AN ABSTRACT OF THE THESIS OF

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Abstract approved: Joseph E. Nixon

Cyclopropenoid fatty acids cause several physiological disorders in rainbow trout and rats. Rainbow trout appear to be more sensitive than rats to the effects of cyclopropenoid fatty acids. Carbon-14 labeled sterculic acid with label in the methylene position of the cyclopropene ring was injected into the stomach of rats and rainbow trout in order to obtain information on the metabolism of sterculic acid in both species. Data were gathered on deposition of carbon-14 label in tissue, distribution of label in liver subcellular fractions and excretion of label in urine, feces and CO_2. Metabolites in excreta were isolated by gas liquid chromatography and identified by infrared spectroscopy, mass spectroscopy and nuclear magnetic reasonance.

The liver was the organ with the highest radioactivity with peak concentrations of 11% of the administered dose in rat liver after four hours and 2.8% in rainbow trout liver after three and five days. Liver microsomal fractions contained 1.1% of the administered dose at peak liver concentrations in both species.

Rainbow trout excreted ^{14}C label at a much slower rate than the rat. Rats eliminated 48% in the urine and 11% in feces after 16 hours.

Rainbow trout eliminated 50% of the administered dose after five days. A significant amount of enterohepatic circulation was indicated in the rainbow trout, since at five days at least 25% of the carbon-14 label injected was found in blood plasma, upper intestine, pyloric ceacum and liver. A maximum of 1% of the label was found in expired CO_2 of the rat and 0.5% in the expired CO_2 of the rainbow trout, suggesting that the cyclopropene ring cannot be metabolized to CO_2.

Cis and trans-3,4-methylene adipic acid and cis-3,4-methylene suberic acid were isolated from rat urine. Sterculic acid would have to undergo beta and omega oxidation plus reduction of the cyclopropene ring to form the metabolites. Cis-3,4-methylene adipic acid was the major metabolite. Metabolites in the rainbow trout excreta could not be identified. However, gas liquid chromatography data indicated rainbow trout metabolites were not the same as those isolated from rat excreta.

Metabolism of ^{14}C-Sterculic Acid in Rats and Rainbow Trout (*Salmo gairdneri*)

by

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LIST OF ABBREVIATIONS

Name	Abbreviation
Acetic acid	HOAC
Cotton seed meal	CSM
Cotton seed oil	CSO
Cyclopropenoid fatty acids	CPFA
Diethylene glycol succinate	DEGS
Gas liquid chromatography	GLC
Gas Chromatography	GC
Gastrointestinal	GI
Infrared	IR
Intragastric	IG
Intraperitoneal	IP
Malvalic acid	MA
Methyl sterculate	MS
Nuclear magnetic reasonance	NMR
Specific activity	SP
Sterculia foetida oil	SFO
Sterculic acid	SA
Thin layer chromatography	TLC
Tricainmethane sulfonate	MS-222

METABOLISM OF ^{14}C-STERCULIC ACID IN RATS AND RAINBOW TROUT (*SALMO GAIRDNERI*)

INTRODUCTION

Twenty years ago cyclopropenoid fatty acids (CPFA) were found in the triglycerides of plants of the order Malvales. The commercially important cotton plant (*Gossypuim hirsutum*) contains a small amount of CPFA in the seed oil. Consumption of this oil ranks third in the U.S. for food fats. Since their discovery in foodstuffs much more emphasis has been placed on studying the biological effects of cyclopropenoid compounds.

Numerous reports have appeared showing that CPFA cause many biological disorders when fed to animals. The earliest work showed that CPFA when fed to hens caused a pink white discoloration in their eggs. Changes in lipid metabolism and reproductive processes were also reported.

Recently, the effects of CPFA were studied in other animals. An increase in the saturated to unsaturated fatty acid ratio, fatty acid infiltration of the liver and pronounced morphological changes of the liver were some of the abnormalities observed in rats, mice, rabbits, and rainbow trout. An accumulation of CPFA in tissue was also observed.

In this laboratory the effects of feeding CPFA to rainbow trout and rats were investigated. One of the more interesting findings

showed that CPFA act as potent co-carcinogens in trout when fed with the carcinogens aflatoxin B_1 or 2-acetylaminofluorene. No conclusive evidence is available showing CPFA as co-carcinogens in rats. Rainbow trout also develop abnormal physiological and histological changes at much lower levels of CPFA in the diet than the rat.

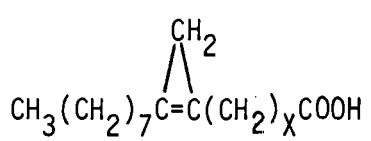
The difference in response of rats and trout to CPFA provides an opportunity to study the mechanisms of action of these compounds. Considerable information is now available on the biological effects of CPFA and their deposition in tissue. Little is known about cyclopropenoid fatty acid metabolism, distribution and excretion of metabolites. It was the purpose of this study to observe the distribution of carbon-14 label and to identify radioactive compounds in tissue and excreta of rats and rainbow trout dosed with carbon-14 labeled sterculic acid (SA).

LITERATURE REVIEW

Occurrence

Halphen (51) in 1897 introduced a color test that detected cotton seed oil (CSO) in mixtures of vegetable oils. Subsequent work indicated that plant oils other than CSO can produce the same color test initially discovered by Halphen. Phelps et al. (107) in a review listed 46 species of plants from four families of the order Malvales that gave a positive Halphen reaction.

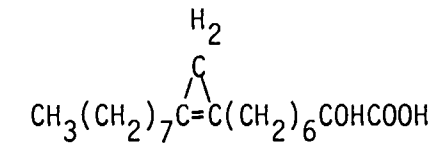
Nunn (98) in 1952 isolated the cyclopropenoid fatty acid, sterculic acid (SA). However, some doubt existed as to whether the compound isolated by Nunn was actually SA. Subsequent work by Faure and Smith (39, 40), Macfarlane et al. (88) and others (18, 19, 121) supplied conclusive support for the structure proposed by Nunn. Faure and Smith (39) in 1956 reported that SA gave a positive Halphen test and Macfarlane et al. (88) showed that malvalic acid (MA) produced a positive Halphen test. Sterculic and malvalic acid are the most common naturally occurring compounds containing the cyclopropene ring.



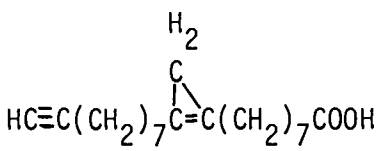
sterculic acid X=7 (SA)
malvalic acid X=6 (MA)

Recently two minor naturally occurring CPFA were found. Morris

and Hall (93) and later Recourt et al. (115) identified 2-hydroxysterculic acid and Jevan and Hopkins (62) identified sterculynic acid in several seed oils.



D-2-hydroxysterculic acid



Sterculynic acid

Some of the seed oils which contain CPFA are important sources of food. Cotton seed oil is used extensively in the United States in salad oils, cooking oils, vegetable shortenings and margarines. Kapok oil (from Eriodendron enfractuosum seeds) is used as the major food oil in Japan (66).

Cotton seed oil contains from 1 to 2% CPFA with MA ranging from 0.7 to 1.5% and SA from 0.3 to 0.5% (139). Cotton seed meal (CSM), an important component in the diet of many farm animals, also contains small amounts of CPFA. By far the richest source of CPFA is the seed oil of the tropical tree Sterculia foetida which is 50 to 70% CPFA. The ratio of SA to MA can be as high as 10 to 1. This is one of the few oils in which SA predominates. In most other cyclopropenoid fatty acid containing oils MA is about 80% of the total CPFA (138).

Cyclopropenoid fatty acids are found principally in seed lipids

though they can exist in other plant tissue (157). They are found mainly in neutral lipid fractions as triglycerides. Some CPFA are also found in diglycerides and phospholipids. Christie (24) investigated the glyceride structure of a seed oil containing CPFA and found the acids in the two position of the triglycerides.

The reviews by Phelps et al. (107) Carter and Frampton (23) and Christie (24) give the best available information on plants that contain CPFA. Analysis, reactions and biological effects of CPFA are also discussed.

Analysis of Cyclopropenoid Fatty Acids

Halphen Test

The Halphen test can quantitatively measure cyclopropenoid compounds by measuring the color developed at 495 nm when an alcohol, carbon disulfide and sulfur are mixed with samples containing cyclopropenoid compounds (Appendix II). Color development depends on the parameters, temperature, concentration of reactants, time and light. Bailey et al. (10) in 1965 heated cyclopropenoid fatty acid containing oils with mixtures of butanol, carbon disulfide and sulfur and observed the changes in absorbance at 495 nm caused by changes in reaction conditions. Coleman (25) in 1970 evaluated five methods of analyzing CPFA. He concluded that the Halphen test as modified by Bailey was the best method for determining low levels of CPFA in fats and oils.

Hammonds et al. (52) in 1971 modified Bailey's procedure by making methyl esters of CPFA and by using sealed reaction tubes during the test. The modifications by Hammonds reduced the variation in response

of different oils to the conditions employed for the Halphen test and increased sensitivity of the test. Coleman and Firestone (28) found the lower limit of sensitivity of the Halphen test to be 18 μg/g of oil. Coleman (27) in 1973 checked the reliability of the Halphen test by having 12 laboratories analyze nine samples of oil. Results from all 12 laboratories were in good agreement. The Halphen test is presently the preferred method of routine analysis of small quantities of CPFA in fats and oils.

HBr-HCl Titration

Smith et al. (143) observed that HBr reacts with CPFA fast enough to interfere with the titration of epoxides in the Durbetaki titration. Smith et al. (14) and Wilson et al. (153) determined cyclopropenoid fatty acid content by titrating with a standard solution of HBr in glacial acetic acid (HOAC) (Durbetaki reagent) after destroying the epoxy acids with lithium aluminum hydride or cold acetolysis.

Harris et al. (53) reported that epoxides could be titrated with Durbetaki reagent at 3°C without interference from CPFA. Cyclopropenoid fatty acids were then titrated rapidly at 55°C. Magne et al. (89) titrated CPFA with HCl instead of HBr. The titration was time consuming because of the slower reaction rate of HCl with the cyclopropene ring. Brown (20) and Feuge et al. (42) observed side reactions in the HBr-titration procedure to the extent of about 15%. Feuge et al, (42) observed that as the concentration of HOAC decreased the side reactions decreased and the concentration of CPFA in the samples increased.

Coleman (26) evaluated several titration procedures and concluded that a HBr-benzene method (125) was the best method for determining CPFA

by titration. HBr-titration of CPFA was not as sensitive as the Halphen test and was time consuming. Coleman recommended that titration methods be used mainly to calibrate standards for the halphen test.

Gas Liquid Chromatography

Wolff and Miwa (154) reported that gas liquid chromatography (GLC) of methyl esters of CPFA depends on the amount and type of immobile phase, column temperature and sample size. Recourt et al. (115) studied the variables involved and found decreased decomposition of CPFA during GLC by injecting directly onto the column and by using lower temperatures and nonpolar immobile phases. Quantitative analysis of CPFA by GLC has not been adequately demonstrated.

Cyclopropenoid fatty acids react with low molecular weight sulfhydryls and the derivatives have been subjected to GLC (110), however, some workers have had difficulty in repeating this work (26). Shenstone et al. (137) demonstrated that hydrogenated CPFA do not decompose on GLC columns and hydrogenation can be used as a step in the analysis of CPFA. Cyclopropenoid fatty acids react with silver nitrate-methanol solutions forming derivatives that can be isolated and quantitated by GLC (135). The silver nitrate-methanol method has been used to calibrate standard solutions of CPFA for the Halphen test.

Mass Spectroscopy

Mass spectroscopy of CPFA and derivatives of CPFA has been used to identify CPFA. Hooper and Law (56) studied the mass spectra of diketo and thiol derivatives of SA and MA. Eisele et al. (35) obtained the mass spectra of CPFA and their silver nitrate derivatives and Pawlowski et al. (104) obtained mass spectra of 1,2-dialkylcyclopropenes.

Nuclear Magnetic Resonance

Pawlowski et al. (102) recently developed a promising nuclear magnetic resonance (NMR) method for quantitative analysis of CPFA. The method measures the cyclopropene ring directly. Percent cyclopropene in a sample was determined by the ratio of terminal methyl protons to methylene protons of the cyclopropene ring. A sample with a ratio of 3:2 would have 100% CPFA. The NMR method has good accuracy for samples with CPFA concentrations of 1 to 100%.

Infrared Spectroscopy

An infrared (IR) method based on the characteristic absorptivity of cyclopropenoids at 9.9 microns was originally investigated with limited success by Varma et al. (148) in 1956. Later Bailey et al. (11) analyzed CPFA by IR. The sensitivity of the assay is low and therefore the assay is used only to a limited extent.

Reactions of Cyclopropenoid Fatty Acids

Cyclopropenoids undergo the same chemical reactions as most olefins but often react more rapidly due to a high energy ground state caused by the unsaturated three membered ring. Carter and Frampton (23) and Christie (24) have reviewed the chemistry of the cyclopropene ring. This section will deal with the reactions of CPFA which relate to their analysis and biological effects. Figure 1 shows the reactions that are discussed below.

Hydrogenation

Nunn (98) hydrogenated SA using a platinum oxide catalyst. For every mole of SA two moles of hydrogen were absorbed yielding n-nonadecanoic acid and two methyl substituted octadecanoic acids.

Malvalic acid undergoes analogous reactions. Cyclopropenoid fatty acids were successfully hydrogenated to the corresponding cyclopropanes in ethanol in the presence of palladized calcium carbonate (98, 138) or palladized carbon (74). Hydrogenation has been used in the analysis of CPFA (138, 153) and for destruction of the cyclopropene moiety for commercial purposes (59, 150, 158).

Oxidation

Products from the oxidation of CPFA were used as proof of structure of SA and MA (98). Pelargonic and azelic acids are the main oxidation products of SA when reacted in acetone with potassium permanganate. Ozonolysis of SA followed by reduction of the ozonide yields a diketone. Further oxidation produces pelargonic and azelic acids again. Other cyclopropenes yield similar oxidation products.

Halogenation

Bailey et al. (12) investigated the reaction of hydrogen halides with the cyclopropene ring and concluded that four monounsaturated monohalo compounds were formed.

Polymerization

In 1952 Nunn (98) theorized that polymerization could occur by addition of a carboxylate group to the cyclopropene ring. Since that time several investigators have observed polymerization of CPFA (70, 71, 120). Kircher (70) studied the reaction of sterculene with HOAC and concluded that two mechanisms were in operation. In the first reaction the olefinic carbon atoms were protonated, and in the second reaction the methylene carbon of the ring was protonated followed by ring opening and addition of HOAC. Polymerization of CPFA could occur by the same

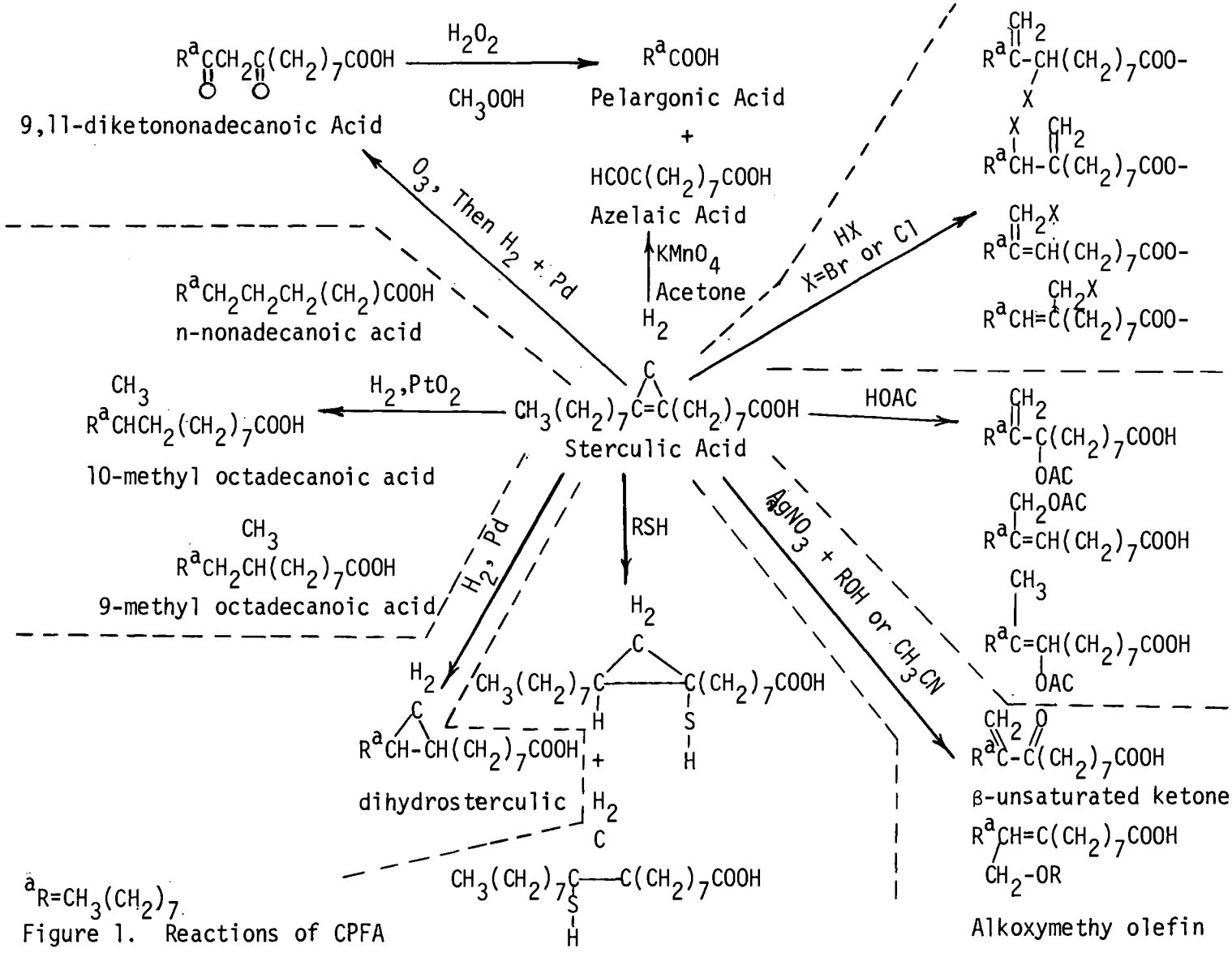
type of reactions with the carboxylate groups of fatty acids adding to the rings of CPFA. The reaction of carboxylic acids with the cyclopropene ring have been used to destroy the ring in commercial processing of cotton seed oil and meal (34, 57, 114, 117).

Reactions with Silver Nitrate

Cyclopropenoid compounds react with silver nitrate solutions (73, 135). The reaction is rapid in alcohols forming alkoxymethyl olefins plus small amounts of α,β-unsaturated ketones. In nonhydrolytic solvents the reaction is slower and α,β-unsaturated ketones are the only products formed.

Reactions with Mercaptans

Mercaptans add readily across the double bond of the cyclopropene ring giving two isomeric thiol ethers (72). Such derivatives are thermally stable and have been used in analysis of CPFA. The reactivity of the cyclopropene ring toward mercaptans may have some biological significance and is discussed later.



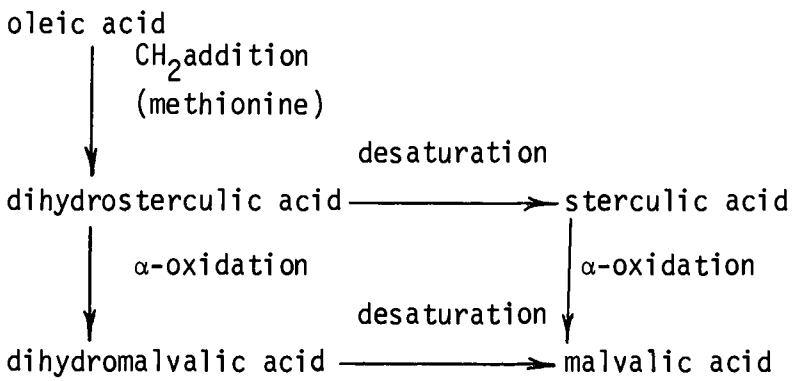
[a] $R=CH_3(CH_2)_7$

Figure 1. Reactions of CPFA

Synthesis of Cyclopropenoid Fatty Acids

Biosynthesis

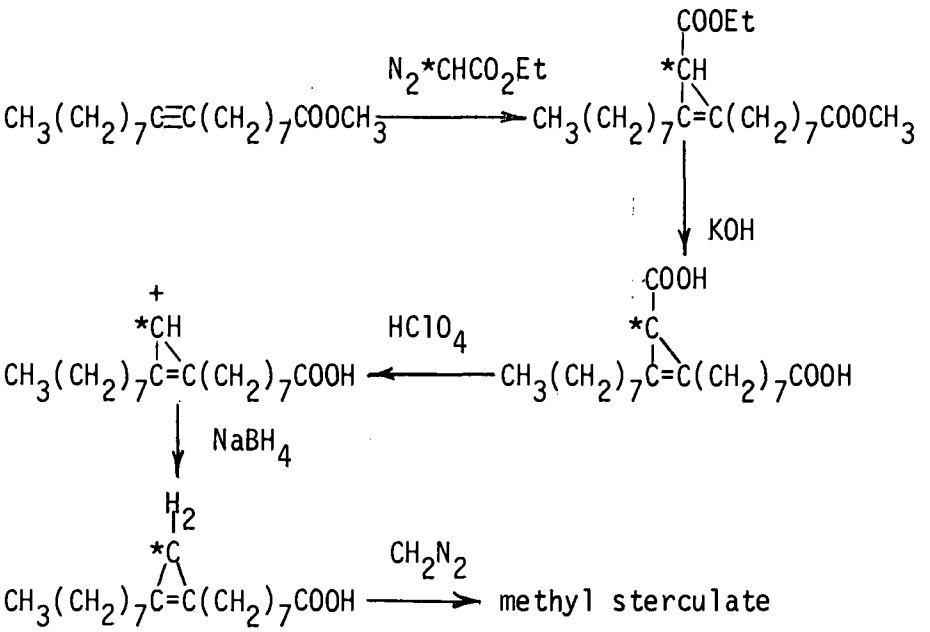
Although CPFA are not found in bacteria the study of the synthesis of cyclopropane fatty acids in bacteria initially lent some insight into the synthesis of cyclopropane and cyclopropene fatty acids in plants (156). In plants the methyl group of S-adenosylmethionine adds to oleic acid forming dihydrosterculic acid (55, 65). The methylene carbon of the ring did not come from acetate (144). In a recent study Yano et al. (156) proposed the scheme of reactions below for the synthesis of CPFA in plants:



Laboratory Synthesis

Several methods have been devised to synthesize CPFA. Gensler et al. (48, 49) synthesized CPFA with an overall yield of 30%. Pawlowski et al. (103) synthesized cyclopropenes in a manner similar to that of Gensler et al. (49). Williams and Spoutas (152) found a three step synthesis of methyl sterculate. Recently the synthesis of 1,2-dialkylcyclopropenes (101), and homologs of CPFA (151) were reported.

Gensler et al. (47) has synthesized methyl malvalate with carbon-14 in the 1 position, the methylene carbon of the cyclopropene ring, and the 10 position. The reaction scheme below is the synthetic route Pawlowski et al. (103) used to synthesize methylsterculate with the methylene carbon of the ring labeled with carbon-14:



Biological Effects of Cyclopropenoid Fatty Acids

Pink White Discoloration

Laying hens fed CSM lay eggs that contain pink whites (127). This pink white discoloration has been found to be caused by Halphen-positive materials in CSM (83, 84, 85). Masson et al. (91) and Shenstone and Vickery (140) have shown that the Halphen-positive materials causing the pink white discoloration are CPFA.

Schaible and Bandemer (129) concluded that iron diffuses from the yolk and chelates with conalbumin in the white forming a pink iron

conalbumin complex in eggs from hens fed CSM. Abou-Ashour and Edward (1) confirmed this conclusion and proposed that the changes in the migration of iron was due to changes in permeability in the vitelline membrane. Increased nonprotein nitrogen in the whites (140), migration of some proteins from white to yolk (38), a build up of water in the yolk (140) and a convergence of pH of the yolk and white on storage were also attributed to changes in the vitelline membrane.

Reproduction, Maturation and Mortality

Cyclopropenoid fatty acids fed to chickens cause changes in their reproductive processes. Egg hatchability has been reduced when relatively high levels of SFO and CSO were fed to laying hens (134, 69). Feeding purified SA caused similar reductions in hatchability (107). Egg production was stopped when 250 mg of SA per day was fed to laying hens (140). Delayed ovary and oviduct development and sexual maturity have also been reported (137).

Austic et al. (9) found that SFO was not toxic when administered directly to chicken embryo via the yolk, but the yolk from eggs from hens fed SFO was highly toxic to young chick embryos. He concluded that the yolk may be the primary egg constituent involved in SFO induced embryo mortality. Schneider (131) reported that feeding oleic acid partially restored egg production in pullets fed SFO. Donaldson and Fites (33) found high levels of embryo mortality in qual fed CPFA. They suggested that embryo mortality associated with ingestion of CPFA was due to the increased ratio of saturated to unsaturated fatty acids in egg yolk and not a direct cycloprdpenoid effect on the embryo.

Cyclopropenoid fatty acids also appear to affect the reproductive

processes in mammals. Sheehan and Vavich (137) fed female rats a diet containing 3% SFO (1.5% CPFA) and found significant retardation of growth, delayed sexual maturity and lengthened estrous cycles. Schneider et al. (133) observed that feeding SFO at 5% and above cause death and lower levels resulted in poor growth in weanling rats. Miller et al. (92) found that SFO fed to female rats caused a decrease in mating behavior, fertility and fetal and newborn viability. At 3% SFO in the diet, reproduction was prevented and at the 1 and 2% levels pre and postpartum death of offspring occurred. Miller suggested that altered cell membrane permeability may be responsible for the detrimental effects of CPFA on pre and postpartum young.

Alterations in Metabolism

Reports on changes in fat composition of domestic animals fed CSO or CSM started to appear in the literature in the 1930's (83, 37, 57, 128). The fat from these animals had higher melting points than animals fed diets that didn't contain CSO or CSM. Subsequent reports show that animals fed CPFA undergo the same changes in fat composition as animals fed CSO or CSM (107).

In the hen, CPFA appear to interfere with deposition of fatty acids in tissue resulting in more saturated and less monenoic fatty acids in egg yolk, liver, blood, ovary and heart (2). Rats also show similar alterations in tissue lipids of the heart, liver and adipose tissue (21, 29, 45, 95, 118). Nixon et al. (95) observed increased saturation of tissue lipids in rats fed 2% SFO (1% CPFA). An increase in ratios of 16:0/16:1, 18:0/18:1 and total saturated to unsaturated fatty acids in liver and depot fat of mice fed 1% SFO (0.5% CPFA) was

observed by Lehman (81). Ferguson (41) reported similar alterations in liver lipids and erythorocycte ghost lipid in rabbits fed 2% SFO. Lee et al. (77) reported increases in endogenous fat in liver tissue of rainbow trout fed 200 ppm CPFA. Roehm et al. (123) found significantly higher 16:0/16:1 and 18:0/18:1 ratios in the hepatic lipids of rainbow trout fed 100 ppm methyl sterculate (MS).

Reiser and Raju (119) in 1964 reported desaturation of stearic acid in rats was inhibited in vivo by feeding 25 mg/day CPFA. Several years later Johnson et al. (63) in an in vitro experiment with hen liver inhibited the fatty acid desaturase system with pure SA and MA. In 1967, Allen et al. (3) reported that SA was more effective inhibitor of the hen liver desaturase system than MA. Johnson et al. (63) and Fogerty et al. (44) demonstrated that CPFA were specific inhibitors of 9-10 desaturation and that the CoA derivatives of CPFA were irreversibly held to the active site on the 9-10 desaturase enzyme. Malvalic acid was a less effective inhibitor because it could not block the desaturation site of the enzyme as completely as SA.

James et al. (61) working with plants found no inhibition of oleic acid formation from acetate but did find inhibition of oleic acid formation from stearate in cyclopropenoid fatty acid treated samples. Some workers (32, 119) found no effect on the synthesis of oleic acid from acetate in animals fed CPFA, but reported inhibition of oleic acid synthesis from stearic acid. Two pathways for the synthesis of oleic acid were proposed one involves the desaturation of stearic acid and an alternate pathway involves de novo synthesis from acetate. Pearson et al. (106), Lehman (81), Coleman and Friedman (29) and Bickerstaff

and Johnson (17) have found no evidence for an alternate pathway for oleic acid synthesis in rats, mice, hens, and goats fed CPFA. Therefore, there is some question as to how CPFA increase the saturated to unsaturated fatty acid ratio in animals fed CPFA.

Raju and Reiser (111) fed lactating mice 2000 ppm CPFA and found the hepatic stearoyl-CoA desaturase activity of their month old pups inhibited. A month after CPFA were removed from the ration desaturase activity was not restored. The Halphen test showed an absorption maximum at 550 nm instead of 495 nm in the liver lipids of the lactating dams at the end of CPFA feeding. Raju and Reiser proposed that this was due to a metabolite of CPFA which may also inhibit stearoyl CoA-desaturase. Raju and Reiser (112) have suggested that CPFA inhibit the desaturase system of rat liver by irreversible binding of sulfhydryl groups on the enzyme to the cyclopropene ring.

By reacting sterculate with mercaptans Kircher (72) was the first to show that CPFA could react with sulfhydryl groups. He postulated that similar reactions may occur with available SH groups of physiologically active proteins. Ory and Altschul (100) lend support to this view since the inhibition of castor bean lipase by CPFA was partially countered by adding cysteine to the system. Taylor et al. (147) observed that CPFA decrease the activity of some dehydrogenases involved in carbohydrate metabolism in rainbow trout fed 100 ppm CPFA.

Goodnight and Kemmerer (50) found that CPFA alter the cholesterol metabolism of white leghorn cockerels. Plasma cholesterol, aortic atherosclerosis and liver weights were increased. Ferguson (41) also found alterations in cholesterol metabolism in rabbits with

increases in plasma and liver cholesterol and liver lipids. Aortic atherosclerosis and alterations in growth rate were also observed in rabbits fed CPFA. Ferguson concluded that CPFA may stimulate cholesterol synthesis.

Rainbow trout appear to be the most susceptible animal to the effects of CPFA. Roehm et al. (123) reported marked alterations in cellular morphology of the liver of rainbow trout fed CPFA at levels as low as 5 ppm. Histological examination of trout fed 50 ppm CPFA revealed extreme liver damage including enhanced deposition of glycogen fatty infiltration, bile duct proliferation and fiber formation in parenchymal cells (80, 123, 141, 145). Rats, however, have been maintained for three generations on diets containing 1000 ppm CPFA (0.2% SFO) without any apparent abnormal effects (94).

Enlarged firm and pale livers have been reported in trout fed CPFA (145). Malevski et al. (90) reported decreases in growth rate, protein synthesis and abnormal lipid synthesis from carbon-14 labeled acetate in livers of trout fed 0.5 mg, CPFA/kg body wt./day. Roehm et al. (124) found that CPFA accumulate with feeding time in the lipids of the muscle, depot fat and liver of cyclopropenoid fatty acid fed rainbow trout.

Struthers (145) reported reduced incorporation of phosphate and fatty acids into liver phospholipids of rainbow trout fed 200 ppm CPFA for two weeks. Major changes in the mitochondria appeared to occur; P/O ratios were significantly lowered and the ability to oxidize oleic acid to CO_2 was reduced.

Lehman (81) reported significant reductions in P/O ratios and the

ability to oxidize carbon-14 labeled palmitate in mice fed 1% SFO. Erythrocytes from the CPFA-fed mice hemolyze more slowly than erthrocytes from controls, suggesting a membrane effect. Nixon et al. (95) reported changes in erythrocyte hemolysis rates, mitochondrial swelling and microsomal codeine demethylase activity in rats suggesting a general alteration in membrane function.

Co-carcinogenesis

Sinnhuber et al. (142) and Jackson et al. (60) in 1968 reported abnormally enhanced development of hepatic carcinoma in rainbow trout fed aflatoxin-containing diets which also contained CSM. Later in the same year Sinnhuber et al. (141) fed rainbow trout 220 ppm CPFA and 4 ppb aflatoxin B_1 and observed a 90% incidence of hepatomas after six months. Trout receiving 4 ppb aflatoxin B_1 with no CPFA developed only 20% incidence after nine months. Lee et al. (79) found that only 50 ppm CPFA fed with 4 ppb aflatoxin B_1 produced 100% incidence of hepatomas in nine months compared to 50% incidence after 12 months in the controls fed 4 ppb aflatoxin. Lee et al. (77) also found that the cyclopropenoid fatty acid containing oil, Hibiscus syriacus oil, promoted hepatic carcinoma in rainbow trout fed aflatoxin B_1 or 2-acetylaminofluorene. Sterculic acid is a much more potent co-carcinogen than MA in rainbow trout (77, 79), and there is no evidence to indicate that CPFA are carcinogenic (142).

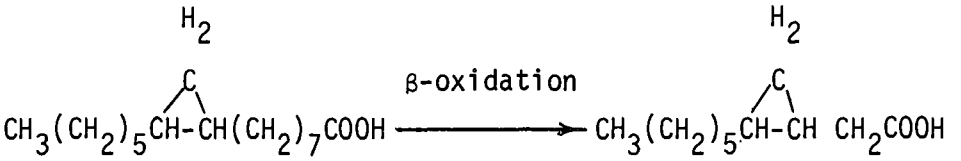
Co-carcinogenic action of CPFA in other species has not been adequately demonstrated. Friedman and Mohr (45) reported no interaction between CPFA and aflatoxin in rats. Conversely Lee et al. (78)

reported a slightly higher incidence of tumors in rats fed aflatoxin and CPFA than those fed aflatoxin alone. The higher incidence of tumors was not significant because of the small number of animals used in the experiment. Nixon et al. (96) found no significant interaction between CPFA and aflatoxin B_1 or diethylnitrosamine in rats.

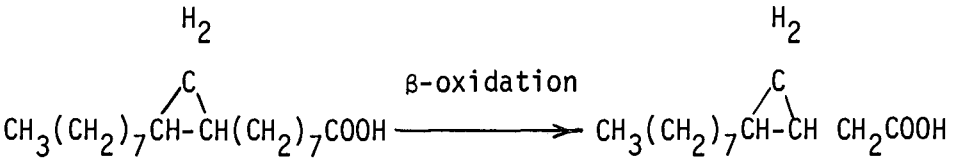
Metabolism of Cyclopropenoid Fatty Acids

Although little is known about the metabolism of CPFA, information is available concerning cyclopropane fatty acid metabolism. Since the structure of the two types of compounds are similar, knowledge of cyclopropane fatty acid metabolism can help to predict cyclopropene fatty acid metabolism. Wood and Reiser (155) fed male weanling rats fat free diets and supplemented the diets with 0.54% methyl-cis-9,10-methylene octadecanoate and methyl-trans-9,10-methylene octadecanoate. Fat metabolism was not affected, however, cis-3,4-methylene dodecanoic and trans-3,4-methylene dodecanoic acids were identified in the adipose tissue. These results tend to indicate that β-oxidation cannot proceed past the cyclopropane ring.

Chung (25) incubated rat liver mitochondria with cis-9,10-methylene hexadecanoic acid labeled with carbon-14 in the methylene position of the cyclopropane ring. Chung (25) also incubated cis-9,10-methylene octadecanoic acid with rat liver mitochondria. No carbon-14 radioactivity was found in the CO_2 and the products formed were similar to those identified by Wood and Reiser (155). The reactions involved are shown below:



cis-9,10-methylene hexadecanoic cis-3,4-methylene decanoic acid



cis-9,10-methylene octadecanoic acid cis-3,4-methylene dodecanoic acid

Coleman and Friedman (29) reported two unknown compounds in fatty acids of tissue lipids from rats fed 2% SFO. Upon GLC their retention times corresponded to C_{13}-C_{14} fatty acids. They speculated that the unknowns were CPFA degradation products comparable to the cyclopropane degradation products reported by Wood and Reiser (155) and Chung (25).

Berry et al. (16) in 1969 fed five month old white leghorn chickens for three days with ring methylene carbon-14 labeled methyl sterculate or carbon-14 labeled methyl oleate. Methyl sterculate was less readily absorbed from the gut, and expired $^{14}CO_2$ was much lower than that observed for methyl oleate. Altenburger (4) in 1971 injected three twelve-month leghorn hens with carbon-14 labeled sterculic acid. More than 50% of the recovered activity was found in fecal samples of two of the hens which were fasted for 24 hours after injection. No activity

was found in abdominal fat. The third hen was fed a normal diet for five days after injection. Only 9.2% of the recovered activity was found in fecal material, however, 68.2% was found in the skin and abdominal fat. The carbon-14 level in the feces of the fasted hens indicates excretion of SA or metabolites of SA from the bile, in the urine or through the intestinal wall. Limited amounts of activity were found in exhaled CO_2.

EXPERIMENTAL

Synthesis of Methyl Sterculate and Sterculic Acid

Carbon-14 labeled MS, 99% chemically pure, with label located in the methylene carbon of the cyclopropene ring was synthesized by Dr. N. E. Pawlowski (103). The methyl ester was saponified to the fatty acid with ethanolic KOH (Appendix I). Percent MS and SA in the synthesized material was checked by the Halphen test (Appendix II) and by NMR (102). Thin layer chromatography (TLC) (Appendix III) of labeled MS and SA was used to determine radiopurity.

Rat Metabolism Studies

Mode of Administration (Experiment I)

This experiment was designed to indicate which derivative of the carbon-14 labeled compound, MS or SA, and which mode of administration of label, intraperitoneal (IP) or intragastric (IG), were best suited for rat metabolism studies. Male 251±8g Wistar strain rats maintained on Purina rat chow were starved for 12 hours before administering IP or IG 4.17±0.45 μci (0.038±-/--4 mmoles)/kg body wt. of labeled MS, SA or uniformly labeled methyl oleate. Refer to Appendix IV for IP and IG injection procedures.

Immediately after injection, the animals were placed in metabolism chambers (Figure 2). Air was pulled through the chambers and through several 1N KOH CO_2 traps. Feces was collected on wire mesh screens and urine flowed into glass tubes packed in ice.

At 6 or 12 hours after injection the animals were decapitated

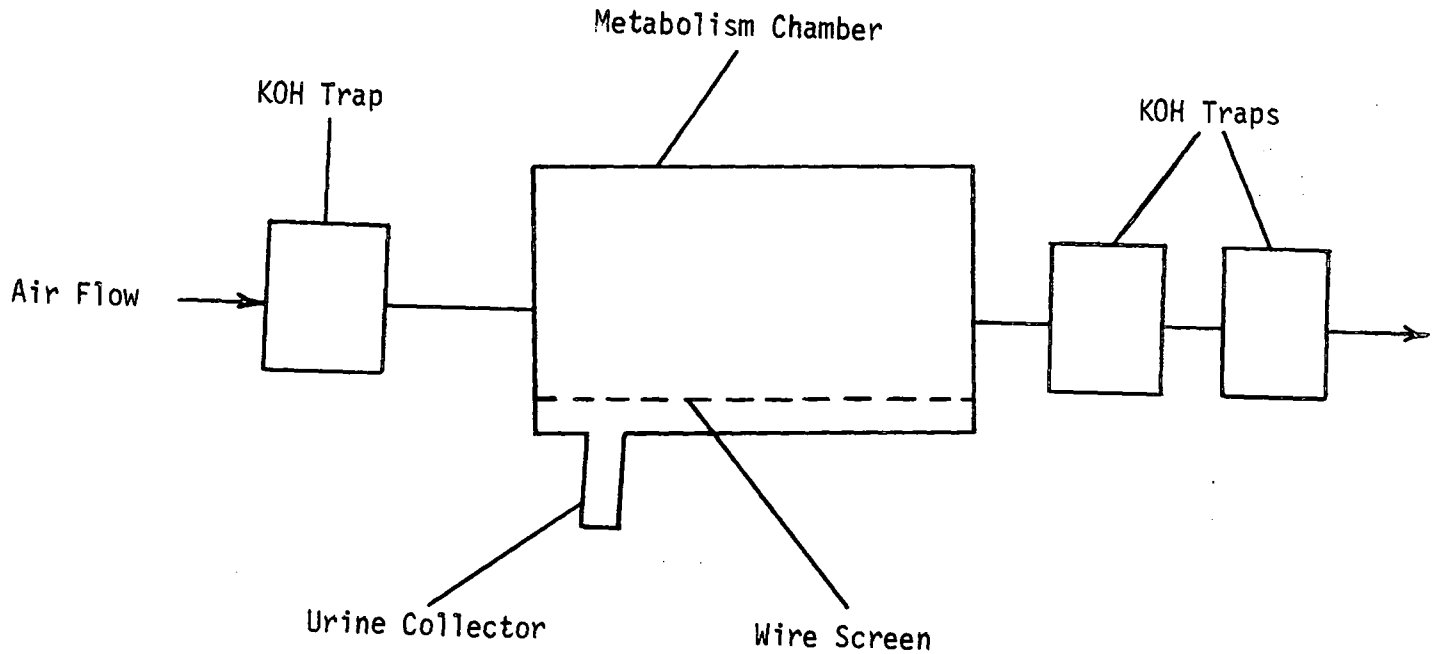


Figure 2. Rat Metabolism Chamber

and blood was collected and immediately centrifuged. Organs and tissue were removed, weighed, wrapped in aluminum foil and stored at -10°C. The feces, urine, carcass and serum were also stored at -10°C. Serum protein was determined by the Lowry method described in Appendix V.

Organs, tissue and feces were homogenized in 3 to 4 volumes (w/v) distilled water with a Tissumizer SDT 100N.[1] Aliquots of the homogenates were digested and counted in a scintillation counter.[2] Refer to Appendix VI for scintillation counting procedures.

The frozen carcass was ground in a meat grinder and digested in approximately 300 ml of 6 N KOH. Aliquots were taken for scintillation counting (Appendix VI).

Deposition and Excretion (Experiment II)

Male Wistar strain rats weighing 252±8g were injected IG (Appendix IV) with 5.66±0.78 μci (0.051±.007 mmoles)/kg body wt. of carbon-14 labeled SA and immediately placed in rat metabolism chambers. After 1, 2, 4, 8, 16 and 26 hours two animals for each time period were removed from the chambers and decapitated. Blood, all organs except the liver and excreta were analyzed as described in ExperimentI.

After decapitation livers were immediately removed, weighed and placed on ice while other organs and tissue were prepared for storage. As quickly as possible, usually one hour after decapitation, livers were minced in four volumes (w/v) of 0.25M refrigerated

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[1]Tekmar Company, Cincinnati, Ohio

[2]Nuclear-Chicago, Des Plaines, Illinois

sucrose and homogenized in a Potter-Elvehjem homogenizer[3] with 11 up and down strokes of a loosely fitting teflon pestile. A model L-2 ultracentrifuge[4] was used to prepare subcellular fractions of 10 ml aliquots of the homogenates. Figure 3 is a flow diagram of the fractionation procedure. Aliquots of the homogenate and subcellular fractions were taken for scintillation counting (Appendix VI).

Rainbow Trout Metabolism Studies

Rainbow trout (Salmo gairdneri) Mt. Shasta Strain, were kept in four foot diameter fiber glass tanks with the flow rate of water at 4 gal/min. at a temperature of 12°C. The trout were fed once a day at the same time of day for two weeks. Three days before injection of material, feeding was stopped. Refer to Appendix VII for diet information.

Intragastric Administration (Experiment III)

To determine whether carbon-14 labeled SA could be placed in gelatin capsules and injected into the stomach of rainbow trout without regurgitation occurring, number 5 gelatin capsules[5] containing Evan's Blue Dye mixed with corn oil, were placed into the stomachs of twelve 300g rainbow trout. Refer to Appendix IV for IG injection procedures for rainbow trout.

Half the trout were starved for three days and the other half were fed normally before injection of the capsules. The two groups of trout

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[3]Kontes Glass Company, Vineland, New Jersey

[4]Beckman Glass Inc., Palo Alto, California

[5]Eli Lilly and Company, Indianapolis, Indiana

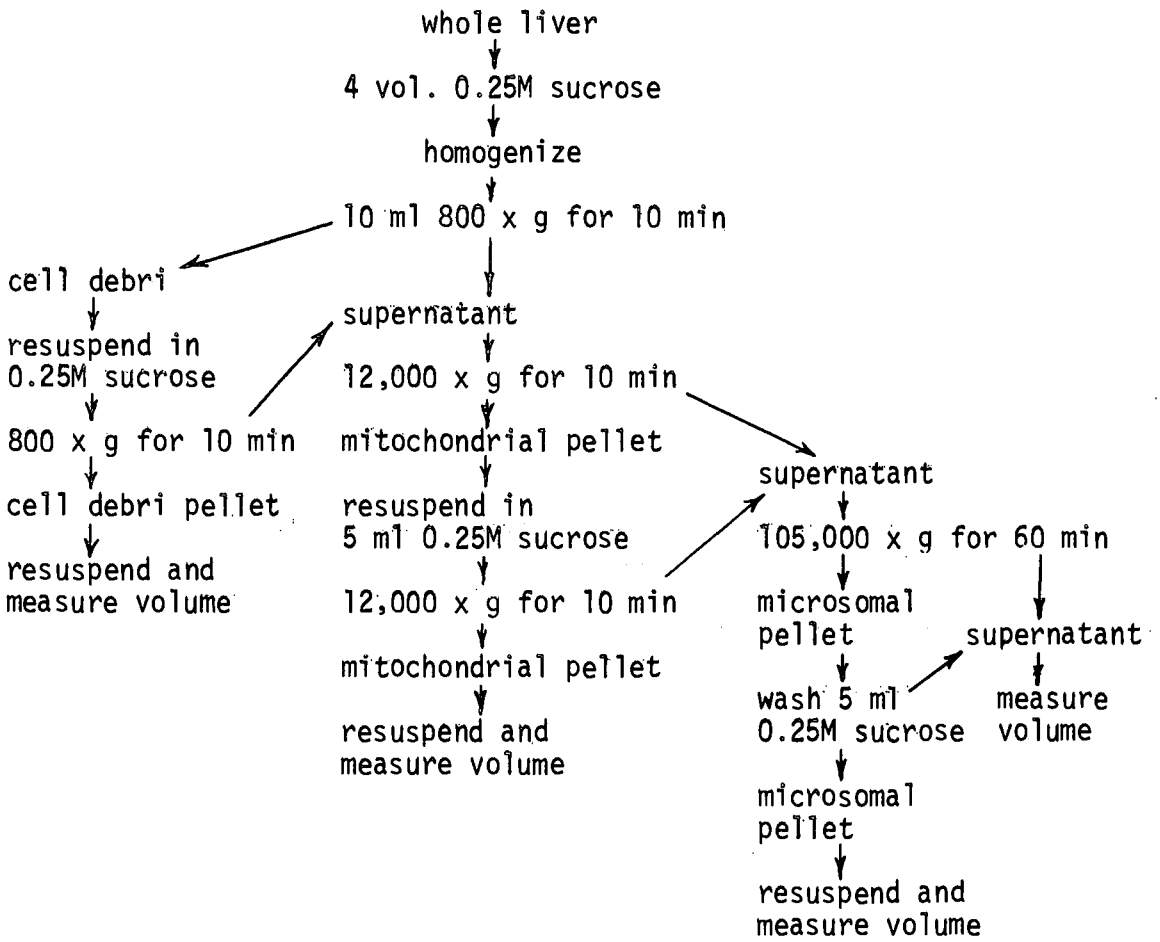

Figure 3. Flow diagram of liver subcellular fractionation procedure.

were placed in two foot diameter plastic buckets that were setting in a fiber glass fish tank. Water in the plastic buckets was aerated. Trout were sacrificed after 24 hours and the location of dye was determined.

Deposition (Experiment IV)

Rainbow trout weighing 356±96g were stomach tubed (Appendix IV) with 3.84±1.41 μci (0.035±0.013 mmoles)kg body wt. of carbon-14 labeled SA. Immediately after injection the trout were placed in 2x2x2 ft. fiber glass tanks and held there for 2, 4, 18, 74, 119 and 168 hours. Water at 12°C was circulated at 2 gal/min, to remove excreta quickly.

Each tank held three trout and at the appropriate time they were removed from the tanks and sacrificed by a blow to the head. A syringe with a 2 inch 22 gage needle was injected into the heart and blood was extracted. Blood was handled as in Experiment I.

Organs and tissue were removed, stored, homogenized, fractionated and analyzed as in experiments I and II. The content of the gall bladder was removered with a 1 ml disposable syringe attached to a 22 gage needle.

Excretion (Experiment V)

To determine the amount of label excreted by rainbow trout, 235±19g trout were fasted for three days before stomach tubing 1.67±0.19 μci (0.015±0.002 mmoles)/kg body wt. of carbon-14 labeled SA (Appendix IV). The fish were placed in metabolism chambers (Figure 4) consisting of 3.5 x 12 inch glass cyclinders partially filled with water and stopped with #14-1/2 rubber stoppers. Water was replaced

at 5 to 12 hour intervals. Air was bubbled through the chambers and then through two 100 ml 1N KOH traps which were also replaced at 5 to 12 hour intervals.

Trout remained in the chambers up to 5 days and water samples containing a significant amount of activity were stored at -10°C. Some water samples were acidified with concentrated HCl and checked for any residual carbon dioxide activity. Carcasses were digested as in Experiment I. Refer to Appendix VI for scintillation counting procedurs.

Isolation and Identification of Metabolites

Excreta was acidified to pH 1 with concentrated HCl and extracted 6 times with ethyl ether. The ether phase and aqueous phase were reduced to known volume and aliquots were taken to determine radioactivity. Some samples were treated with β-glucuronidase Type H-1[6] (Appendix VIII) or saponified in ethanolic KOH at 50°C for four hours before extraction. The ether extracts were methylated with diazomethane as described by Vogel (149). Excreta ether extracts and methylated extracts were developed in various solvent systems via TLC (Appendix III) and analyzed on a radiochromatogram scanner.[7]

Gas liquid chromatography (Appendix IV) of the methylated extracts was used to isolate metabolites. Figure 5 is a flow diagram of the GLC isolation procedure. Peaks in the chromatograms containing carbon-14 activity were found by trapping the effluent on 5% silcon-

[6]Sigma Chemical Company, St. Louis, Missouri

[7]Varian Aerograph, Walnut Creek, California

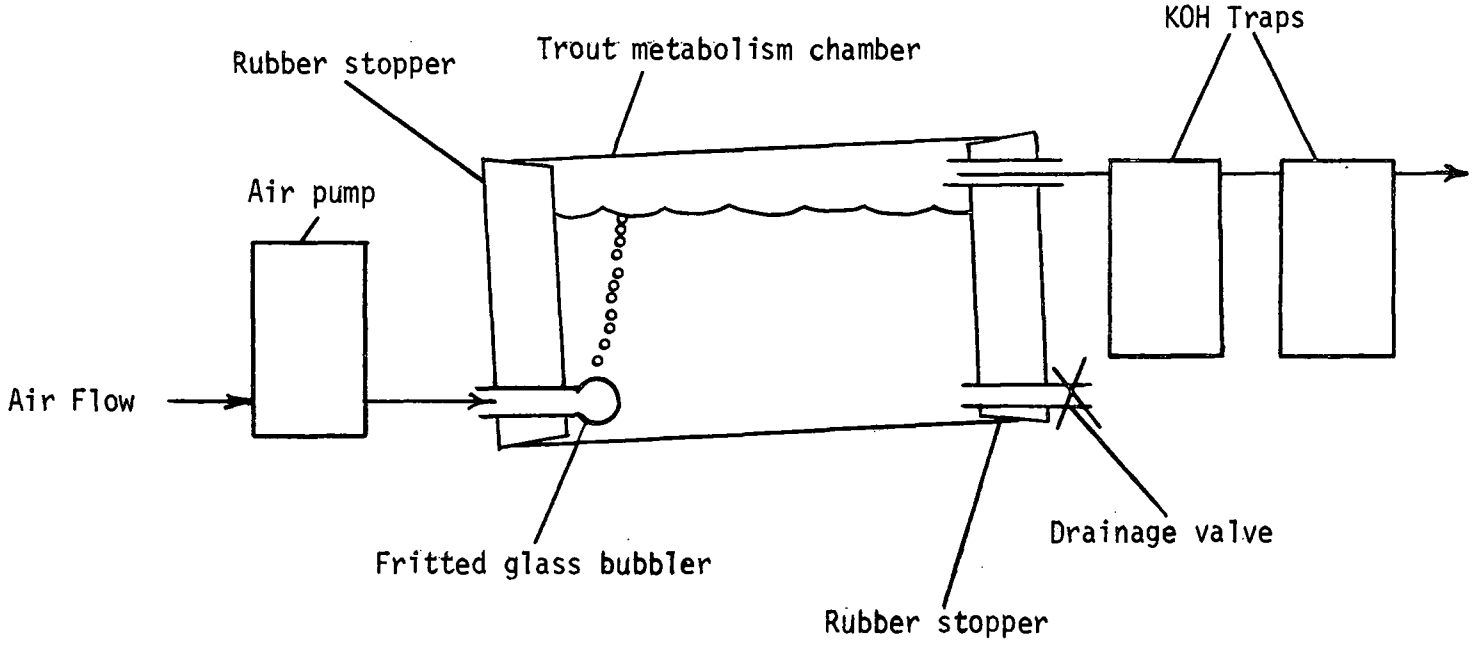

Figure 4. Rainbow trout metabolism chamber.

celite traps (Appendix X). Every two minutes a new trap was connected to the effluent port. Refer to Appendix VI for scintillation counting procedures for the 5% silicon-celite traps.

Radioactive peaks were trapped in capillary tubes (Appendix X) for IR, NMR and mass spectra analysis (Appendix XI).

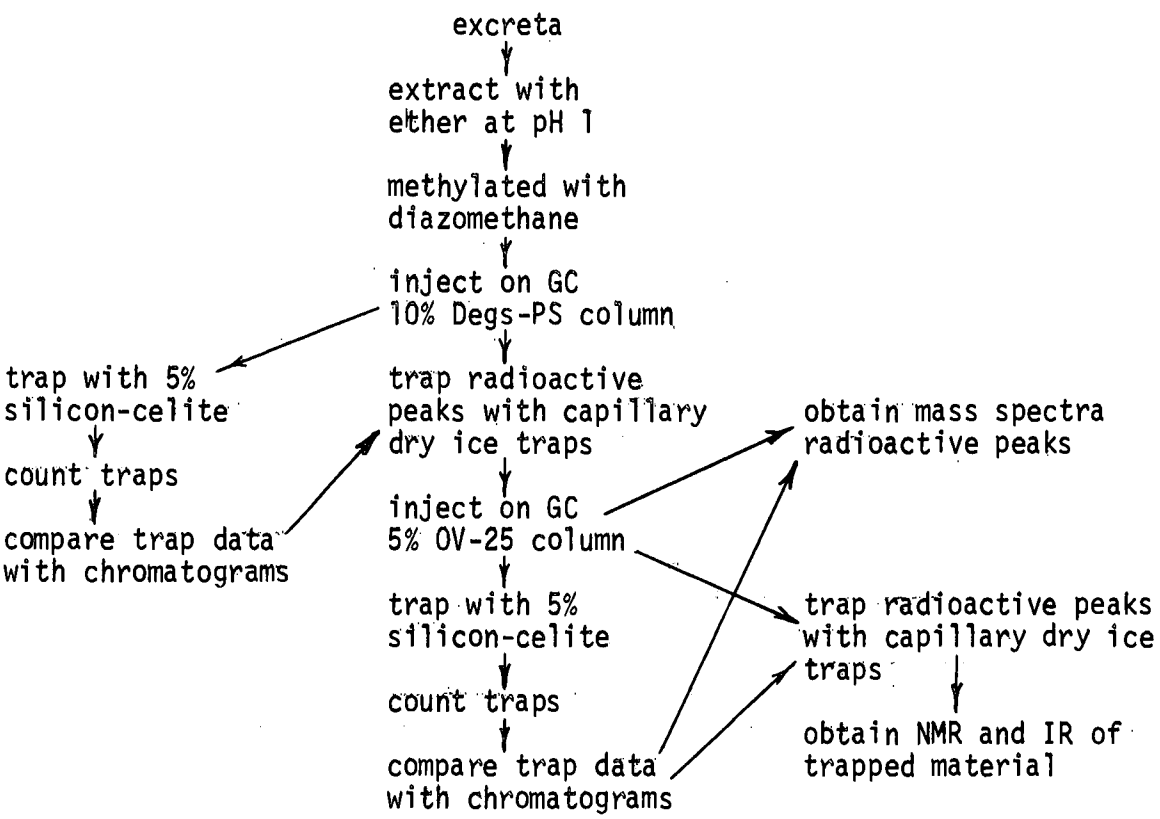

Figure 5. Flow diagram of metabolite isolation procedure.

RESULTS AND DISCUSSION

Purity of Carbon-14 Label

The cyclopropene ring because of its highly strained unsaturated structure is readily destroyed by strong acid and base (23, 24). The chemical purity of carbon-14 labeled methyl sterculate was checked before and after saponification (Appendix I). Table I shows the percent MS and SA in samples before and after saponification as determined by the Halphen test (Appendix II) and NMR (102). The Halphen test results are an accumulation of data obtained from six different saponifications run over a two year period. Saponification destroyed little SA, and methyl sterculate appeared to be stable over the two year period. Label was always injected immediately after saponification.

Table I. CHEMICAL PURITY OF CARBON-14 LABELED MA AND SA.

	percent[a]	
	methyl sterculate	sterculic acid
Halphen test	96.8 ± 2.5	93.2 ± 1.1
NMR	99.0	96.7

[a]percent ± standard deviation

The radiopurity of labeled MS and SA was checked by TLC (Appendix III) in a hexane:ether:benzene (70:30:1) solvent. All the radioactivity was near the origin in the SA sample (R_F=0.097). Radioactive MS was found in a spot further up the plate (R_F=0.605).

Rat Metabolism

Mode of Administration

Different routes of administration of a compound do not always result in the compound producing the same pharmacologic response (75). Derivatives of compounds are not always absorbed and metabolized at the same rate (105). Because of these variables an initial study was planned to determine the route of administration and form of compound best suited for the deposition and metabolite identification studies in the rat. The results of the study are shown in Table II.

Intraperitoneal injection of MS was a poor mode of administration because very low levels of carbon-14 label were incorporated into organs and excreta. Much of the label was not recovered after IP injections of the carbon-14 labeled methyl ester. The unaccounted for radioactivity was probably retained in the peritoneal cavity unabsorbed and was lost during the removal of the animals' organs. Uniformly labeled methyl oleate was also injected IP. Again carbon-14 label deposition and excretion were low. Intragastric administration of MS yielded higher incorporation of label in organs. Urine contained 4.2% of the label after 6 hours. A slow rate of absorption was indicated since 55.2% of the administered dose was still in the stomach and small intestine after 6 hours.

Carbon-14 labeled SA injected IP and IG produced higher levels of carbon-14 label in rat organs and excreta than when carbon-14 labeled MS was injected IP and IG. Higher levels of radioactivity in the 6 hour IG SA injected animal than in the 6 hour IP SA injected

animal indicates that higher rates of absorption and metabolism occur with IG injection of the fatty acid.

Deposition and Excretion

The location of radioactivity was determined at various times after rats were subjected to IG injections of carbon-14 labeled SA. The percent of administered carbon-14 label expressed as a function of time in the stomach and small intestine is shown in Figure 6. After 2 hours only 26% of the label could be found in the stomach and at 8 hours 1% was found in the stomach. Figure 6 shows that after 8 hours 94% of the label was absorbed by the small intestine or passed down the gastro intestinal (GI) tract. Only 11% of the label was in the lower intestinal tract after 8 hours (Figure 10), indicating that the majority of the radioactivity was absorbed by the small intestine. The small intestine is the organ normally involved in adsorption of fatty acids (8).

The concentration of label in blood serum expressed as a function of time is shown in Figure 7. The peak concentration of label at 2 hours and the rapid decrease at longer time periods indicates a relatively fast transfer of label from the small intestine to and from the liver with a minimal amount of enterohepatic circulation.

Figure 9 shows that carbon-14 label in the liver and kidney reached maximums of 11% and 0.8% of the administered dose respectively 4 hours after an IG injection of carbon-14 labeled SA. At this same time little activity was found in the spleen, heart, lung and testes (Figure 8).

The distribution of label in the lower GI tract and feces is

TABLE II. DISTRIBUTION OF RADIOACTIVITY IN RATS INJECTED IP AND IG WITH CARBON-14 LABELED MS AND SA.

	Percent of Administered Carbon-14 Activity							
	Methyl Oleate		Methyl Sterculate			Sterculic Acid		
	6hr,IP	12hr,IP	6hr,IP	12hr,IP	6hr,IG	6hr,IP	12hr,IP	6hr,IG
Stomach	0.2	0.2	0.1	0.2	7.7	0.5	0.8	6.8
Intestine	1.3	1.9	0.7	0.1	47.5	7.1	18.9	19.5
Liver	3.5	0.9	0.4	1.0	8.0	2.6	8.2	9.3
Spleen	0.8	1.1	0.2	0.5	0.1	0.0	0.1	0.1
Heart	0.4	1.0	0.1	0.0	0.3	0.0	0.1	0.2
Lung	0.8	0.7	0.5	0.0	0.1	0.1	0.1	0.1
Testes	0.2	0.4	0.1	0.3	0.0	0.1	0.6	0.1
Kidney	0.3	0.1	0.2	0.0	0.8	0.3	0.6	0.6
Urine	0.2	0.4	0.2	0.4	4.2	6.7	43.0	29.3
Feces	0.3	0.3	0.1	2.9	0.4	0.0	4.9	0.0
CO_2	1.4	4.4	0.1	0.0	0.1	0.3	0.6	0.4
[a]Total Recovered	---	64.5	---	40.6	82.2	26.0	91.4	83.3

[a]Activity recovered in organs listed in table plus carcass.

shown in Figure 10. At 16 hours, 11% of the label was found in the feces and 6% was found in the lower GI tract. After 26 hours, 15% was found in the lower GI tract and feces. Figure 10 also shows excretion of label in urine and respired air. The rat apparently cannot metabolize the cyclopropene ring to CO_2, since only 1% of the administered carbon-14 label which was present in the methylene carbon of the cyclopropene ring was trapped as CO_2 in respired air.

Excretion of label in the urine leveled off after 16 hours after almost 48% of the label had been excreted. Figure 10 indicates that 59% of the administered dose was excreted in the urine and feces after 16 hours. The high concentration of label in liver, kidney, urine and feces and low concentration of label in expired CO_2 indicates that SA was metabolized in the liver to a metabolite or metabolites that were excreted in the urine and feces retaining the methylene carbon of the cyclopropene ring.

Label gradually increased in the fatty tissue of the rats with time (Figure 9). After 26 hours roughly 8% of the label accumulated in fatty tissue.

Table III is a summary of the data obtained in the rat deposition and excretion studies.

Liver Subcellular Distribution

Distribution of radioactivity in subcellular fractions of the liver is shown in Figure 11 and Table IV. High activity in the supernatant was probably due to the movement of SA or metabolites of SA in the cytosol. The microsomal fractions contained a significant amount of label, 1.1%, after 4 hours, and the time of peak concentration

of label in the microsomal fractions came before the time of peak concentration of label in the mitochondrial fractions.

Metabolite Isolation and Identification

Extraction

The carbon-14 label in rat urine, injected IG as carbon-14 labeled SA, was not very extractable in ether under alkaline conditions. At pH 7 only 10% was extracted from rat urine, but as pH 1, 70% of the label was found in the ether extract. Rat feces had similar extraction characteristics. Approximately 70% of the carbon-14 label was found in the ether extract when rat feces was extracted at pH 1. Halphen tests (Appendix II) of the ether extracts were negative.

Thin layer chromatography (Appendix III) of urine ether extracts developed in hexane:ether:HOAC (80:20:1) had one radioactive spot near the origin (R_F=0.09). A SA standard migrated further up the TLC plate (R_F=0.42).

Ether extracts and methylated portions of the extracts were spotted on TLC plates and developed in methanol:chloroform:benzene: HOAC (40:60:1:1). Radioactive spots of the nonmethylated samples (R_F=0.66 and 0.71) did not migrate as far as the spots of the methylated samples (R_F=0.85 and 0.88).

To determine whether the metabolites in the urine and feces were conjugated, rat urine and feces were treated in 1N KOH or β-glucuronidase (Appendix VIII). The extraction characteristics of the carbon-14 labeled metabolites and migration patterns on TLC plates

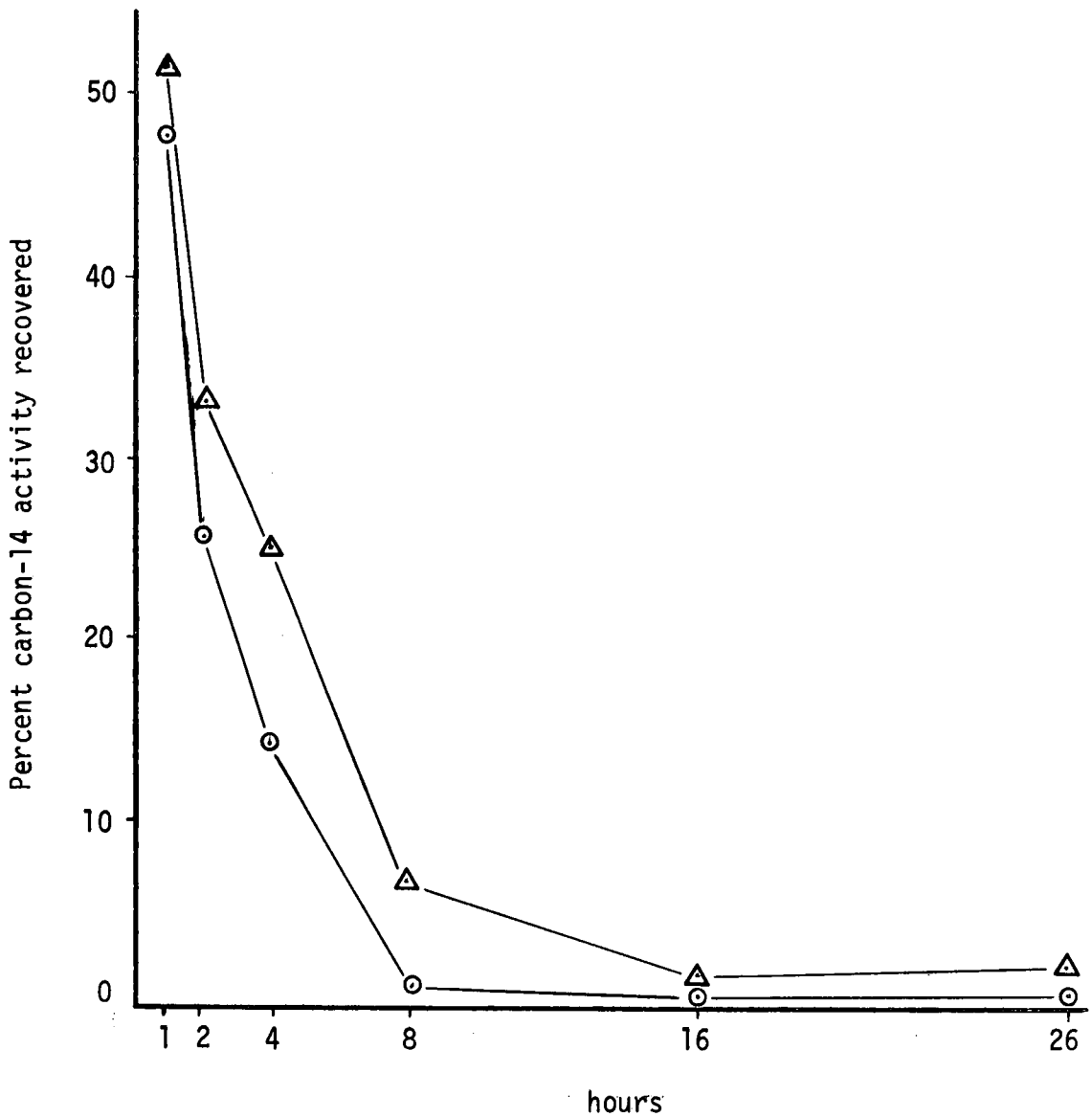

Figure 6. Percent of administered radioactivity recovered in the stomach (- ⊙ -) and small intestine (- △ -) of rats expressed as a function of time after an IG injection of carbon-14 labeled SA.

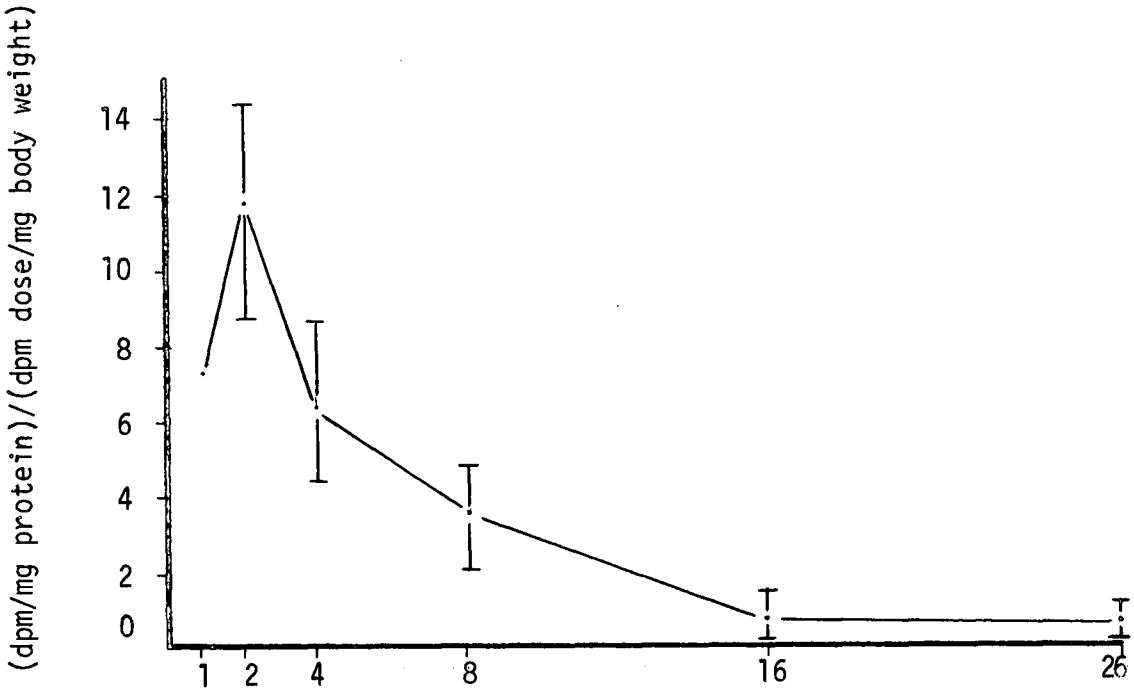

Figure 7. Concentration of radioactivity in blood plasma of rats expressed as a function of time after an IG injection of carbon-14 labeled SA.

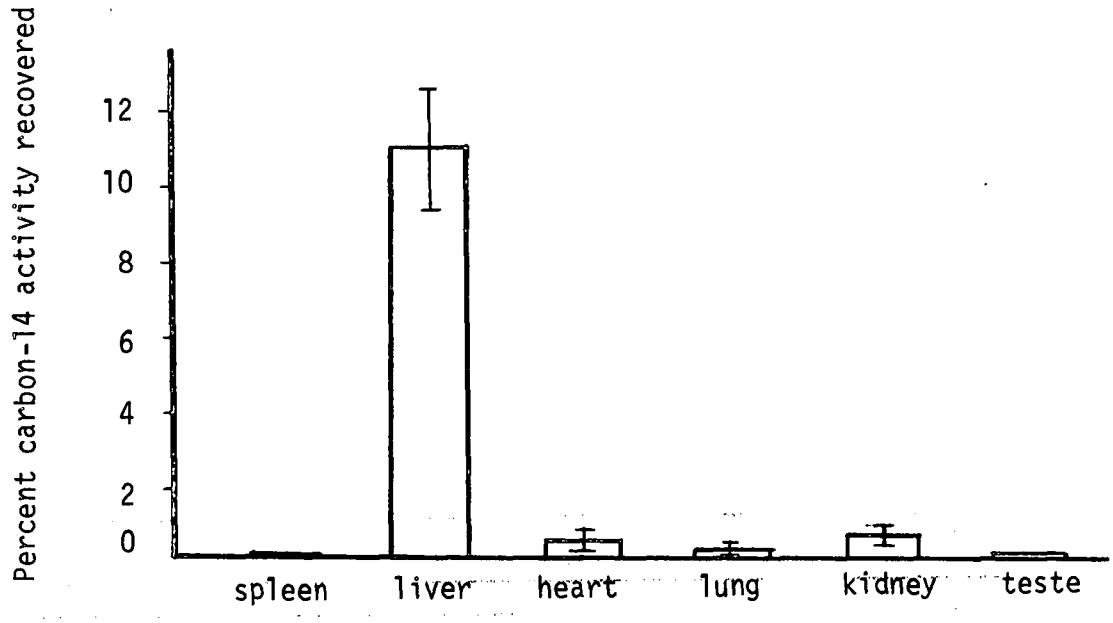

Figure 8. Percent of administered radioactivity recovered in organs of rats four hours after IG injections of carbon-14 labeled SA.

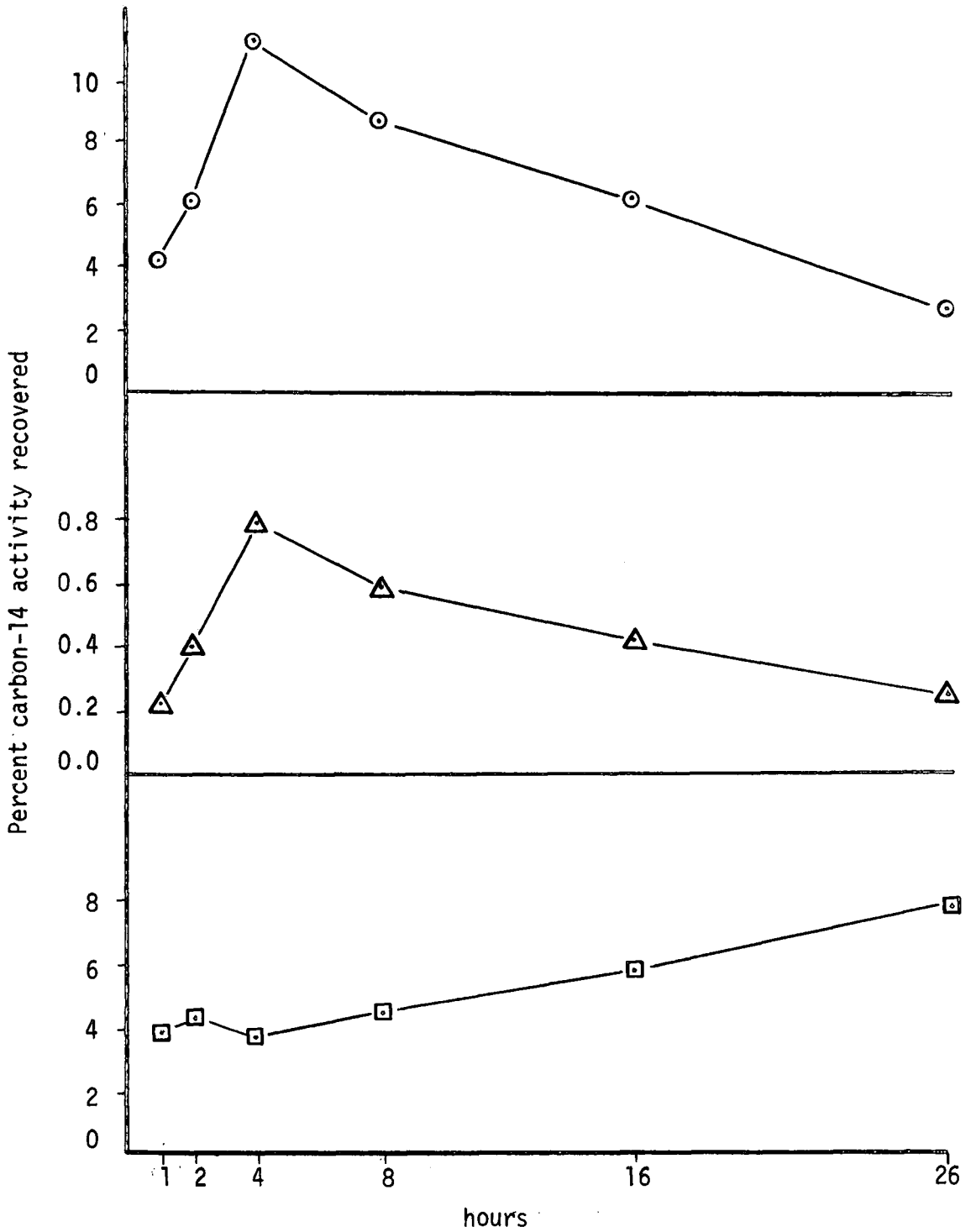

Figure 9. Percent of administered radioactivity recovered in the liver (- ⊙ -), kidney (- △ -), and fatty tissue (- ⊡ -) of rats expressed as a function of time after an IG injection of carbon-14 labeled SA.

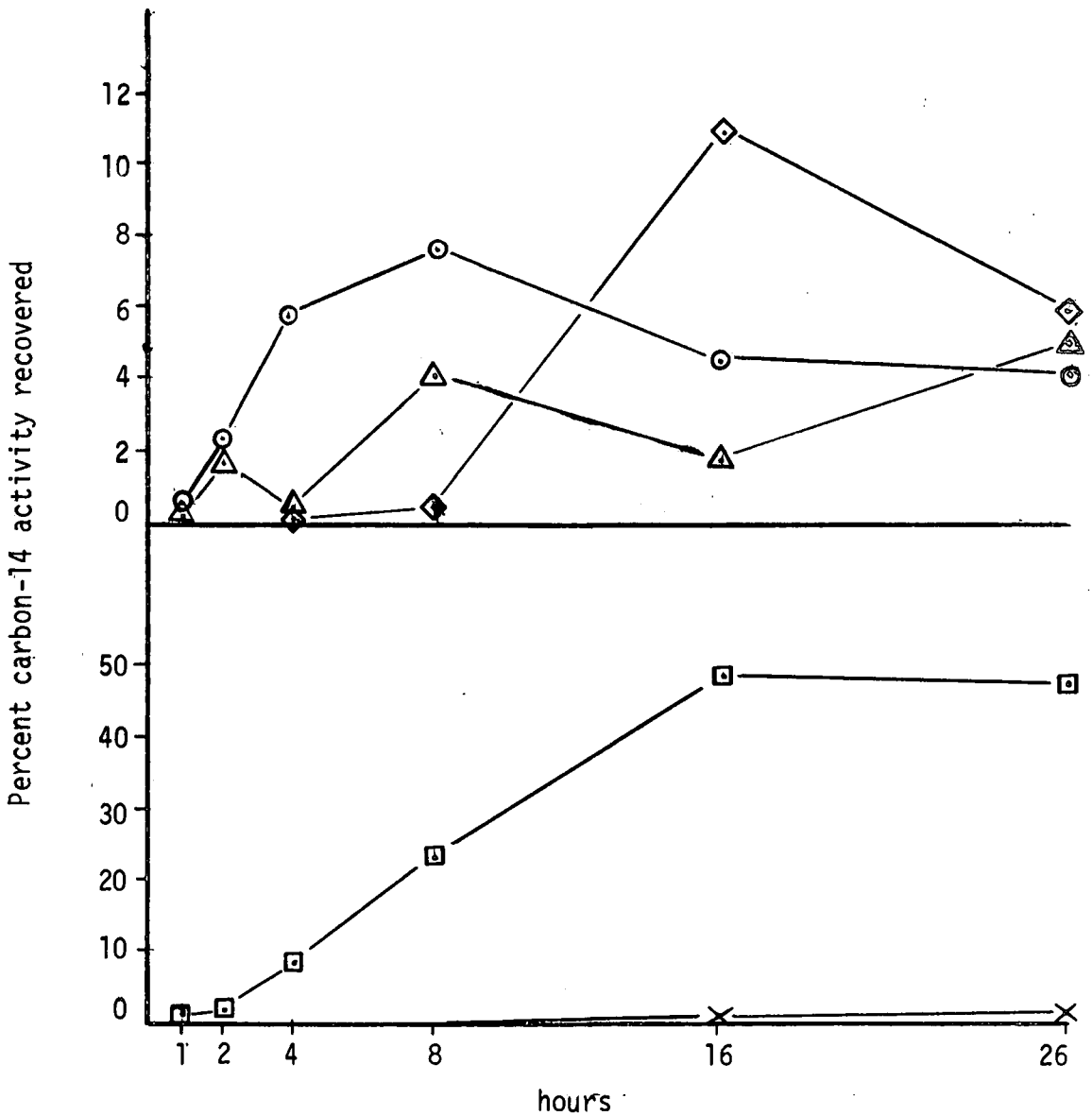

Figure 10. Percent of administered radioactivity recovered in the ceacum (- ⊙ -), colon (- △ -), feces (- ◇ -), urine (- ⊡ -) and expired CO_2 (- X -) of rats expressed as a function of time after an IG injection of carbon-14 labeled SA.

TABLE III. PERCENT OF ADMINISTERED RADIOACTIVITY RECOVERED IN RAT TISSUE AND EXCRETA.

	hours[a]					
	1[b]	2	4	8	16	26
stomach	47.4	25.9 ±5.2	14.2±10.0	1.17±0.19	0.20±0.18	0.33±0.16
small intestine	50.7	33.2 ±7.5	25.8± 8.0	6.34±0.85	1.94±1.69	2.34±0.07
liver	4.30	5.90±3.37	10.9± 1.6	8.28±0.77	6.13±0.12	2.56±0.20
kidney	0.22	0.39±0.22	0.78± 0.13	0.58±0.12	0.46±0.13	0.27±0.00
fat	3.85	4.33±2.16	3.38± 0.63	4.29±0.56	5.75±0.84	7.78±2.11
ceacum	0.04	1.92±1.65	5.82± 0.19	7.51±0.14	4.08±1.40	4.67±0.68
colon	0.14	1.76±1.36	0.26± 0.04	3.81±1.13	1.63±0.02	5.07±0.27
feces	0.00	0.00	0.00	0.20±0.07	11.1 ±0.8	5.71±2.16
urine	0.42	2.19±1.14	8.89± 4.52	23.9 ±3.6	48.3 ±1.9	47.9 ±4.0
CO_2	0.00	0.00	0.00	0.00	0.36±0.00	1.04±0.05

[a]Data expressed as the average of two rats ± the range.

[b]Data from one rat.

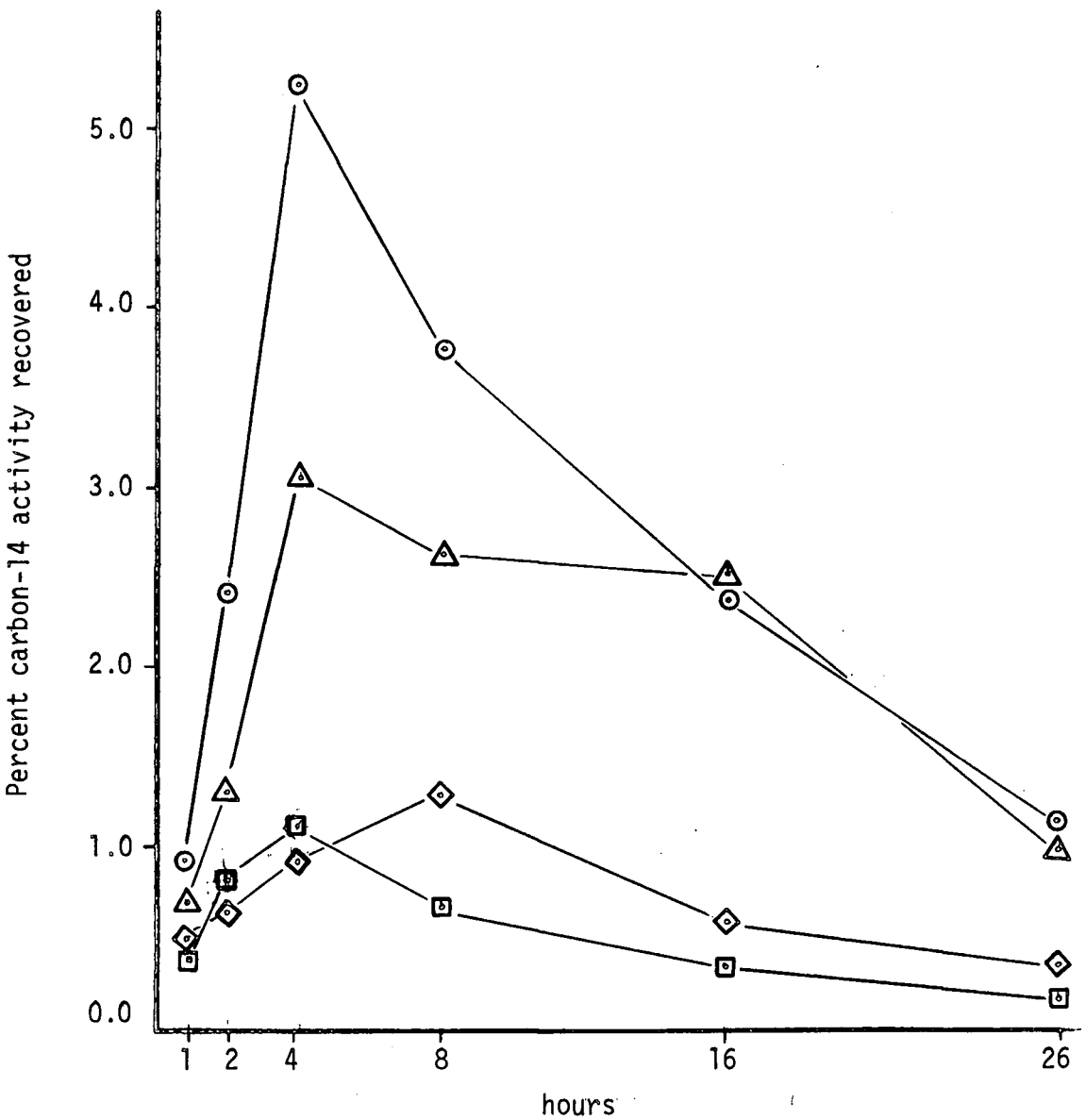

Figure 11. Percent of administered radioactivity recovered in rat liver supernatant (- ⊙ -), 800xg cell debri (- △ -), 12,000xg mitochondrial (- ◇ -) and 105,000xg microsomal (- ⊡ -) subcellular fractions expressed as a function of time after an IG injection of carbon-14 labeled SA.

TABLE IV. PERCENT OF ADMINISTERED RADIOACTIVITY RECOVERED IN RAT LIVER SUBCELLULAR FRACTIONS.

Subcellular Fractions	hours[a]					
	1[b]	2	4	8	16	26
800xg cell debri	0.74	1.18±0.73	3.10±0.58	2.69±0.22	2.51±0.002	1.01±0.01
12,000xg mitochondrial	0.28	0.59±0.30	0.96±0.11	1.35±0.13	0.61±0.04	0.37±0.06
105,000xg microsomal	0.26	0.71±0.34	1.13±0.11	0.67±0.18	0.35±0.05	0.17±0.02
supernatant	0.91	2.46±1.02	5.29±0.51	3.67±2.09	2.45±0.03	1.06±0.10

[a]Data expressed as the average of two rats ± range.

[b]Data from one rat.

were not changed. It is not likely that the labeled metabolites were conjugated, since KOH or β-glucuronidase would have changed the extraction characteristics and migration patterns on TLC plates of the carbon-14 labeled metabolites of SA.

Gas Liquid Chromatography

Figure 12-1 is a typical gas chromatogram (Appendix IX) of pooled methylated ether extracts of rat urine collected 16 and 26 hours after IG injections of carbon-14 labeled SA. Peaks A through E in the chromatogram contained a significant amount of radioactivity (Table V) with 34.1% of the activity found in the silicon-celite traps (Appendix X) of the effluent which correspond to peak B in the chromatogram. The radioactive material in peaks A through E were trapped in capillary tubes (Appendix X) and reinjected on a second column (Appendix IX). Figure 13 shows the gas chromatograms obtained. Figure 12-2 is the gas chromatogram obtained from a methylated ether extract of rat feces and Table V shows the amount of radioactivity found in peaks F, G and H.

Spectra

Figures 14, 15, and 16 are the IR spectra of peaks A' through E' in Figure 13 which are rechromatographed material from peaks A through E in Figure 12 respectively. All spectra contain intense carbonyl absorption at 1745 cm^{-1}. Peaks A', B' and D' show cyclopropane absorption at 1024 cm^{-1} (24, 25). Peaks C' and E' have no cyclopropane or cyclopropene absorption.

Figures 17, 18 and 19 are mass spectra of peaks A' through E' in Figure 13. The metabolites appear to be methyl esters of

dicarboxylic acids. Table VI shows some characteristic peaks in mass spectra of methyl esters of dicarboxylic acids (126). All spectra contain the M-30, 60, 63, 64, 73, 91, 92, 105 and 106 fragment loss.

The NMR spectra of peak B' (Figure 20) has multiplets at 10.0 and 9.2 indicating methylene protons of a cis-cyclopropane (82). Methine protons of the cyclopropane ring are found at 8.8τ. The number of methoxyl protons at 6.4τ to the number of methylene protons next to carboxyl groups at 7.8τ was 6 to 4, indicating two methyl ester groups. Infrared and NMR data plus the mass spectra showing a molecular weight of 186 indicates that the radioactive material in peak B' was cis-methyl-3,4-methylene adipate.

The mass spectra of peaks A' and B' in Figure 13 are shown in Figure 17. Peaks A' and B' have the same molecular weight (M=186) and almost identical fragmentation patterns. Infrared spectra of peaks A' and B' (Figure 14) are also almost identical with both peaks showing absorptions for the cyclopropane ring. Since peak B' is cis-methyl-3,4-methyl adipate peak A' is probably the trans isomer. Peak A' could not be trapped in sufficient quantity to obtain an NMR spectra.

Figure 15 shows the IR spectra of peak C'. No absorption for cyclopropane or cyclopropene rings is indicated. The mass spectra of peak C' (Figure 18) indicates a dibasic acid with a molecular weight 14 mass units higher than the compound found in peak B'. Peak C' could not be trapped in sufficient quantity to obtain an NMR spectra.

The characteristic cyclopropane absorbance at 1024 cm^{-1} appears in the IR spectra of peak D' (Figure 15). Mass spectra of peak D'

(Figure 18) indicates a dibasic acid with a molecular weight of 214, 28 mass units higher than peak B'. Multiplets at 10.6τ and 9.7τ in the NMR spectra of peak D' (Figure 21), indicate methylene protons of a cis-cyclopropane ring. Methylene protons are found at 8.7τ and the quintet at 8.3τ represents a methine group with four adjacent protons. Methylene protons next to carbonyl groups appear at 7.8τ and methoxyl protons are indicated at 6.4τ. The spectral data points to cis-methyl-3,4-methylene suberate as the material in peak D'. The peaks at 7.9τ and 9.1τ are impurities which were seen in the solvent.

No cyclopropane absorbance appeared in the IR spectra of peak E' (Figure 16). The mass spectra indicates a methylester of a dicarboxylic acid with a molecular weight of 228, 14 mass units higher than peak D'.

The mass spectra of peak G (Figure 12) of methylated fecal extract has a parent peak at 186 and a fragmentation pattern identical to peak B' (Figure 13). The compound in peak G is probably the same compound that is in peak B, cis-methyl-3,4-methylene adipate.

Rainbow Trout Metabolism

Intragastric Administration

In order to attempt the trout metabolism studies a reliable method was needed for injecting carbon-14 labeled SA into the stomach of the test animals without the animals regurgitating the injected material. An initial study was designed to check the feasibility of placing SA into gelatin capsules and then forcing the capsules into the stomach of the rainbow trout without regurgitation occurring (13).

TABLE V. AMOUNT OF CARBON-14 ACTIVITY RECOVERED IN THE GAS CHROMATOGRAMS OF RAT URINE AND FECAL METHYLATED ETHER EXTRACTS (FIGURE 12).

Peaks	A	B	C	D	E	F	G	H
% of injected radioactivity	5.7	34.1	12.4	9.3	6.7	4.0	38.1	2.7

TABLE VI. CHARACTERISTIC PEAKS IN THE MASS SPECTRA OF METHY ESTERS OF DICARBOXYLIC ACIDS (125).

m/e	Fragment lost	Remarks
M-31	$-OCH_3$	Characteristic of all methyl esters of dicarboxylic acids.
M-60	$-COOCH_3 + H$	Prominent only for short chain esters.
M-63	$-(OOCH_3)_2 + H$	
M-64	$-(OOCH_3)_2 + 2H$	Prominent in all but methyl adipate.
M-73	$-CH_2COOCH_3$	Characteristic of all esters.
M-91	$COOCH_3 + -OCH_3 + H$	M-91 higher than M-92 from methyl-azelate upwards.
M-92	$COOCH + -OCH + 2H$	
M-105	$CH_2COOCH_3 + -OCH_3 + H$	Low for methyl adipate, high for higher esters.
M-106	$CH_2COOCH_3 + -OCH_3 + 2H$	Marked only for long chain esters.

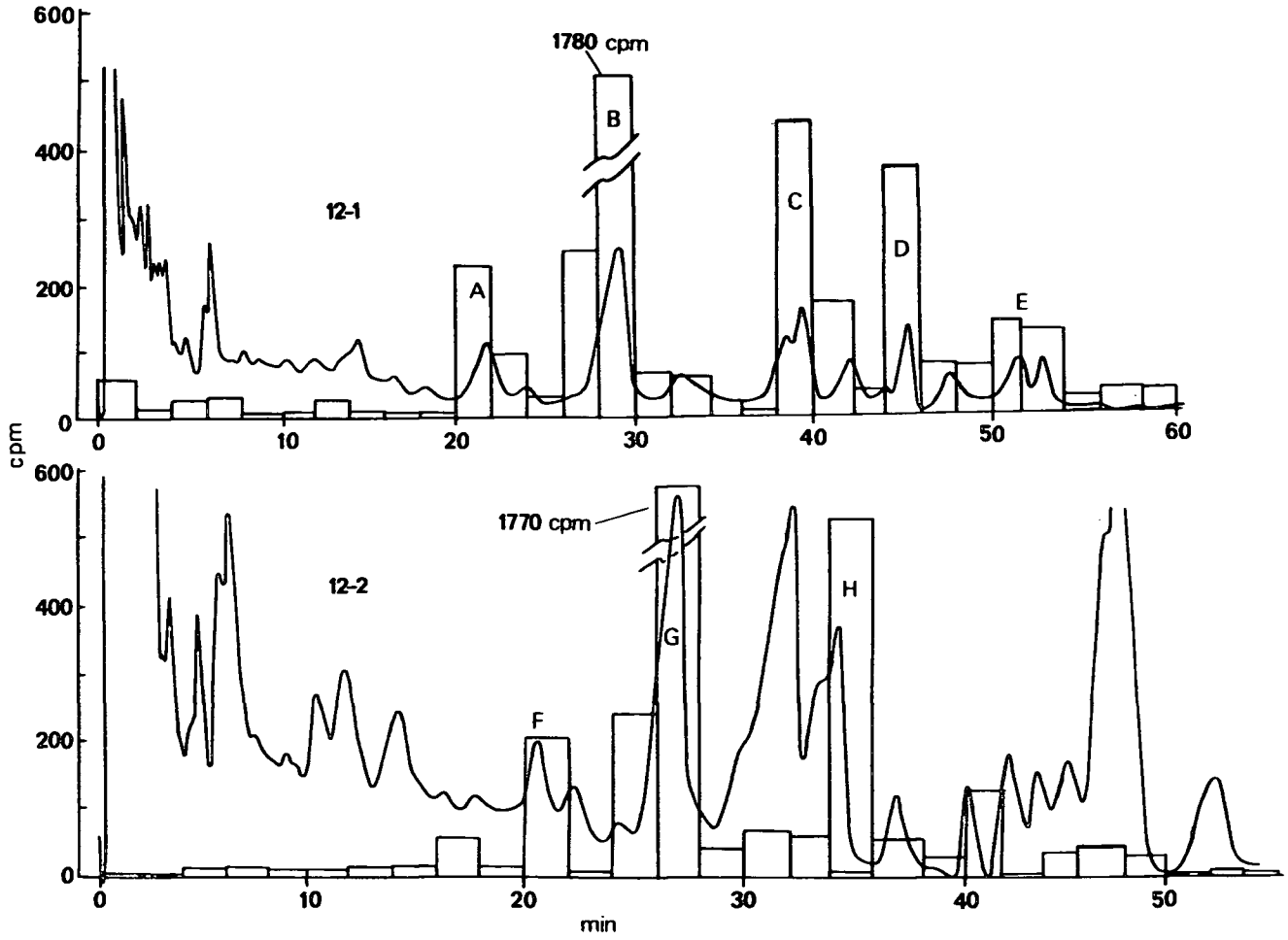

Figure 12. Gas chromatograms of rat urine (12-1) and fecal (12-2) methylated ether estracts on a 8' x 1/8" 10% Degs-PS column. Radioactivity in the effluent, collected in silicon-celite traps (Appendix X), is shown corresponding to the elution time. Peaks in the chromatograms containing significant radioactivity were assigned letters A through H.

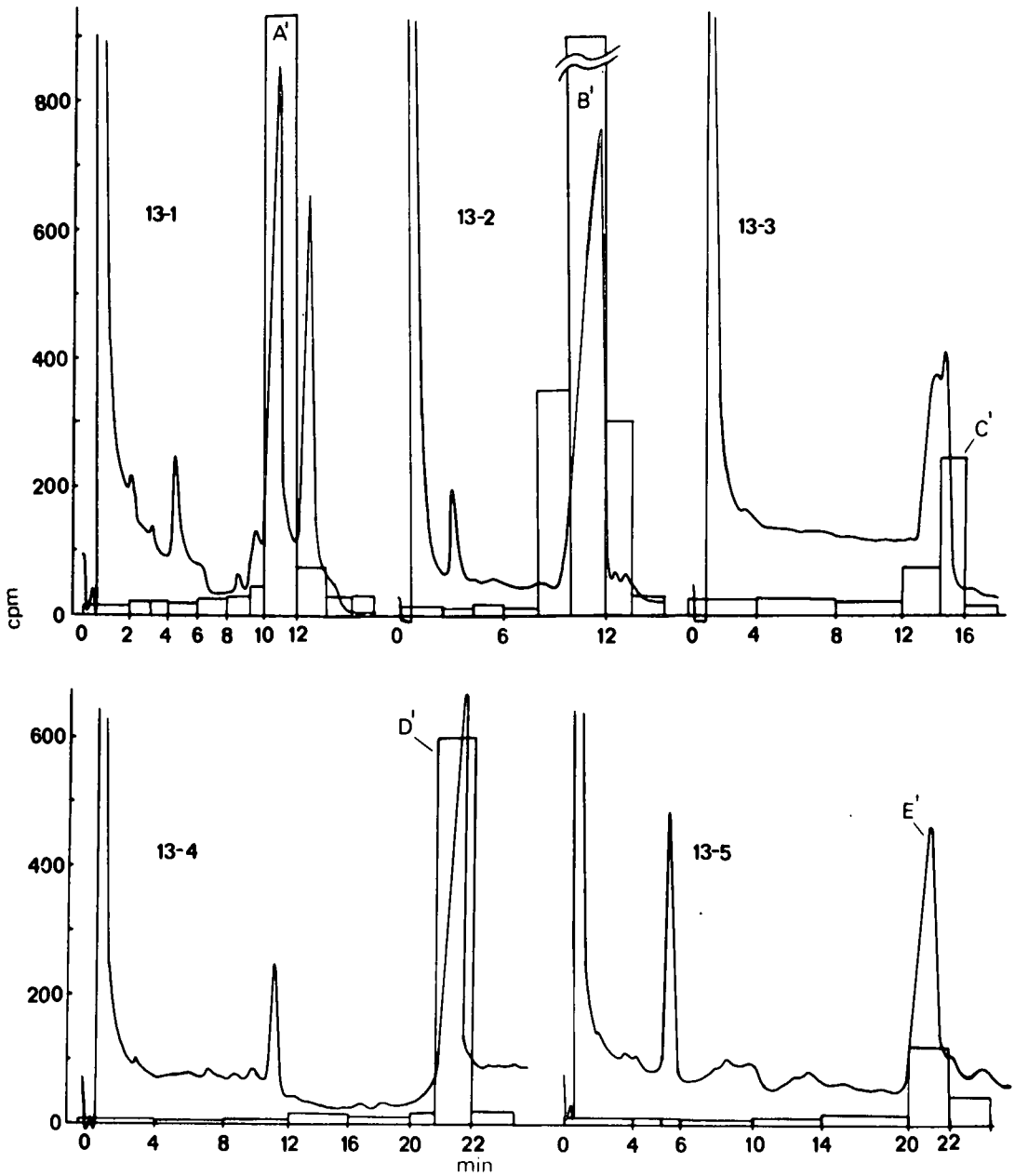

Figure 13. Gas chromatograms of radioactive peaks A through E in Figure 12 on a 8' x 1/8" OV-25 column. Radioactivity in the effluent, collected in silicon-celite traps (Appendix X), is shown corresponding to the elution time. Peaks in the chromatograms containing significant radioactivity were assigned letters A' through E'.

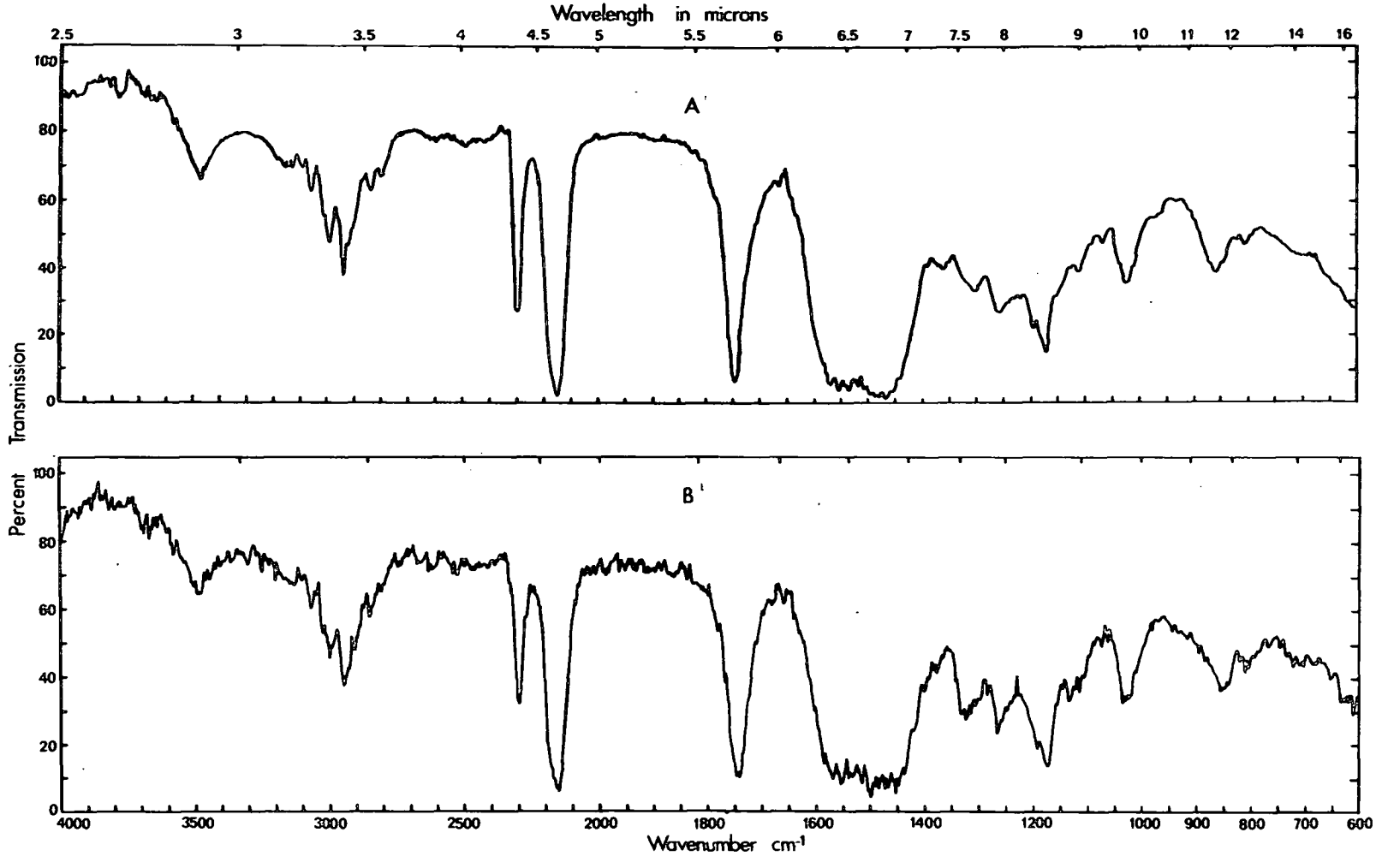

Figure 14. Infrared spectra of peaks A' and B' in Figure 13.

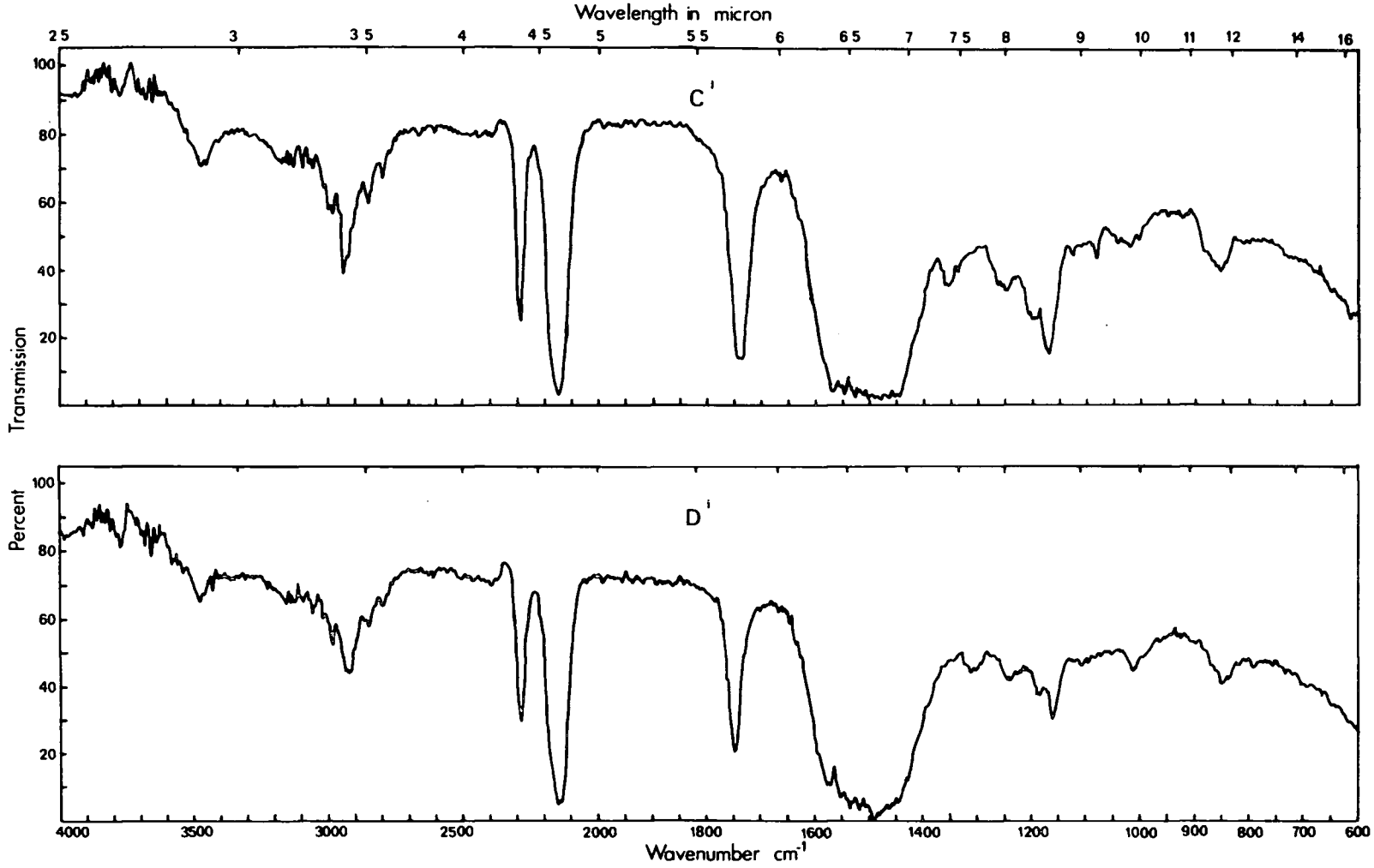

Figure 15. Infrared spectra of peaks C' and D' in Figure 13.

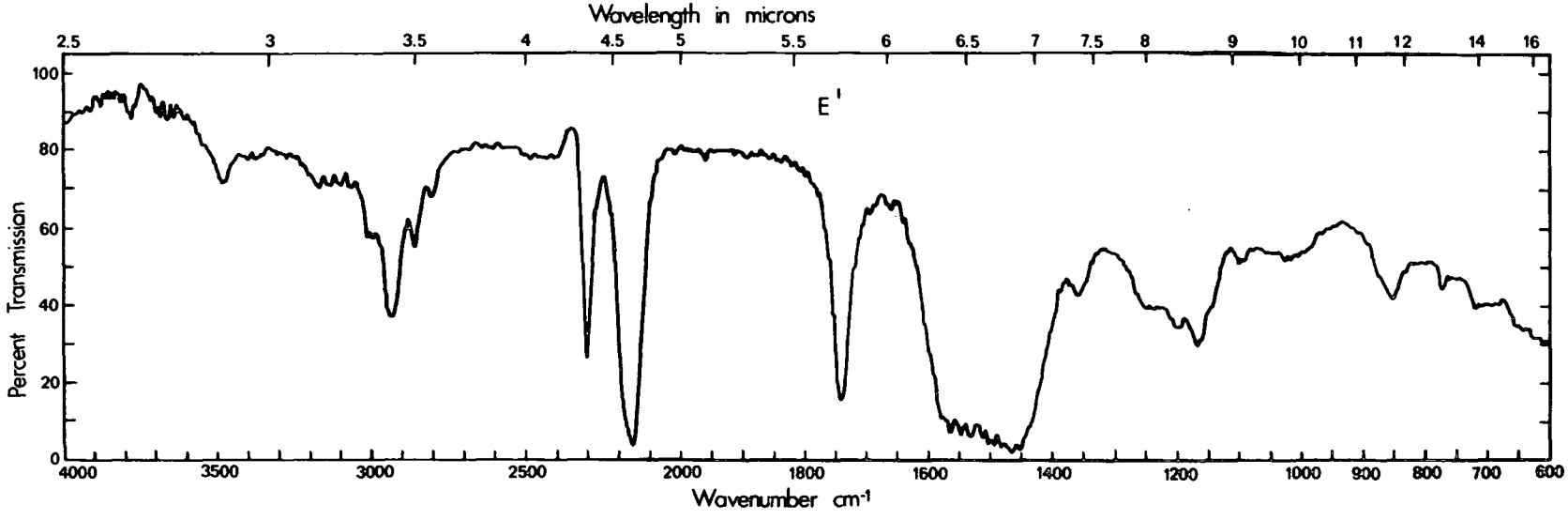

Figure 16. Infrared spectra of peak E' in Figure 13.

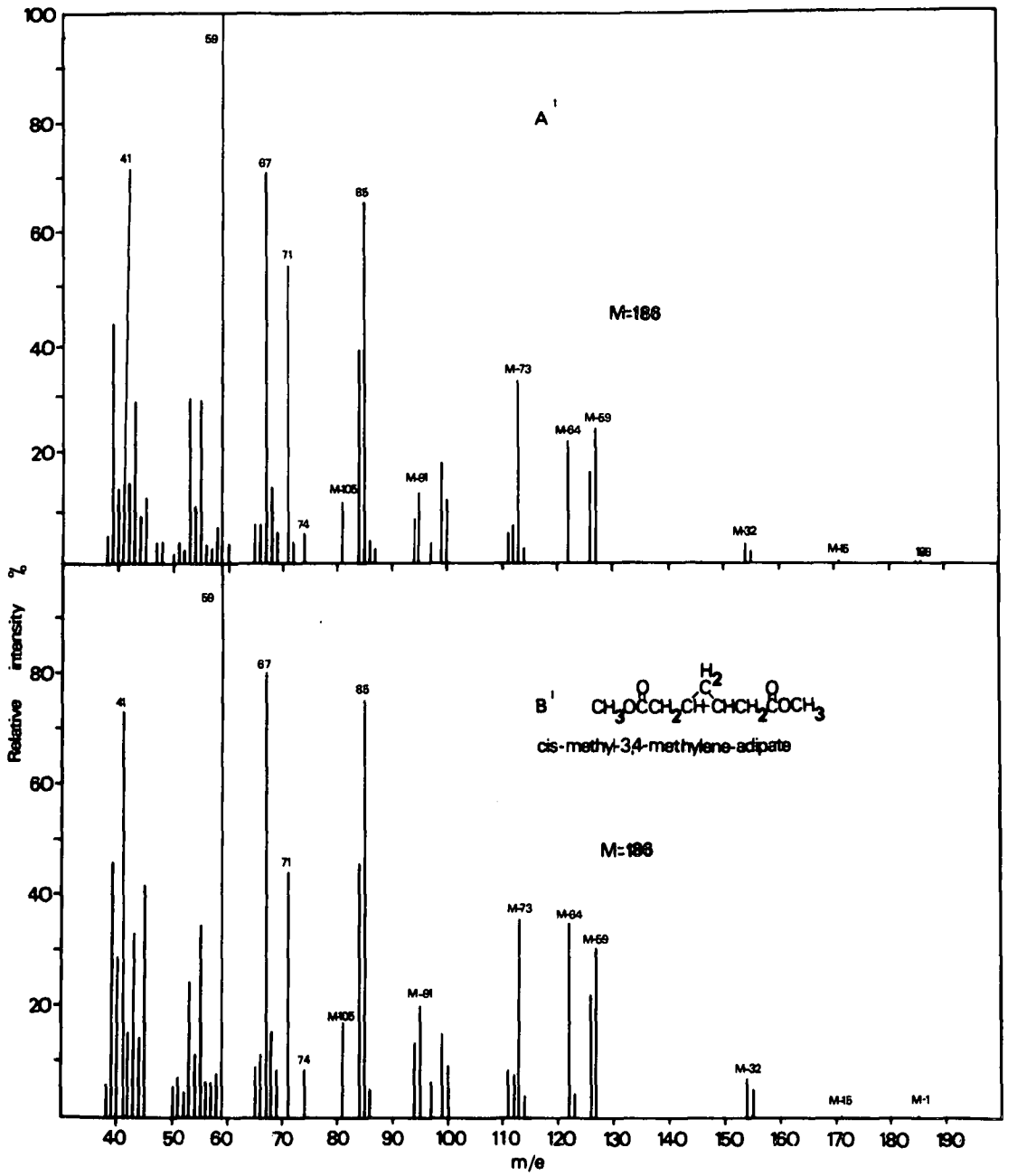

Figure 17. Mass spectra of peaks A' and B' in Figure 13.

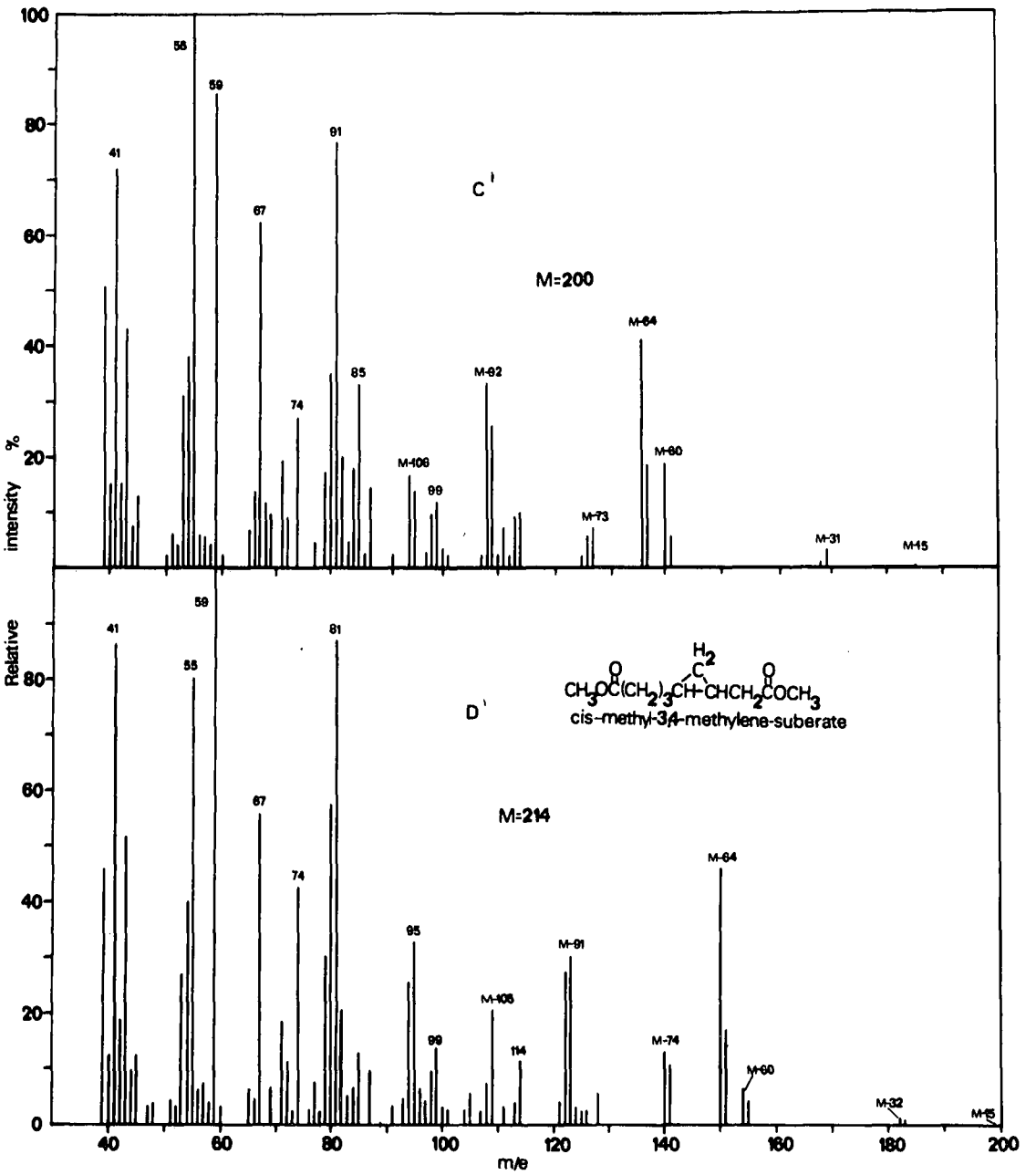

Figure 18. Mass spectra of peaks C' and D' in Figure 13.

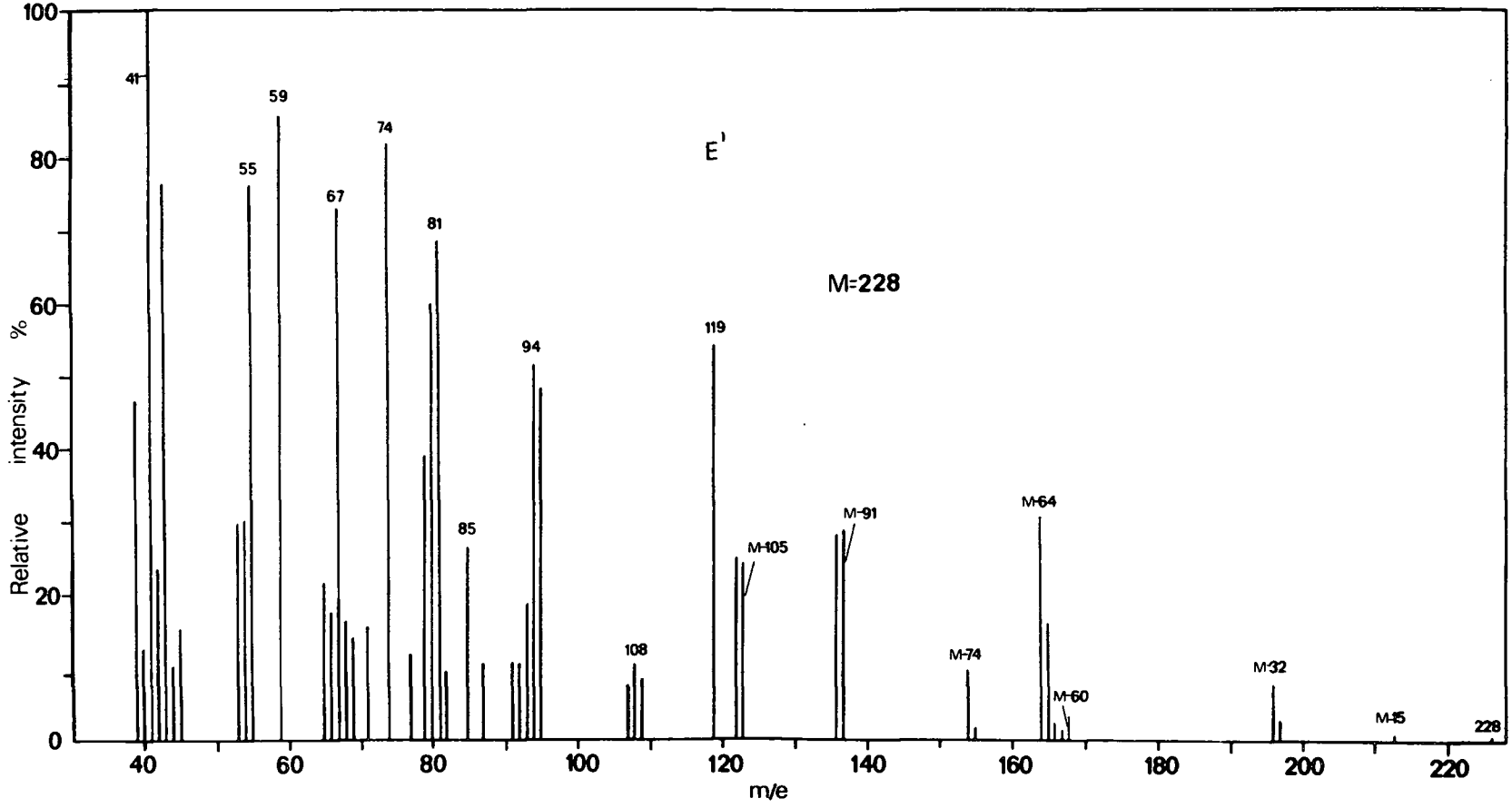

Figure 19. Mass spectra of peak E' in Figure 13.

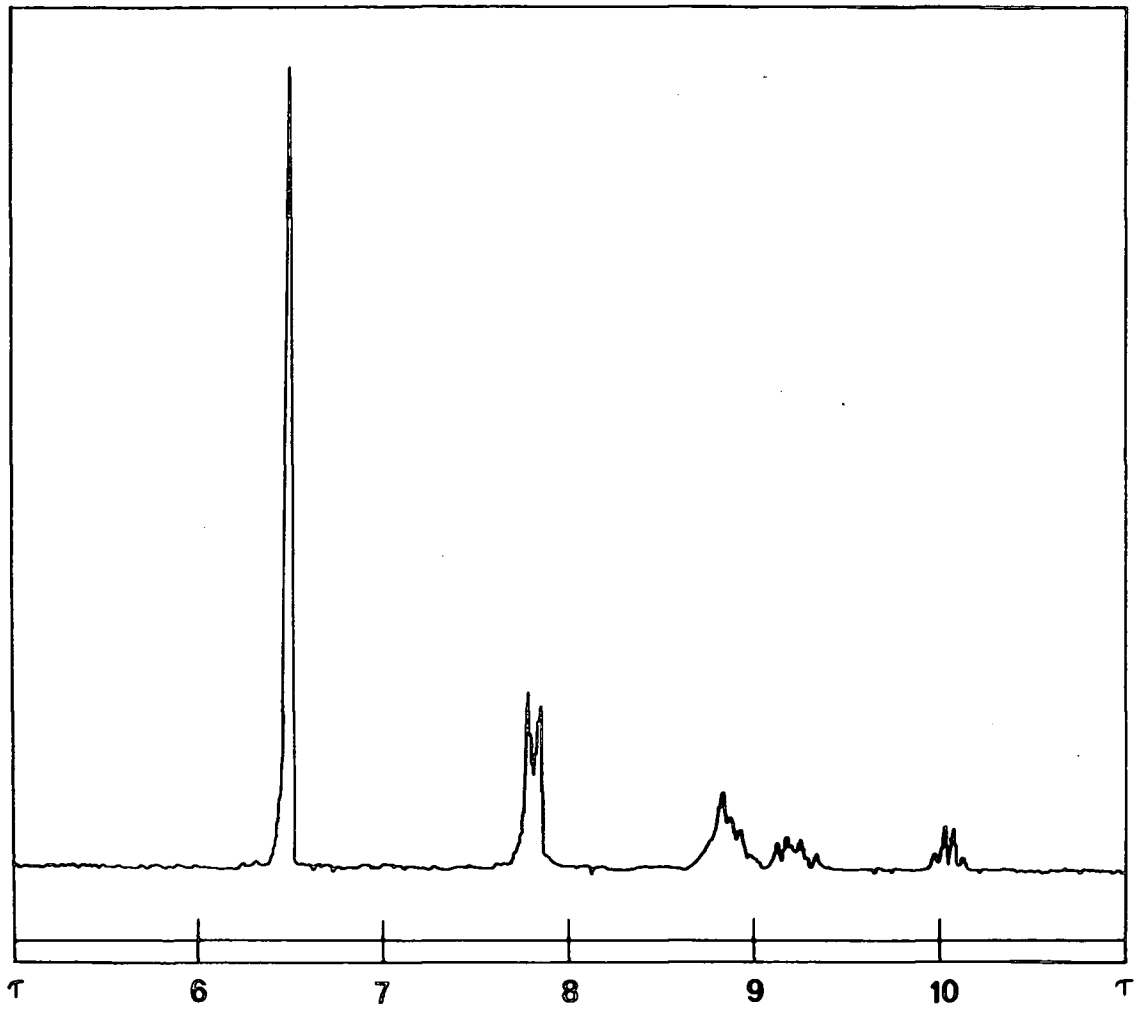

Figure 20. Nuclear magnetic resonance spectra of peak B' in Figure 13.

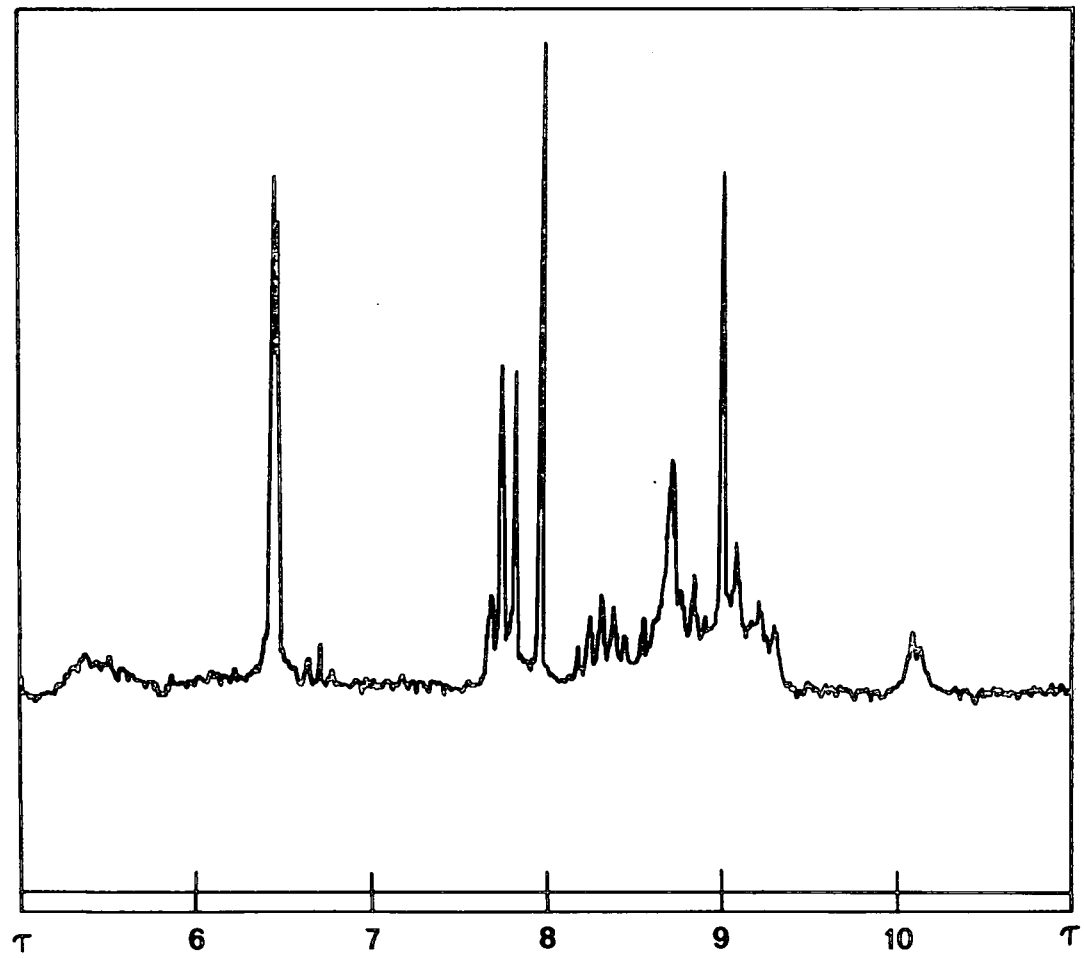

Figure 21. Nuclear magnetic resonance spectra of peak D' in Figure 13.

Twelve rainbow trout orally dosed with capsules containing blue dye plus corn oil (Appendix IV) did not regurgitate the capsules or the dye. There was enough dye in just one capsule to produce a noticable change in the color of the water if regurgitation had occurred. Dye was not found in the stomach but was found all along the intestinal tract. All twelve trout appeared to be in good health after 24 hours.

Deposition

The deposition of label in the tissue and organs of rainbow trout was determined at various times after intubating carbon-14 labeled SA into the stomach. Radioactivity in tissue is shown in Table VII. The movement of label in the stomach and GI tract is shown in Figure 22. After 4 hours 50% of the label remained in the stomach but after 18 hours only 11% could be found. Unlike the rat where the stomach and upper intestine curves roughly paralleled each other (Figure 6), label in the upper intestine of the trout gradually increases to a maximum of 30% of the administered dose at 3 days and then decreases to 82% after 7 days. At 2 hours the gelatins capsules that were inserted into the stomach of the rainbow trout were partially dissolved. Almost all the label (98.8%) was found in the upper intestine and stomach. After 3 days 70% of the label was recovered in tissue, organs and carcass of the trout. The unaccounted for activity was probably lost because the excreted metabolites ended up in the water of the metabolism tank which was circulated at 2 gallons per minute.

The concentration of label in blood plasma and gall bladder is shown in Figures 23 and 24. At least 10% of the administered activity

was found in the gall bladder after 5 days.

Movement of label in the liver is shown in Figure 25. Peak concentration of label approached 3% at 3 and 5 days. Radioactivity in organs at 5 days is shown in Figure 26. The GI tract contained the majority of the activity with the heart and spleen containing the least. Most organs reached a maximum concentration of radioactivity at 3 to 5 days. The kidney contained 0.4% of the administered dose after 5 days.

The relatively high percent of carbon-14 activity in the intestinal tract, blood, liver and gall bladder at 3 and 5 days indicate a substantial amount of enterohepatic circulation, and that the trout retained SA or metabolites of SA much longer than the rat.

Liver Subcellular Distribution

Distribution of label in liver subcellular fractions is shown in Figure 27 and Table VIII. As in the rat the supernatant fraction contained a relatively high amount of activity. The microsomes had much more activity than the mitochondria, 6.2 times higher activity at 3 days and 2.6 times higher at 5 days.

Excretion

The trout deposition study was not designed to determine the amount of carbon-14 label in CO_2, urine and feces. To determine the amount of label excreted, rainbow trout were orally dosed with carbon-14 labeled SA (Appendix IV) and placed in metabolism chambers (Figure 4).

Less than 0.5% of the administered dose was trapped as CO_2 (Figure 28). The trout excreted approximately 30% of the label after 12 and 36 hours. Label was then excreted at a constant rate up to

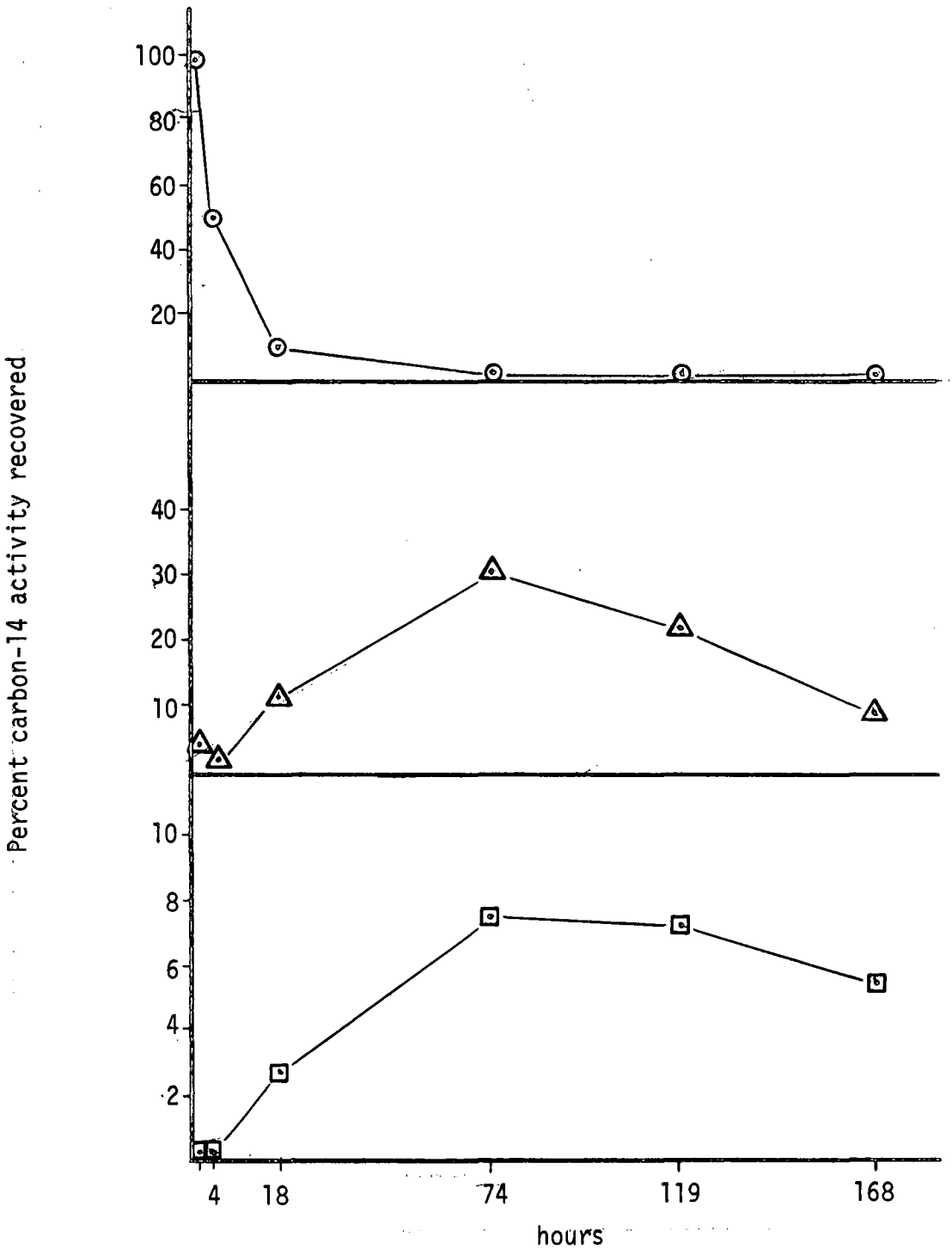

Figure 22. Percent of administered carbon-14 activity recovered in the stomach (- ⊙ -), pyloric ceacum + upper intestine (- △ -) and lower intestine (- ⊡ -) in rainbow trout expressed as a function of time after an IG injection of carbon-14 labeled SA.

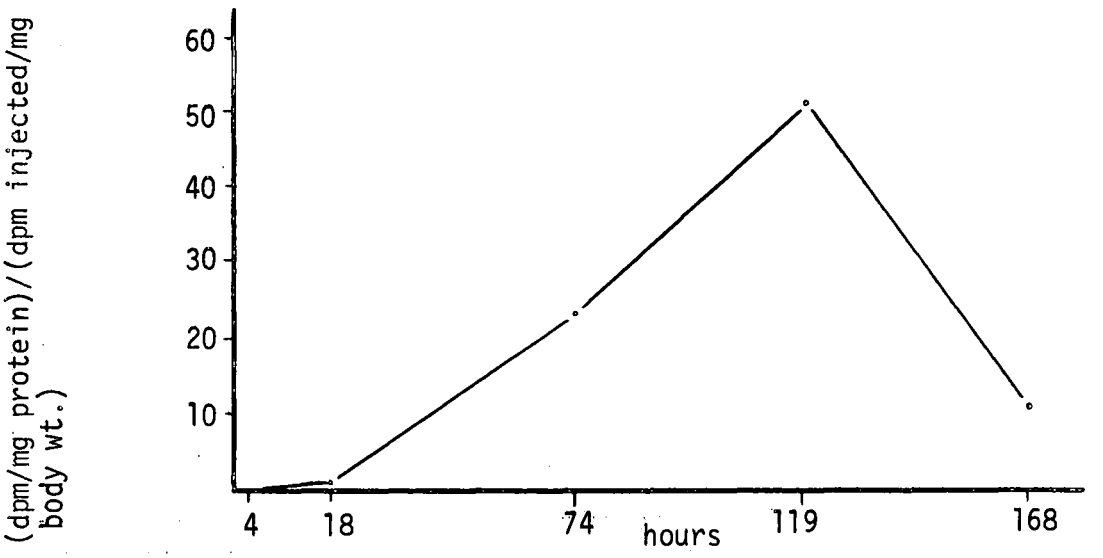

Figure 23. Concentration of radioactivity in blood plasm of rainbow trout expressed as a function of time after an IG injection of carbon-14 labeled SA.

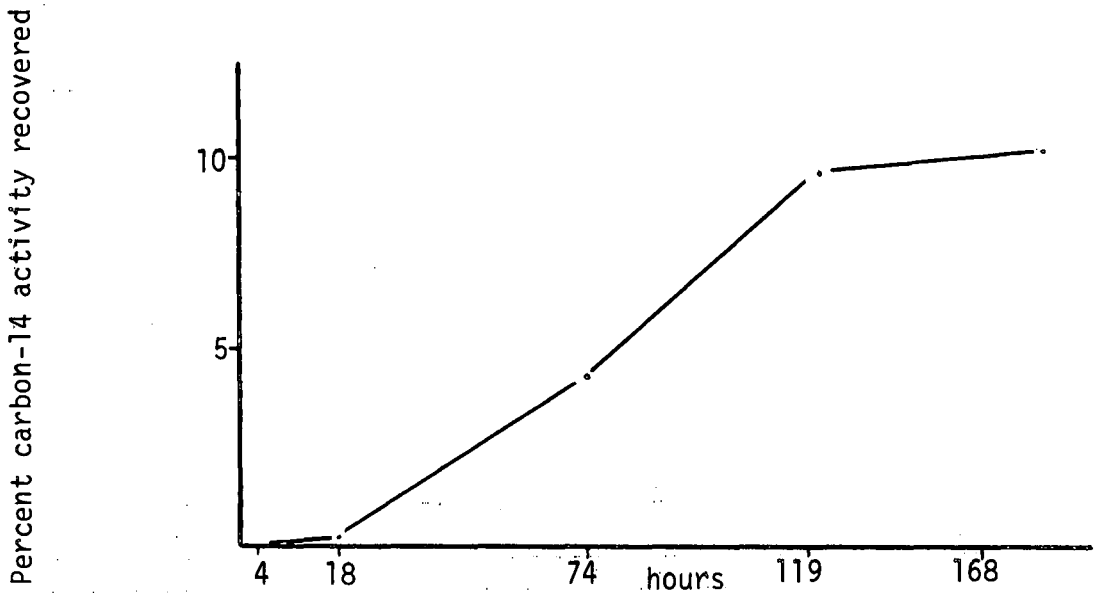

Figure 24. Percent of administered radioactivity recovered in the bile of rainbow trout expressed as a function of time after an IG injection of carbon-14 labeled SA.

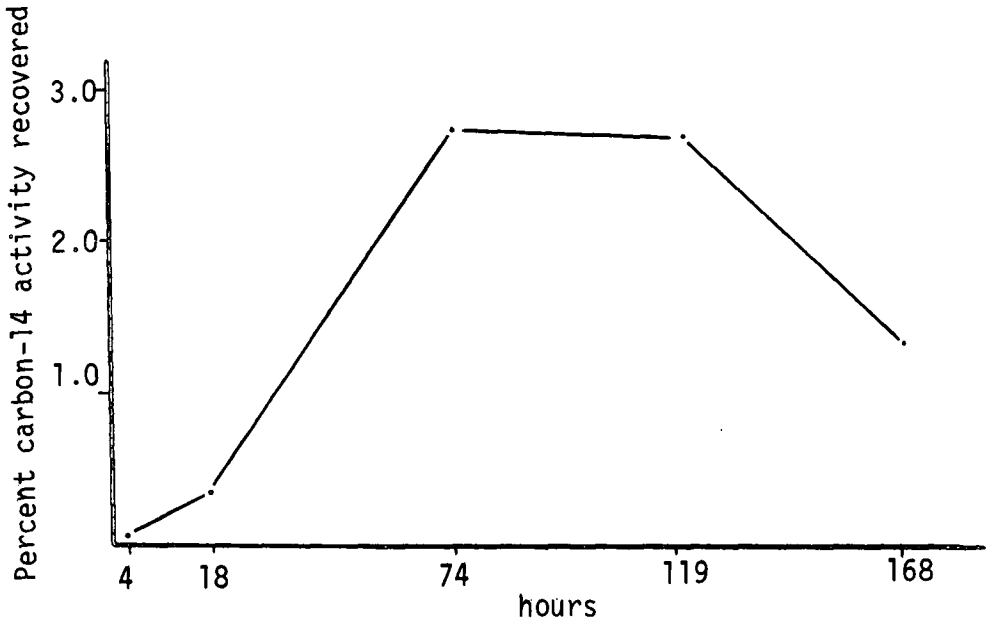

Figure 25. Percent of administered radioactivity recovered in the liver of rainbow trout expressed as a function of time after an IG injection of SA.

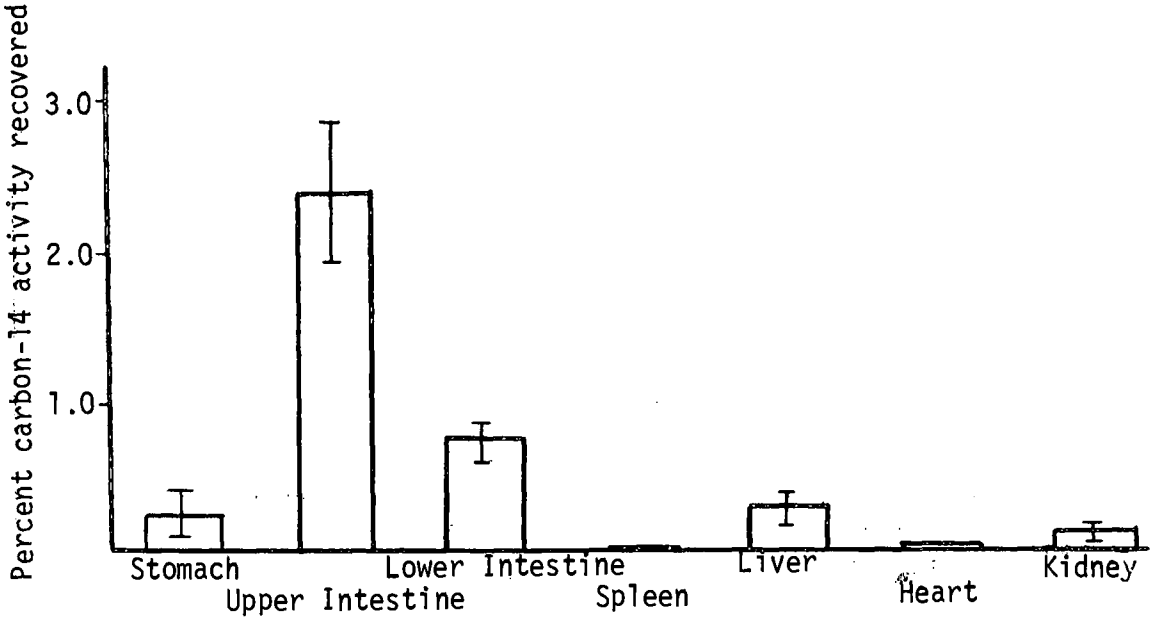

Figure 26. Percent of administered radioactivity found in the organs of rainbow trout five days after an IG injection of carbon-14 labeled SA.

TABLE VII. PERCENT OF ADMINISTERED RADIOACTIVITY RECOVERED IN TISSUE OF RAINBOW TROUT.

	hours					
	2[a]	4[a]	18[a]	74[b]	119[a]	168[a]
stomach						
range	91.8-110.7	44.6-54.9	5.0-16.8	0.3-2.6	0.8-5.2	0.3-0.9
mean	98.8	49.8	10.9	1.5	2.3	0.6
pyloric ceacum						
range	0.1-7.3	0.0-2.9	3.2-18.8	20.2-40.1	19.2-28.0	6.2-11.6
mean	3.7	1.0	10.9	30.2	21.7	8.2
lower intestine						
range	0.0-0.2	0.0-0.3	0.2-5.7	6.8-8.0	6.2-7.2	2.6-9.2
mean	0.1	0.1	2.9	7.4	7.0	5.3
bile						
range			0.0-0.5	2.3-7.0	6.4-14.5	8.7-12.6
mean	0.0	0.0	0.3	4.7	10.0	10.6
liver						
range			0.2-0.5	2.6-2.8	2.4-3.5	0.9-2.0
mean	0.0	0.0	0.4	2.7	2.8	1.3

[a]Data from three rainbow trout.

[b]Data from two rainbow trout.

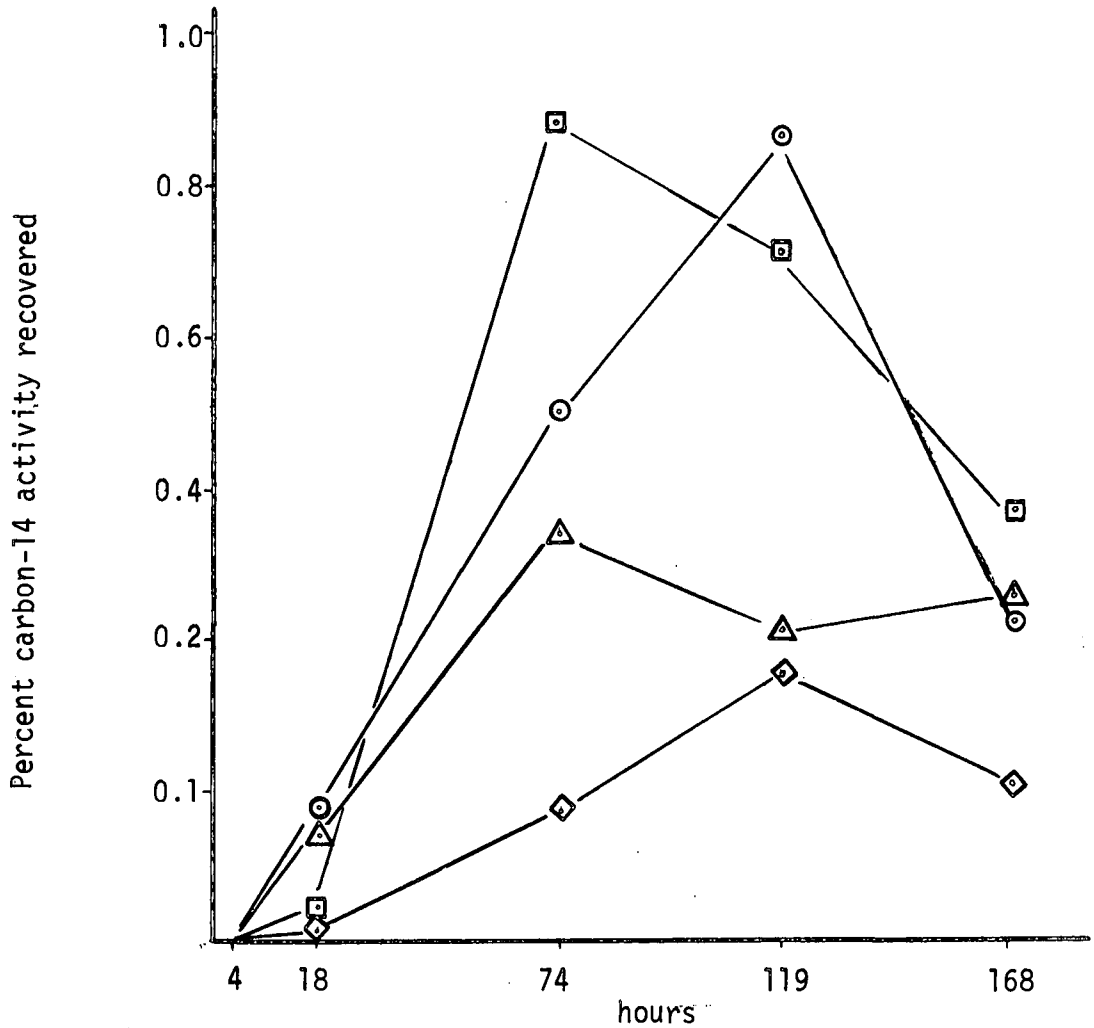

Figure 27. Percent of administered radioactivity recovered in rainbow trout supernatant (- ⊙ -), 800xg cell debri (- △ -), 12,000xg mitochondrial (- ◇ -) and 105,000xg microsomal (- ⊡ -) subcellular fractions expressed as a function of time after an IG injection of carbon-14 labeled SA.

TABLE VIII. PERCENT OF ADMINISTERED RADIOACTIVITY IN RAINBOW TROUT LIVER SUBCELLULAR FRACTIONS.

Subcellular Fractions	hours					
	2[a]	4[a]	18[a]	74[b]	119[a]	168[a]
800xg cell debri						
range			0.09-0.21	0.42-0.65	0.38-0.44	0.22-0.74
mean	0.00	0.00	0.14	0.54	0.41	0.45
12,000xg mitochondrial						
range			0.01-0.04	0.15-0.18	0.23-0.51	0.19-0.26
mean	0.00	0.00	0.02	0.17	0.35	0.21
105,000xg microsomal						
range			0.02-0.11	0.82-1.83	0.74-1.21	0.33-0.98
mean	0.00	0.00	0.05	1.08	0.91	0.57
supernatant						
range			0.08-0.27	0.69-0.70	0.94-1.30	0.27-0.54
mean	0.00	0.00	0.17	0.70	1.06	0.43

[a]Data from three rainbow trout.

[b]Data from two rainbow trout.

five days. The trout that was in the metabolism chamber five days (Figure 28) excreted 50% of the administered dose. As in the rat less than 1% of the carbon-14 label administered as SA was trapped as $^{14}CO_2$.

Trout excreted label at a much slower rate than the rat. The rat excreted 60% of the administered dose in 16 hours. Five days were required for the trout to eliminated 50% of the administered dose.

Metabolite Characteristics

Carbon-14 label collected in the trout metabolism chambers, after IG injections of carbon-14 labeled SA (Figure 28), was not extractable in ether under alkaline conditions. At pH 1, 76% of the excreted label was extracted with ether. Heating the excreted carbon-14 label in KOH did not increase the amount of ether extractable label, indicating little conjugation. The ether extracts gave negative Halphen tests.

Only 40% of the carbon-14 label in the bile was extracted in ether at pH 1. After incubation in β-glucuronidase (Appendix VIII) 44% of the label was extracted. When the bile was placed in ethanolic KOH 63% of the carbon-14 label was extracted at pH 1, indicating that some conjugation of the labeled compounds had occurred. The bile extracts also gave negative Halphen tests.

Radioactive spots on thin layer chromatograms of rainbow trout excreta ether extracts did not migrate as far as the SA standard (Table IX). The TLC data indicates that both the rat and trout metabolites are very polar compounds since they do not migrate very

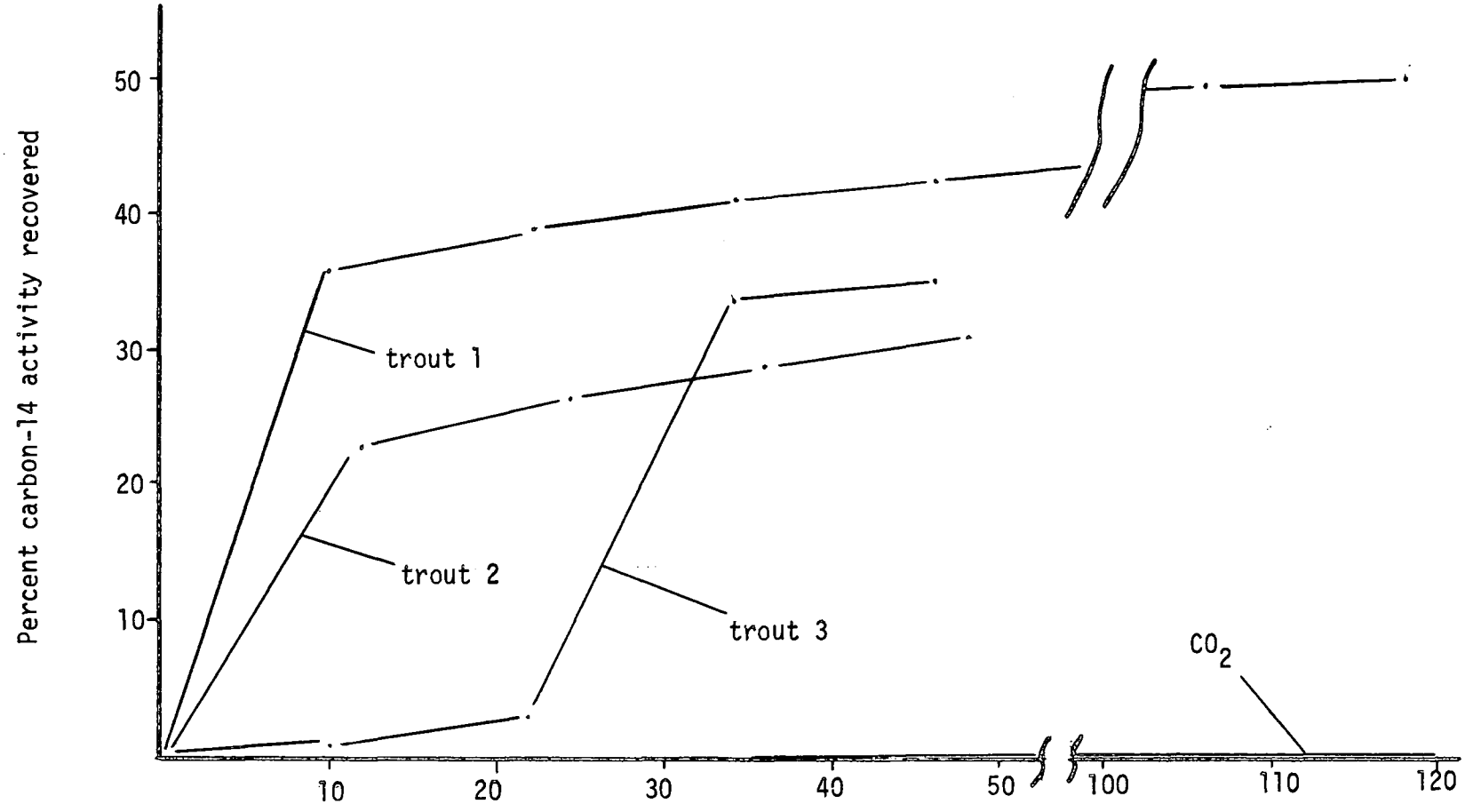

Figure 28. Percent of administered radioactivity recovered in excreta from three rainbow trout expressed as a function of time after an IG injection of carbon-14 labeled SA.

far on TLC plates even when using very polar solvent systems.

TABLE IX. RAINBOW TROUT AND RAT METABOLITE TLC DATA.

	R_F[a]		R_F[b]	
	ether extract	sterculic acid	methylated ether extract	methyl sterculate
rainbow trout	0.04, 0.12 0.16, 0.34	0.23	0.03, 0.07 0.29	0.52
rat	0.04, 0.11 0.12, 0.22	0.23	0.04, 0.10	0.52

[a]Chlorophorm:methanol:benzene (80:20:1)

[b]Hexane:ether:benzene (80:20:1)

When the fish methylated excreta extracts were injected on the GLC columns used in the rat metabolite identification experiments (Appendix IX), there was no correlation with the radioactive peaks found in the gas chromatograms of the rat urinary metabolites. Unfortunately, identification of trout metabolites was not accomplished because an insufficient quantity of material was trapped in the effluent of the gas chromatograms of the rainbow trout methylated excreta extracts to run IR, Mass spectrographic and NMR analysis.

Significance of Sterculic Acid Metabolism

Infrared spectroscopy, mass spectroscopy and NMR, of several carbon-14 labeled rat urine metabolites of SA, indicate that the rat metabolizes SA to cis-3,4-methylene adipic and cis-3,4-methylene suberic acids. Both metabolites have cyclopropane IR absorption at

1024cm^{-1}, and their methyl ester derivatives have mass spectra with fragmentation patterns characteristic of methyl esters of dicarboxylic acids. The methyl ester derivatives of the metabolites have molecular weights of 186 and 214. Nuclear magnetic resonance of the methylated metabolites show methylene absorption at 10.0τ and 9.2τ characteristic of cis-cyclopropanes. Methine absorption appears at 8.8τ. The number of methoxyl protons to the number of methylene protons next to carboxyl groups is 6 to 4, indicating two methyl ester groups.

In order to form these metabolites SA had to undergo oxidation from both the carbonyl and the terminal methyl ends. Oxidation of fatty acids from the carbonyl end involves β-oxidation which occurs in the mitochondria (76). Normally straight chained fatty acids undergo β-oxidation (6), however, in some cases straight chained (107, 121), branched chained (107) and substituted fatty acids (67) have been oxidized from their terminal methyl end. Bergstrom et al. (15) reported that in rats 2,2-dimethyl stearic acid was extensively converted to urinary 2,2-dimethyl adipic acid by oxidation starting at the terminal methyl end of the molecule. The urinary metabolite found by Bergstrom et al. (15) is very similar to the rat SA urinary metabolites reported in this thesis.

Oxidation of fatty acids at the ω-carbon atom involves hydroxylation to a ω-hydroxy acid followed by successive oxidations to the aldehyde and dicarboxylic acid (99). The hydroxylation reaction can be catalyzed by the liver and kidney cortex microsomes (99, 36). The microsomal enzyme system involved in ω-hydroxylation requires oxygen and reduced pyridien nucleotides and contains cytochrome P-450,

cytochrome P-450 reductase and a heat stable lipid component (87). The enzyme system is inhibited by CO and induced when phenobarbital is administered (87, 36).

The oxidation step from the ω-hydroxy derivatives to the dicarboxylic acids occurs in the cytosol and requires NAD+ (99). The dicarboxylic acids formed are then β-oxidized from both ends in the mitochondria.

The rainbow trout and rat metabolism studies show that a significant amount of carbon-14 activity was found in the liver microsomes and mitochondria (Figures 11 and 27). In both species carbon-14 activity was initially higher in the microsomal fractions, indicating that ω-hydroxylation was initially occurring in the microsomes before β-oxidation was occurring in the mitochondria. The trout and rat had significant radioactivity in the supernatant fractions (Figures 11 and 27) where oxidation of the ω-hydroxy derivatives of fatty acids to dicarboxylic acids occur (99).

Hepatic toxicity (115) and carcinogenicity (54) of aromatic hydrocarbons and aflatoxin have been reported to require microsomal activation. Microsomes isolated from several species of animals convert aflatoxin B_1 to an active metabolite toxic to Salmonella typhimurium TA 1530 (46). The activation is dependent on oxygen and NADPH and is sensitive to inhibitors of the microsomal mixed function oxidase system. Sc hoenhard (135) found that rainbow trout liver microsomes activate aflatoxin B_1 to products lethal to Bacillus subtiles GSY 1057. The microsomal enzyme system which metabolizes aflatoxin B_1 is the same system which makes ω-hydroxy derivatives of

fatty acids since it requires oxygen and reduced pyridien nucleotides contains cytochrome P-450 and cytochrome P-450 reductase, is inhibited by CO and is activated by phenobarbital (22, 108). Sterculic acid may be acting as a co-carcinogen by inducing the enzyme systems responsible for converting aflatoxin B_1 to an ultimate carcinogen, since in the microsomes aflatoxin B_1 and SA are metabolized by the same type enzyme system.

The differences in sensitivity of CPFA may in part be explained by the slower rate of metabolism of SA in the trout with a higher degree of enterohepatic circulation. Sterculic acid or its metabolites remain in the liver of the trout for a longer period of time allowing SA or its metabolites a longer time to react.

Cyclopropenoid fatty acids appear to cause major changes in the mitochondria (144, 81). The urinary metabolites of SA identified resemble intermediates of the Krebs cycle. The 3,4-methylene adipic and suberic acids may be acting as inhibitors to enzymes of the Krebs cycle and causing some of the changes that have been observed.

Mass spectra of the radioactive peaks B' to E' in Figure 13 show molecular weights increasing by increments of 14 mass units. If just β- and ω-oxidation were occurring there would be increases of 28 mass units instead of 14. If α-oxidation, a microsomal system, was involved in the metabolism of SA, metabolites would increase by increments of 14 mass units. Therefore, the possibility exists that α-oxidation has a role in the metabolism of SA.

Areas of Future Study

A more detailed look at the metabolism of SA in rainbow trout is needed. Identification of metabolites of SA in rainbow trout excreta is of prime importance. Knowledge of rainbow trout metabolites will lead to better understanding of the metabolic process involved and help point out differences in the way SA is metabolized by rats and rainbow trout.

Dialkyl cyclopropenes, SA and MA should be synthesized with carbon-14 label in the methylene carbon of the ring and in several positions located on the chains of the compounds. The significance of α, β and ω-oxidation pathways in the rainbow trout and rats can be determined by monitoring expired CO_2 and by identifying the metabolites formed in both in vitro and in vivo experiments.

It would be of interest to determine the biological effects of feeding 3,4-methylene adipic and suberic acids to rats and rainbow trout. Since SA causes impairment of mitochondrial function and the metabolites isolated are similar to substrates in the Krebs cycle, experiments should be designed to show whether 3,4-methylene adipic and suberic acids act as inhibitors of the Krebs cycle.

Rainbow trout could be fed rations containing aflatoxin B_1 plus the SA metabolites to determine whether the metabolites are co-carcinogens. Finally, experiments with the objective of determining whether SA can increase the microsomal enzymes needed for conversion of aflatoxin B_1 to a active carcinogen are needed.

SUMMARY AND CONCLUSIONS

1. Intragastric administration of carbon-14 labeled MS and SA in rats yielded higher levels and faster absorption of carbon-14 label in tissue and excreta than in the IP mode of administration.
2. The liver was the organ which contained the highest radioactivity after absorption of carbon-14 labeled SA in rats and rainbow trout. In both species microsomal subcellular fractions of the liver contained a significant amount of label, pointing to a contribution of the microsomes in SA metabolism.
3. Little radioactivity was found expired as CO_2 in rats and rainbow trout. Since the carbon-14 label was in the methylene carbon of the cyclopropene ring, the cyclopropene ring was not metabolized to CO_2.
4. Rats excreted label at a much faster rate than rainbow trout. Rats having a 60% turnover of label at 16 hours after administration of SA. Rainbow trout excreted 50% after five days. The majority of label excreted by the rat was found in the urine. Rainbow trout appeared to have a significant amount of enterohepatic circulation, with approximately 40% of the administered dose in the GI tract, liver, and bile five days after administering SA.
5. The major metabolite isolated in rat urine was 3,4-methylene adipic acid. Sterculic acid must undergo β- and ω-oxidation plus reduction of the cyclopropene ring in order to form the metabolite.

6. Metabolites from SA metabolism in rainbow trout were not identified. However, enough data was obtained to indicate rianbow trout metabolize sterculic acid to metabolites that are not the same as those found in the rat.

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APPENDICES

APPENDIX I

SAPONIFICATION OF METHYL STERCULATE

Approximately 4 μci (0.036 mmoles) of pure MS 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. Refrigerated distilled water (300 mls) was added before the acidity of the reaction mixture was changed to pH1 with HCl. The saponified fatty acid was removed from the aqueous phase by three extractions with anhydrous ether. The ether phase was evaporated to dryness in a Buchler Flash-Evaporator[8] and stored at -30°C in ether.

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[8]Buchler Instruments, Fort Lee, New Jersey

APPENDIX II

HALPHEN TEST

This procedure is essentially that of Hammonds et al. (52). Between 150,000 and 200,000 dpms (188 to 251 μg) of carbon-14 labeled MS or SA was placed in 15 x 415 mm screw-cap tubes[9] and mixed with 0.1 ml of 4% morpholine in n-butanol and 4.9 ml of n-butanol. Exactly 1.0 ml of 1% sulfur in carbon disulfide (prepared just before use) was added under subdued light. The tubes were sealed with teflon lined caps and the contents thoroughly mixed before heating in the dark in a glycerine bath at 110°C for 110 min.

Since the specific activity (SP) of the carbon-14 labeled samples in dmp/μg of oil was known, the % CPFA in the sample was calculated using the following formula.

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

[9]Kimble, Toledo, Ohio

APPENDIX III

THIN-LAYER CHROMATOGRAPHY

Sluries of silica gel G[10] in distilled water 45:85 (w/v) were spread with a plate spreader[11] adjusted for spreading the slurries with a 250 μ thickness. The plates were air dried and heated at 130°C for 30 minutes.

Plates were scored before spotting with 2 to 10 λ disposable micropipets. Chromatography tanks were lined with paper and the plates were developed in several solvent systems. Spots were visualized in an iodine saturated chromatography tank, and the location of label determined by scrapping the spots and counting in a scintillation counter (Appendix VI). Location of label was also determined by scanning the TLC plates with a radiochromatogram scanner.

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[10] EM Laboratories, Inc. Elmsford, New York

[11] C. Desage, Heidelburg, West Germany

APPENDIX IV

INJECTIONS IP AND IG

Rat IP Injections

Labeled MA or SA (3 to 4 μci) was added to approximately 0.8 ml of corn oil. A B-D Yale luer-lock 1 cc syringe fitted with a 27 gage needle was filled with 0.4 ml of the labeled oil and weighed. The material was injected IP and the syringe was weighed again. Specific activity in dmp/mg of injected oil was determined and dpm of injected label was calculated.

Rat IG Injections

A 1 cc luer-lock syringe fitted with 8 inches of PE-90 intramedic[12] tubing was filled with labeled oil. The tubing was pushed into the stomach of the rat and approximately 0.4 ml of oil was injected into the stomach. From the weigh and specific activity of material injected dpm of injected label was calculated.

Rainbow Trout IG injections

Number 5 gelatin capsules, containing evans blue dye or labeled SA diluted corn oil, were fitted into a dry plastic tube. The tube with the capsule inside was pushed into the stomach of the fish which were anesthetized with tricainmethane sulfonate (MS-222) (58). A glass rod was inserted into the tube and the capsule was pushed into

[12] Clay-Adams, Inc., New York

the stomach of the trout. After removing the tube and rod 0.5 ml of corn oil was injected into the stomach. It took 1-2 minutes for MS-222 to act, 15-20 seconds to stomach tube the trout and 1-2 minutes for the trout to recover and start swimming. The weight and specific activity of the oil injected was used to calculate dpm injected.

APPENDIX V

LOWRY PROTEIN DETERMINATION

Reagents

A. 2% sodium carbonate in 0.1 N sodium hydroxide

B. 0.5% copper sulfate pentahydrate in 1% sodium tartrate

C. 50 ml A. plus 1 ml B.

D. Commercial Fishers Phenol Reagent[13] diluted 1:1 with distilled water

Procedure

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

Reference (86)

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[13]Folin-Ciocalteau, Fisher Scientific Co., Fair Lawn, New Jersey

APPENDIX VI

LIQUID SCINTILLATION COUNTING

Sample preparation

Whenever possible samples were counted in disposable Kimble 1-dram Opticlear glass vials (15 x 45 mm) with polyethylene caps.[14] The vials were placed in 20 ml screw-cap vials.

Tissue, homogenates and other samples that needed to be digested were solubilized in NCS tissue solubilizer. Wet samples were digested at 40°C with 3 to 6 parts NCS (w/v or v/v) in the 1-dram or 20 ml vials. Toluene fluor was added after digestion.

Aquasol is a xylene based fluor capable of solubilizing organic and aqueous solutions. A clear thixotropic gel forms with aquasol in about 25% or more aqeuous solution. Experimentation was necessary to find the proper ratio of carcass KOH digest in aquasol so that a gel was formed.

Counting procedures

Quench correction curves for the toluene and aquasol fluor solutions were used to calculate dpm in samples. In some cases high quenching was observed. A drop of 30% H_2O_2 was added to reduce color quenching. A toluene internal standard was used to calculate dpm.

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[14] Kimble, Toledo, Ohio

Fluor Solutions

Name	Fluor[a] Solution Components	Solution[b] Volume (ml)	Sample Volume[c] (ml)	Sample Type
Toluene	1 liter toluene 6 g PPO 50 mg POPOP	3-15	0-1	organic
Toluene digest	1 liter toluene 6 g PPO 50 mg POPOP	4-18	0-0.5 + 3 to 6 time volume NCS	Homogenized samples, Tissue, Blood, Urine and Bile
Toluene gel	600 ml toluene 400 ml ethanol 4 g PPO 15 mg POPOP	10 ml + full vial of Cab-o-sil	0-2	CO_2 trap
Aquasol	Xylene based	3	0-1	TLC-scrappings, Silicon-celite trap
Aquasol gel	Xylene based	6	2 or more	KOH Carcass digest

[a]PPO: 2,5-diphenyloxazole (Scintillation grade); POPOP: 1,4-bis-2-(5-phenyloxazoly-benzene) (Scintillation grade); Aquasol: New England Nuclear, Boston, Massachusetts.

[b]Cab-o-sil: Godfrey L. Cabot, Inc., Boston, Massachusetts.

[c]NCS: Amersham/Searle Corporation, Arlington Heights, Illinois.

APPENDIX VII

RAINBOW TROUT DIET

Ingredient	Diet (%)
Casein	49.5
Gelatin	8.7
Dextrin[a]	15.6
Mineral mix[b]	4.0
CMC[c]	1.0
Alpha cellulose	8.2
Choline chloride (70%)	1.0
Vitamin mix[d]	2.0
Herring oil	10.0

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

[b]Calcium carbonate ($CaCO_3$) (2.100%); calcium phosphate ($CaHPO_4.2H_2O$) (73.500%); potassium phosphate (K_2HPO_4) (8.100%); potassium sulfate (K_2SO_4) (6.800%); sodium chloride (NaCl) (3.060%); sodium phosphate ($Na_2HPO_4.6H_2O$) (2.140%); magnesium oxide (MgO) (2.500%); ferric citrate ($FeC_6H_5O_7.3H_2O$) (0.558%); manganese carbonate ($MnCO_3$) (0.418%); cupric carbonate ($2CuCO_3Cu(OH)_2$) (0.034%); zinc carbonate ($ZnCO_3$) (0.081%); potassium iodide (KI) (0.001%); sodium fluoride (NaF) (0.002%); cobalt chloride ($CoCl_2$) (0.020%); and citric acid (0.686%). Modified from Bernhart and Tomarelli (29) by addition of the NaF and $CoCl_2$.

[c]Carboxymethyl cellulose, Hercules Powder Co., San Francisco, California.

[d]Thiamine hydrochloride (0.3200%); riboflavin (0.7200%); niacinamide (2.5600%; biotin (0.0080%); Ca-pantothenate (D) (1.4400%); pyridoxine hydrochloride (0.2400%); folic acid (0.0960%); menadione (0.0800%);

B_{12} (cobalamine-3,000 µg/g) (0.2667%); i-inositol (meso) (12.5000%); ascorbic acid (6.0000%); para-amino-benzoic acid (2.0000%); vitamin D_3 (500,000 usp/g) (0.0400%); vitamin A (250,000 IU/g) (0.5000%); DL alpha tocopherol acetate (2.5000%); and celite (70.7293%).

APPENDIX VIII

B - GLUCURONIDASE ASSAY

Enzyme

2 mg/ml β-glucuronidase from Helix pomatia also containing some sulfatase activity. Type H-1.

Substrates

0.01M phenolphthalein glucuronic acid.[15]

Excreta from rat and rainbow trout.

Buffer

0.1M sodium acetate, pH 4.5.

Procedure

Mix 0.2 ml of substrate, 0.8 ml of acetate buffer and warm to 37°C. Add 0.2-0.4 ml of enzyme. After 2 hours cool the samples and acidify with concentrated HCL. Extract with ether and count extract for radioactivity. Check activity of enzyme by adding KOH to samples with phenolphthalein glucuronic acid and observe color of solution.

Reference (43).

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[15] Sigma Chemical Company, St. Louis, Missouri

APPENDIX IX

GAS LIQUID CHROMATOGRAPHY

Apparatus

Aerograph autoprep A-700[16]

Detector

Thermal conductivity

Columns

Initial separation

8 ft x 1/8 in 10% Degs-PS on 80/100 mesh Supelcoport.[17]

Final separation

8 ft x 1/8 in 5% OV-25 on 100/120 mesh chromosorb G.[18]

Conditions

Initial separation

Flow - 20 cc/min
Injector temperature - 190°C
Detector temperature - 220°C
Collector temperature- 210°C
Column temperature - 142°C for first 32 to 36 minutes
then programmed up to 180°C

Final separation

Flow - 20 cc/min
Injector temperature - 190°C
Detector temperature - 220°C
Collector temperature- 210°C
Column temperature - Peak A' - 130°C
Peak B' - 130°C
Peak C' - 140°C
Peak D' - 140°C
Peak E' - 150°C

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[16]Aerograph, Walnut Creek, California
[17]Supelco, Inc., Bellefonte, Pennsylvania
[18]Applied Science Laboratories, Inc., State College Pennsylvania

Syringes

Pressur-Lok Liquid syringes[19] (10 or 100 µl) were used to inject samples on the gas chromatograph.

[19]Precision Sampling Corporation, Baton Rouge, Louisiana

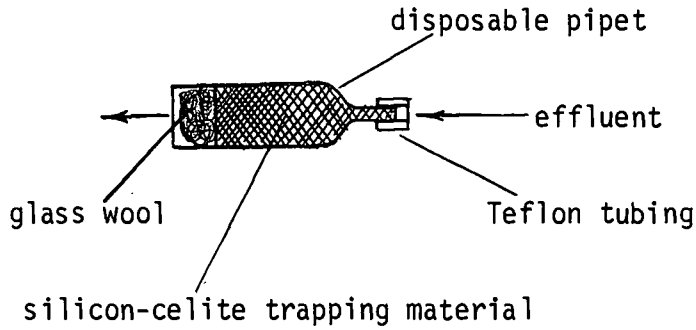
APPENDIX X

GLC TRAPS

Silicon-celite traps

The silicon-celite traps are similar to those described by Arthur (7). Celite (120-140 mesh) was added to 150 ml of Hexane containing silicon fluid SWS-101.[20] The mixture was stirred and hexane was evaporated under vacuum. Enough silicon fluid was added so 5% of the trapping material by weight was silicon fluid.

Disposable capillary pipets 9" x 7.0-7.4 mm O.D. were filled with trapping material. During collection GLC traps were attached to the collector exit port and effluent was trapped. The entire trap was counted with the scintillation counter (Appendix VI). A diagram of a trap is shown below:



[20] Stauffer Wacker Silicone Corporation, Adrian, Michigan

Capillary traps

Glass capillary tubing (1.5 mm O.D.) was attached to the collector exit port of the GC when radioactive peaks were eluted. A section of the tubing was cooled with dry ice and a heat gun was used to drive condensed material into the cooled section of the capillary tube. The tubes were sealed and stored at -10°C.

APPENDIX XI

IR, NMR AND GC/MS OPERATING CONDITIONS

Infrared

Instrument: Beckman IR-18_A[21] with beam condenser.

Cell: 0.1 mm salt cell.

Solvent: CS_2

Nuclear Magnetic Resonance

Instrument: Varian HA 100[22]

Solvents: $CDCl_3$, or CCl_4

Internal standard: Benzene

Gas Chromatography/Mass Spectroscopy

Instrument: Finnigan 105C GC/MS[23]

Vacuum: 10^{-7} Torr

Ionization voltage: 70 ev.

Scan speed: 1 sec.

Column: 8 ft x 1/8 inch 5% OV-25 on 100/120 mesh chromosorb G.

Column temperature:
Peak A' - 130°C
Peak B' - 130°C
Peak C' - 140°C
Peak D' - 140°C
Peak E' - 150°C

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[21] Beckman Instruments, Inc., Palo Alto, California
[22] Varian, Walnut Creek, California
[23] Finnigan Instruments Corp., Sunnyvale, California