fatty acid and glycerol fractions compared to the CO fed rats. The method used in fractionating the lipid would result in the steroid fraction containing the free n-octadecane (see Appendix VII). Also, the SFO fed rats excreted label in the urine. The identity of the metabolite has not been determined. In conclusion, CPFA seems to increase n-octadecane metabolism in rats, but the evidence was not very conclusive for the time period used.

Effects of Sterculic Acid Metabolism on the Rat

Tricarboxylic Acid Cycle Inhibitors

The major urinary metabolite of sterculic acid is 3, 4-methylene adipic acid which closely resembles the tricarboxylic acid cycle intermediates. These metabolites may be acting as inhibitors of the TCA cycle, thus causing some of the observed physiological effects of CPFA (38, 50). Also, Pettersen et al. (63) demonstrated that dicarboxylic acids compete with mono-carboxylic acids for the same ATP-activation enzymes that form CoA derivatives, and consequently,

may inhibit the overall lipid-energy balance of the organism.

Renal Tubular Degeneration

Weitzel et al. (94), when feeding β-substituted short chain dicarboxylic acids to dogs, observed the following "tolerances" (gm/kg body wt/day) for the following acid derivatives: n-adipic acid (6.27), β-methyl adipic acid (0.16), and β-ethyl adipic acid (0.15). Also, Rose (70) tested many dicarboxylic short chain fatty acids on rabbits. He noticed a failure in renal function, marked retention of nitrogenous waste products, and nephrosis, which is the degeneration of the renal tubules without inflammation. Thus, the evidence strongly suggests that excessive amounts of dicarboxylic acids can cause physiological changes in animal species. A rough calculation of the amount of 3,4-methylene adipic acid excreted from a rat fed CPFA diet in this thesis (Appendix VIII) amounts to approximately 0.15 gm/kg body wt/day. Nixon et al. (50) reported that focal degeneration of the kidney tubules was common in CPFA fed rats; hence CPFA metabolites may very well cause renal damage.

SUMMARY

Very little activity was expired as labelled carbon dioxide in CO and SFO fed rats given an injection of [9, 10-methylene-^{14}C] sterculic acid. The majority of the label was excreted in the urine as short-chain dicarboxylic acids with an intact cyclopropane ring. The rat apparently does not metabolize the ring, but can reduce (hydrogenate) it. The major urinary metabolites were cis-3, 4-methylene adipic acid and cis-3, 4-methylene suberic acid. Sterculic acid must undergo β- and ω-oxidation to form these metabolites and be transported through both mitochondrial and microsomal systems. α-oxidation played a minor role. SFO fed rats could metabolize sterculic acid at a faster rate than CO fed rats, but both produce the same urinary metabolites.

There was a tendency for SFO fed rats to metabolize n-[1-^{14}C] octadecane faster than CO fed rats. CPFA may induce ω-hydroxylation reactions; however, the evidence was not conclusive.

Rats are able to metabolize sterculic acid satisfactorily, but to do so must contend with cyclopropene's physiological effects. It is these physiological effects that are a possible health hazard to man and animal. It is hoped these results will make possible a more accurate evaluation of cyclopropene fatty acids as a world health hazard.

AREAS OF FUTURE WORK

A detailed look into the metabolism of other cyclopropene fatty acids, in particular malvalic acid, is needed. One would expect basically the same type of metabolites since malvalic differs from sterculic only in chain length. Lee et al. (36) reported that the co-carcinogenic response of malvalic with aflatoxin B_1 was negative while sterculic acid produced more and larger tumors. Therefore, it would be interesting to determine the reason for this difference.

Carcinogenicity of aflatoxin and other aromatic hydrocarbons has been reported to require microsomal activation (23). Schoenhard (78) reported that trout liver microsomes activated aflatoxin B_1 to products lethal to Bacillus subtiles GSY 1057, and Garner et al. (18) noticed that isolated microsomes of several animal species also converted aflatoxin B_1 to metabolites toxic for Salmonella typhimurium TA 1530. Thus, the same microsomal system that ω-hydroxylated sterculic acid and n-octadecane may also metabolize aflatoxin B_1 to carcinogenic metabolites. There was weak evidence shown that CPFA induced this system.

To further test for this induced hydroxylation effect by CPFA, an ω-labelled [^{14}C] fatty acid, such as [11-^{14}C]-3, 3-dimethyl undecanoic acid (91), which cannot be β-oxidized from the carboxy end (90) should be synthesized. The mammalian body does not store

short chain fatty acids to any extent, and would metabolize the compound readily. The labelled carbon dioxide which would be proportional to the amount of hydroxylation, could be collected and used as an index of ω-oxidation. Animals fed CPFA could then be tested for induced hydroxylation with a compound similar to cyclopropene rather than a straight chain hydrocarbon such as n-octadecane used in this study.

Yoss (97) found different GLC patterns of the urinary metabolites in CPFA fed rainbow trout than in rats. These metabolites should be identified, thus helping characterize the carcinogenic effect of CPFA in trout.

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APPENDICES

APPENDIX I

Rat Diet Composition

Modified Krishnarao and Draper Diet (33)

Premix	9280	92.8%
Vitamin Mix[a/]	220	2.2%
Corn Oil	500	5.0%
	10000	

Premix	% of Diet	g/30 kg
Cornstarch	32.17	9650
Cerelose	34.60	10380
Promine D[b/]	20.00	6000
Cellulose	2.50	750
Methionine	0.05	15
$CaCO_3$	0.75	225
$Ca(H_2PO_4)_2 \cdot H_2O$	2.01	600
Mineral Mix	0.73	220
	92.80	27840

[a/] U. S. Biochemicals vitamin fortification mixtures.

[b/] Isolated soy protein, Central Soya Co.

Mineral Mix	g/kg Mix
NaCl	105.52
Na_2CO_3	220.38
K_2CO_3	484.94
$MgCO_3$	142.98
$ZnCO_3$	5.27
$FeSO_4 \cdot 7H_2O$	17.02
$CuSO_4 \cdot 5H_2O$	2.69
$MnSO_4 \cdot H_2O$	21.13
KI	0.05

The diet is made into a solid form with a 3.0% aqueous agar solution. The premix, vitamin mix and corn oil are thoroughly mixed to form the complete dry diet. The dry diet and hot agar (80°C) solution are mixed 50/50 (g/ml) and allowed to cool at 2-3°C.

APPENDIX II

Saponification of [9, 10-methylene-^{14}C]methyl sterculate

Approximately 8 μCi (110 μCi/mmole) of pure methyl sterculate was added to 100 ml of 0.5N 95% ethanolic KOH. The solution was stirred at 45-50°C for 1.5 hours and then cooled in an ice bath. 300 mls refrigerated distilled water was added and the pH was adjusted to 1.0 with conc. HCl. The saponified fatty acid was extracted three times with 20 mls of ethyl ether and combined in a pear shaped flask. The ether phase was removed in a rotary vacuum evaporator, and the fatty acid stored at -30°C in ethyl ether until used.

APPENDIX III

Halphen Test

This procedure is essentially that of Hammonds et al. (22). 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 a 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 percent 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 IV

Preparation of Diazomethane (93) and Fatty Acid Methyl Esters

Dissolve 2.14 gm of p-tolysulphonylmethylnitrosamide in 30 mls of ethyl ether, cool in ice, and add a solution of 0.4 gm of potassium hydrocide in 10 mls of 96% ethanol. If a precipitate forms, add more ethanol until it just dissolves. After 5 minutes, distill the etheral diazomethane solution from a water bath. The etheral solution contains 0.32-0.35 gm of diazomethane.

Add dropwise to urine ethyl ether extract until bubbling stops and etherate is slightly yellow. (Indicates reaction is completed.) Evaporate off ether under nitrogen gas flow. Sample is ready for gas chromatography.

APPENDIX V

Liquid Scintillation Counting Procedures

Fluor Solutions

Toluene: 6 gm 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, IL) 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 was necessary to find the proper ration 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, urine, and liver fractions were counted directly in either Aquasol or toluene fluor solution.

Carbon dioxide trap samples were counted by mixing 1.0 ml

trapping solution with 0.5 ml water and 2.5 ml Aquasol in a disposable vial. The solution was a milky liquid at room temperature and a semi-clear gel at counting temperature, 38°C. Samples were dark-adapted overnight at this temperature before counting.

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

APPENDIX VI

Procedure for the Separation of Liver Tissue into Acid Soluble, Lipid, and Protein Fractions (81)

Liver (approximately 1.0 gm)

1. Homogenize (1/8 w/v) in 5% cold perchloric acid (PCA)
2. Centrifuge 10 min at 2000 rpm
3. Wash pellet twice with (1.8 w/v) portions of 5% PCA
4. Centrifuge 10 min at 2000 rpm

Supernatant (Acid Soluble Fraction)

Pellet

1. Suspend in 10 ml of 0.35% PCA in ethanol
2. Centrifuge 10 min at 2000 rpm
3. Resuspend in 10 ml ethanol/chloroform (3:1)
4. Centrifuge 10 min at 2000 rpm
5. Resuspend in 5 ml ethanol/ether (3:1), heat 15 min at 37°C, and add 5 ml pet ether
6. Centrifuge 10 min at 2000 rpm
7. Resuspend in 10 ml ethyl ether
8. Centrifuge 10 min at 2000 rpm

Supernatant (Lipid Fraction)

Pellet (Protein Fraction)

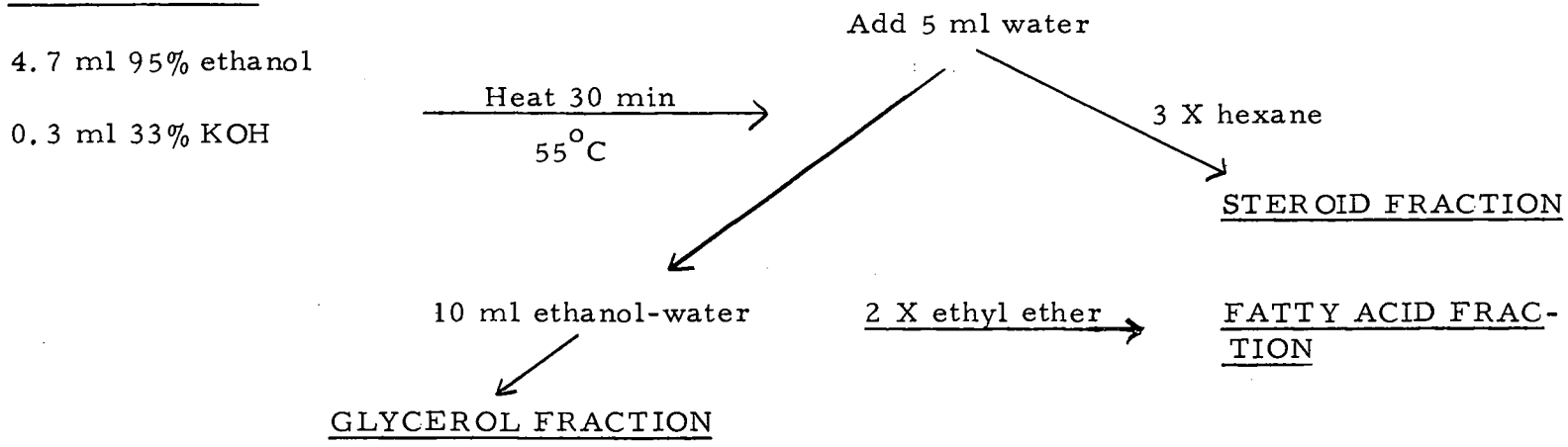
1. Suspend in 5% NaOH 2 x (1/8 w/v) mls
2. Heat at 37°C until dissolved

APPENDIX VII

Procedure for the Separation of Liver Lipids into Steroid, Fatty Acid, and Glycerol Fraction (28)

The solvent from a 10 ml aliquot of the lipid fraction was removed by rotary evaporation under vacuum. The lipid 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^{o}C water bath for 30 min and cooled. Five ml water was added and the unsaponified material extracted with three 10 ml volumes of hexane. The hexane extracts were combined and aliquots of this solution was counted in toluene fluor. The water layer was acidified with con. HCl to pH 3-4, and extracted twice with 10 ml portions of ethyl ether. Aliquots of ethyl ether were counted in toluene fluor and the water layer was counted in Aquasol. Activity was expressed as percent of recovered [^{14}C] dose given to the animal. The next page shows a skematic outline of the fractionation procedure.

Procedure for the Separation of Liver Lipids into Steroid, Fatty Acid, and Glycerol Fractions.



APPENDIX VIII

Calculated Amount of Dicarboxylic Acid Excreted in the Urine of an SFO Fed Rat

Assumptions:

Body weight of rat = 250 gm

Rat consumes 30 gm diet per day

In the SFO diet in this thesis, the wet diet contains 0.25% CPFA. If a rat consumes 30 gm diet per day, this amounts of 0.075% CPFA per day. If the rat weighs 250 gm, it is consuming 0.30 gm CPFA/kg body wt/day. Since roughly 50% of the metabolites consists of 3,4-methylene adipic acid, the rat is excreting about 0.15 gms/kg body wt/day.