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**STUDIES ON EFFECTS OF DIET AND OTHER CONDITIONS
ON
CHOLESTEROL CATABOLISM IN RATS**

by

Jae Jin, Kim

Department of Biochemistry

**Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy**

**Faculty of Graduate Studies
The University of Western Ontario**

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CONTENTS

	Page
Acknowledgements -----	iv
List of Tables -----	viii
List of Figures -----	x
Abstract -----	xiii
INTRODUCTION -----	1
HISTORICAL REVIEW -----	5
1. Relation of Cholesterol to Bile Acids -----	5
2. Regulation of Cholesterol Metabolism by Cholesterol and Bile Acids -----	11
A. By Cholesterol -----	12
(i.) Effect of Dietary Cholesterol on Cholesterol Synthesis -----	12
(ii.) Effect of Dietary Cholesterol on Cholesterol Catabolism -----	17
B. By Bile Acids -----	20
(i.) Effect of Bile Acids on Cholesterol Catabolism -----	20
(ii.) Effect of Bile Acids on Cholesterol Synthesis -----	24
3. Involvement of Intestinal Microorganisms in Cholesterol and Bile Acid Metabolism -----	29
A. Transformation of Bile Acids -----	29
B. Transformation of Cholesterol -----	33

4. Effect of Dietary Factors on Cholesterol Metabolism -----	34
A. On Cholesterol Synthesis -----	35
B. On Cholesterol Catabolism -----	37
5. Other Factors Influencing Cholesterol Metabolism -	43
A. Suckling Rats -----	43
B. Fasting, Triton-Treatment and X-Irradiation -	44
PRELIMINARY STUDIES -----	47
1. Studies on Intestinal Bacteria in Relation to Cholesterol Metabolism -----	47
Experimental -----	48
Discussion -----	55
2. Studies on the Distribution of 4- ¹⁴ C-Cholesterol Administered Orally to Rats -----	57
Experimental -----	57
Results and Discussion -----	58
MAIN STUDIES: OXIDATIVE CATABOLISM OF CHOLESTEROL	65
Materials and Experimental Procedures -----	65
1. Materials -----	65
2. Experimental Procedures -----	67
Experimental Results -----	75
1. Studies on Effect of Diets -----	75
2. Studies on Suckling and Recently-Weaned Rats -	96
3. Studies on Fasting, X-Irradiation, Triton-Treatment -----	104

DISCUSSION -----	116
1. Validity of the Methods -----	116
2. Studies on Effect of Diets -----	128
3. Studies on Effect of Other Conditions -----	140
CONCLUSIONS -----	149
References -----	152
Appendix I (a) -----	183
Appendix I (b) -----	184
Appendix II (a) -----	185
Appendix II (b) -----	186
Vita -----	187

LIST OF TABLES

Table		Page
i	Oxidation of Cholesterol by Bacteria Isolated From Rat Feces Using A Respirometer -----	54
ii	Distribution of Radioactivity at Different Time Intervals After Feeding 4- ¹⁴ C-Cholesterol to Rats Fed on Commercial Diets -----	62
iii	Distribution of Radioactivity at Different Time Intervals After Feeding 4- ¹⁴ C-Cholesterol to Rats Fed on Semisynthetic Diets -----	63
I	Distribution of ¹⁴ C in Expired Air and Total Lipid Extracts of Tissues After Injection of 26- ¹⁴ C-Cholesterol in Rats Fed Different Diets -----	76
II	Quantitative Estimation of Conversion of Cholesterol to Bile Acid in Rats Fed Different Diets -----	91
III	Concentrations of Cholesterol in Plasma and Liver of Rats Fed Different Diets -----	92
IV	Distribution of 26- ¹⁴ C-Cholesterol to the Whole Intestine in Rats Fed Different Diets -----	95
V	Distribution of ¹⁴ C in Expired Air and Total Lipid Extracts of Tissues After Injection of 26- ¹⁴ C-Cholesterol in Rats of Different Ages -----	98
VI	Quantitative Estimation of Conversion of Cholesterol to Bile Acid in Rats of Different Ages -----	102
VII	Cholesterol Concentrations of Plasma and Liver of Rats of Different Ages -----	103
VIII	Distribution of 26- ¹⁴ C-Cholesterol to the Whole Intestine in Rats of Different Ages -----	105
IX	Distribution of ¹⁴ C in Expired Air and Total Lipid Extracts of Tissues after Injection of 26- ¹⁴ C-Cholesterol in Rats Under Different Treatments ---	107

Table		Page
X	Cholesterol Concentrations in Plasma and Liver in Rats Under Different Treatments -----	111
XI	Quantitative Estimation of Conversion of Cholesterol to Bile Acid in Rats Under Different Treatments ---	114
XII	Distribution of 26-¹⁴C-Cholesterol to the Whole Intestine in Rats Under Different Treatments -----	115
XIII	Effects of Diet, Age and Treatment on Distribution of Injected 26-¹⁴C-Cholesterol to Liver in Rats ----	119
XIV	Percentage of ¹⁴C in Esterified Cholesterol of Plasma After Injection of 26-¹⁴C-Cholesterol -----	125
XV	Percentage of ¹⁴C in Esterified Cholesterol of Liver After Injection of 26-¹⁴C-Cholesterol -----	135

LIST OF FIGURES

Figure		Page
1	Pathway of the Side Chain Degradation -----	10
A & B	Recovery of Radioactivity Following Oral	
C & D	Administration of 4- ¹⁴ C-Cholesterol -----	60
2	Schematic Diagram of Metabolism Cage -----	71
3	Levels of Radioactivity in Liver and Plasma Following Injection of 26- ¹⁴ C-Cholesterol Fat-Free Commercial Diet Fat-Free Semisynthetic Diet -----	78
4	Levels of Radioactivity in Liver and Plasma Following Injection of 26- ¹⁴ C-Cholesterol Commercial Diet with 15% Butter Semisynthetic Diet with 15% Butter -----	79
5	Levels of Radioactivity in Liver and Plasma Following Injection of 26- ¹⁴ C-Cholesterol Commercial Diet with 15% Corn Oil Semisynthetic Diet with 15% Corn Oil -----	80
6	Rate of ¹⁴ CO ₂ Production From Injected 26- ¹⁴ C-Cholesterol Fat-Free Commercial Diet High-CellufLOUR Diet Fat-Free Semisynthetic Diet -----	82
7	Rate of ¹⁴ CO ₂ Production from Injected 26- ¹⁴ C-Cholesterol Commercial Diet with 15% Butter Semisynthetic Diet with 15% Butter -----	83
8	Rate of ¹⁴ CO ₂ Production from Injected 26- ¹⁴ C-Cholesterol Commercial Diet with 15% Corn Oil Semisynthetic Diet with 15% Corn Oil -----	84
9	Rate of ¹⁴ CO ₂ Production from Injected 26- ¹⁴ C-Cholesterol for 48 Hours -----	86

Figure		Page
10	Comparison of Rate of $^{14}\text{CO}_2$ Expiration with Radioactivity Level of Liver -----	88
11	Calculation Method for Average Liver Level -----	89
12	Liver Levels of Radioactivity Following the Injection of 26- ^{14}C -Cholesterol Young Adult Rats Recently-Weaned Rats Suckling Rats -----	99
13	Rate of $^{14}\text{CO}_2$ Production from Injected 26- ^{14}C -Cholesterol Young Adult Rats Recently-Weaned Rats Suckling Rats -----	101
14	Liver Levels of Radioactivity Following the Injection of 26- ^{14}C -Cholesterol Control Rats Fasted Rats X-Irradiated and Fasted Rats Triton-Treated Rats -----	108
15	Plasma Levels of Radioactivity Following the Injection of 26- ^{14}C -Cholesterol Triton-Treated Rats Normal Rats -----	109
16	Rate of $^{14}\text{CO}_2$ Production from Injected 26- ^{14}C -Cholesterol Normal Rats Fasted Rats X-Irradiated and Fasted Rats Triton-Treated Rats -----	112
17 a	Acetate-1- ^{14}C Incorporation into Cholesterol by Liver Slices (3 Hour Incubation) -----	132
17 b	Conversion of Cholesterol to Bile Acids -----	132

Figure		Page
18	Free- and Esterified- ^{14}C -Cholesterol in Plasma and Liver Following Injection of $26\text{-}^{14}\text{C}$ -Choles- terol Fat-Free Commercial Diet Fat-Free Semisynthetic Diet -----	137
19	Free- and Esterified- ^{14}C -Cholesterol in Plasma and Liver Commercial Diet with 15% Butter Semisynthetic Diet with 15% Butter -----	138
20	Free- and Esterified- ^{14}C -Cholesterol in Plasma and Liver Commercial Diet with 15% Corn Oil Semisynthetic Diet with 15% Corn Oil -----	139

ABSTRACT

ABSTRACT

The rate of conversion of cholesterol to bile acids has been studied in rats fed on different diets which had been shown previously in our laboratory to affect liver cholesterol biosynthesis.

Subsequent to a single injection of 26-¹⁴C-cholesterol intravenously, the measurement of radioactivity in CO₂ expired by the rats and in the liver during the experimental period made it possible to calculate the total cholesterol transformed daily into bile acids. From the experience obtained from this study, a modified simpler method of quantitative estimation of bile acid formation after a single injection of 26-¹⁴C-cholesterol was proposed.

The results obtained from dietary experiments indicated that the rate of bile acid formation is greatly enhanced in rats fed on commercial diet compared to those fed on semisynthetic diets. This could explain the observation that liver cholesterol synthesis was higher in rats on commercial diets than in those on semisynthetic diets. Contrary to the marked effect of the two different basic diets, the addition of different fats or of cellulflour does not have a significant effect on the conversion of cholesterol to bile acids.

However, different fats did show marked effects on the excretion of plasma cholesterol through intestinal wall. In addition, feeding corn oil in either diet seems to increase the influx rate of plasma cholesterol into liver as compared with feeding no fat or butter. Determination of cholesterol concentration in liver suggests that the rate of appearance of cholesterol in the body is greater than the rate of the disappearance from the body in rats fed corn oil in either diet. This led to the question whether lowering effect of corn oil in serum cholesterol level has any significant measure in the treatment of atherosclerosis.

Some preliminary experiments were also carried out to investigate a possible utilization of cholesterol by any species of intestinal bacteria and effect of diets on this utilization and to investigate rate of absorption of 4-¹⁴C-cholesterol administered orally to rats depending on different diets. Intestinal bacteria isolated from feces of rats raised on laboratory chow or the semisynthetic diet containing butter or corn oil did not grow on media enriched only with cholesterol as a sole source of carbon and did not oxidize it for their energy source. The rate of absorption studied in rats fed ad libitum was not possible to be compared between groups fed different diets because of great variation in individual values.

The rate of conversion of cholesterol to bile acids was also studied in other conditions which have been shown to influence liver

cholesterol biosynthesis as measured by 1-¹⁴C-acetate incorporation into ¹⁴C-cholesterol.

In suckling rats the rate of bile acid formation is lower than in weaned or young adult rats. This is related to the lower rate of liver cholesterol synthesis. There seems to be simultaneous development of the transformation of cholesterol to bile acids with the secretion of synthesized cholesterol. Since two-thirds of the liver-plasma cholesterol pool in suckling rats is supplied by milk and since there was no significant effect of different fats on the conversion of cholesterol to bile acids it is suggested that hypercholesterolemia in suckling rats could be due to milk cholesterol.

Fasting, in which cholesterol biosynthesis in the body is decreased, also inhibited rate of bile acid formation. This makes it possible to maintain relatively constant cholesterol concentration in the fasted condition.

In X-irradiated-fasted rats, the rate of bile acid formation was almost the same as in fasted rats. Even though plasma cholesterol concentration was increased, the liver-plasma cholesterol pool was not increased compared with controls, since liver cholesterol concentration was slightly decreased. Based on several findings it was concluded that acetate incorporation into liver cholesterol cannot be representative of a true picture of cholesterol synthesis in X-irradiated-fasted rats. The decreased rate of bile acid formation in the presence of a normal rate of cholesterol synthesis may be a

contributor to an increased plasma cholesterol level in X-irradiated-fasted rats.

Triton-treatment brings a tremendous net increase in the cholesterol pool in the body but did not increase the rate of bile acid formation. Therefore, the rate of appearance of cholesterol is much greater than the rate of the disappearance from the body. The transfer of circulating cholesterol to liver and intestine does not increase proportionately to an increased plasma cholesterol concentration but remains relatively constant. It was also observed that the concentration of esterified cholesterol in plasma and liver was far smaller than that observed in controls. These results may be related to the effect of Triton on lipoprotein structure and to an anti-atherogenic effect of Triton in cholesterol-fed rabbits.

INTRODUCTION

INTRODUCTION

Cholesterol metabolism has been extensively studied since Anitschkow (4) observed that cholesterol feeding to rabbits resulted in a picture of atherosclerosis similar to that found in the human. In addition, since Schoenheimer (173) in 1926 proposed that cholesterol and cholesterol esters in human lesions accumulated through deposition from plasma, many investigators have paid most attention to the lowering of serum cholesterol level as a means for the prevention and treatment of atherosclerosis.

Much excitement came from Kinsell's finding (117) that polyunsaturated oils dramatically lower the serum cholesterol level but saturated fats do not. This stimulated many investigators to study the mechanism by which the oils exert their effect on serum cholesterol concentration and on the benefit of polyunsaturated oils for the treatment of atherosclerosis. None of these problems has yet been solved and they are still a subject of controversy.

A previous study in our laboratory showed that cholesterol synthesis by liver slices was greatly enhanced in rats fed on commercial diet but inhibited in those fed on semisynthetic diet. The addition of corn oil to either diet stimulated liver cholesterol synthesis more than

butter. These findings stimulated us to study the effect of these different diets and fats on cholesterol catabolism.

It has been suggested for a long time that changes in cholesterol metabolism caused by different diets may be mediated through quantitative or qualitative changes in intestinal bacteria. Most of the studies on the involvement of intestinal microflora in cholesterol metabolism have dealt with transformation of bile acids or cholesterol in germ-free rats or animals treated with antibiotics or chemotherapeutic agents. Since some bacteria are known to utilize cholesterol as a sole source of carbon, an attempt was made to investigate whether any species of intestinal bacteria is able to utilize cholesterol as a sole source of carbon and whether this is affected by different dietary treatment. Since the results obtained from this study were essentially negative, another aspect of the metabolism of cholesterol was investigated.

In this study, the purpose was to see whether the absorption rate of 4-¹⁴C-cholesterol administered orally to rats is different, depending on the different diets. Some of the cholesterol excreted through the intestinal lumen is transformed by intestinal bacteria into a form which is absorbed poorly or not at all from intestinal wall by which re-utilization is decreased or prevented. The intention was to carry out the experiments in rats fed ad libitum. This resulted in great variation and poor reproducibility of individual values which made it difficult to compare the rates of absorption in rats fed different diets.

A further attempt to explain the effect of different diets on liver cholesterol synthesis was made by studying the conversion of cholesterol to bile acids.

Cholesterol is excreted partly as sterols, in which the cholesterol skeleton of 27 carbon atoms is retained, and partly as bile acids, in which the terminal three carbon atoms of the side chain have been removed. In rats, the conversion of cholesterol to bile acids is much the more important of these two pathways, more than 80% of the circulating cholesterol eventually appearing in the bile as bile acids. Therefore, based on the present understanding of the conversion of cholesterol to bile acids in liver and degradation of neutral and acidic sterols to a multitude of steroidal secondary products within the intestinal lumen, the daily fecal excretion of bile acids represents a large proportion of the cholesterol synthesized daily in the body.

In addition, there is already experimental evidence of a direct correlation between rate of bile acid excretion and cholesterol biosynthesis in the body. Thus, an increased rate of removal of bile acids from the body by several means, leads to an increased rate of cholesterol synthesis in order to maintain a constant cholesterol concentration in the body. On the other hand, an increased intake of cholesterol in the diet by rats also increases the rate of bile acid excretion from the body. This evidence prompted us to carry out experiments to measure the rate of bile acid formation in order to clarify the effects of different diets on liver cholesterol biosynthesis.

Several different methods for estimating rate of the conversion of cholesterol bile acids have been applied to rats by many investigators. However, the size of the rat and the complexity of the mixture of fecal bile acids make it difficult to achieve the complete measurement. This led us to adopt a method which is based on the measurement of radioactivity in respiratory CO₂ after the intravenous injection of cholesterol labeled with ¹⁴C at C-26.

Since dietary experiments in this study showed a positive correlation between liver cholesterol synthesis and bile acid formation, we further investigated the rate of conversion of cholesterol to bile acid formation in suckling and fasted rats, in which acetate incorporation into liver cholesterol is shown to be inhibited and in X-irradiated and Triton-treated rats, in which the incorporation is known to be stimulated.

HISTORICAL REVIEW

HISTORICAL REVIEW

1. Relation of Cholesterol to Bile Acids

It has been known for many years that cholesterol can be synthesized and also degraded in the organism. This was shown by balance methods in experimental animals by several workers (50, 155, 174). Since the fundamental structure of bile acids had long been known to be similar to that of cholesterol, a biological transformation of cholesterol into bile acids seemed probable and had often been discussed (174). In 1942, Bloch et al (22) demonstrated that acetate is utilized for the synthesis of cholesterol (21).

Bloch, Berg and Rittenberg (20) first demonstrated in 1943 that bile acids are end products of cholesterol metabolism in the body. They injected deuterium-labeled cholesterol into a dog and recovered a high content of deuterium in cholic acid, thus providing evidence for the conversion of cholesterol to cholic acid. The same transformation was shown to occur in the rat by Byers and Brigg in 1952 (30), employing cholesterol labeled with tritium. This was also confirmed by Bergstrom (14) using ^{14}C -labeled cholesterol in rats.

In 1952, the quantitative importance of the pathway of cholesterol metabolism in the rat was established by Siperstein et al (194) and

Bergstrom (14), and Bergstrom and Norman (17). Using 4- ^{14}C -cholesterol and 26- ^{14}C -cholesterol, Chaikoff et al (37) found that none of the 4th carbon of cholesterol was converted to CO_2 whereas the 26th carbon was rapidly oxidized and excreted as respiratory CO_2 . This finding led to the view that the sterol ring system is not broken down to fragments that can be oxidized (45). Further studies by Siperstein and Chaikoff (180) and by Siperstein et al (184) presented evidence that cholesterol is excreted mainly in the bile as bile acids and that bile acids are finally eliminated from the body in the feces. Subsequent to the intravenous injection of 4- ^{14}C -cholesterol, as much as 95% of the dose injected could be recovered in the feces during 15 days. Of this recovered activity, 10% of the original amount was found in the unsaponifiable fraction and 85% in the saponifiable fraction, which was later identified by the above authors as bile acids. Furthermore, it was shown that most of the ^{14}C recovered in the feces came via bile since in bile-cannulated rats, the radioactivity recovered in the feces amounted to only one-tenth of that observed in the intact rat. This conclusion was supported by Bergstrom (14) who demonstrated that cholic acid, the chief bile acid excreted in rat bile, accounted for most of the radioactivity found in the bile after intraperitoneal injection of 4- ^{14}C -cholesterol. Bergstrom (14) and Bergstrom and Norman (17) showed that after intraperitoneal administration of 4- ^{14}C -cholesterol to rats, the main excretory products in bile were cholic acid and chenodeoxycholic acid,

present as taurine conjugates. Friedman et al (74) showed that a marked increase in biliary excretion of cholic acid occurred during 72 hours following the injection of excess cholesterol in the form of hypercholesterolemic serum. Fully 60% of the injected cholesterol could be accounted for in this way, appearing as excess cholate in the bile.

The degradation of cholesterol was shown to be a process occurring mainly in the liver. Thus, Robbins et al (164) demonstrated that even the isolated perfused liver of the rat maintained its ability to degrade 4-¹⁴C-cholesterol to cholic acid. This finding was confirmed by Harold et al (95) who studied the fate of the 4-¹⁴C- and 26-¹⁴C-cholesterol in the perfused rat liver. They found that ¹⁴C of the ring labeled cholesterol was excreted almost exclusively via bile, with about 80% of the ¹⁴C being found in the bile acid fraction, the 26-¹⁴C-cholesterol was readily oxidized to ¹⁴CO₂. Thus, it was concluded by the above authors that the similarity of the results obtained in studies with perfused rat liver to those in the intact rat indicate that cholesterol degradation may be regarded, qualitatively at least, as a result of a process occurring in the liver. Using different types of solutions of cholesterol, Danielson et al (54) also found that the main acid product in the bile in the isolated perfused liver of a rat was taurocholic acid and the proportion of taurocholic to taurochenodeoxycholic acid in the bile was approximately the same as that found in the intact rat, viz 8:2.

It has been shown that the last reaction step in the conversion of

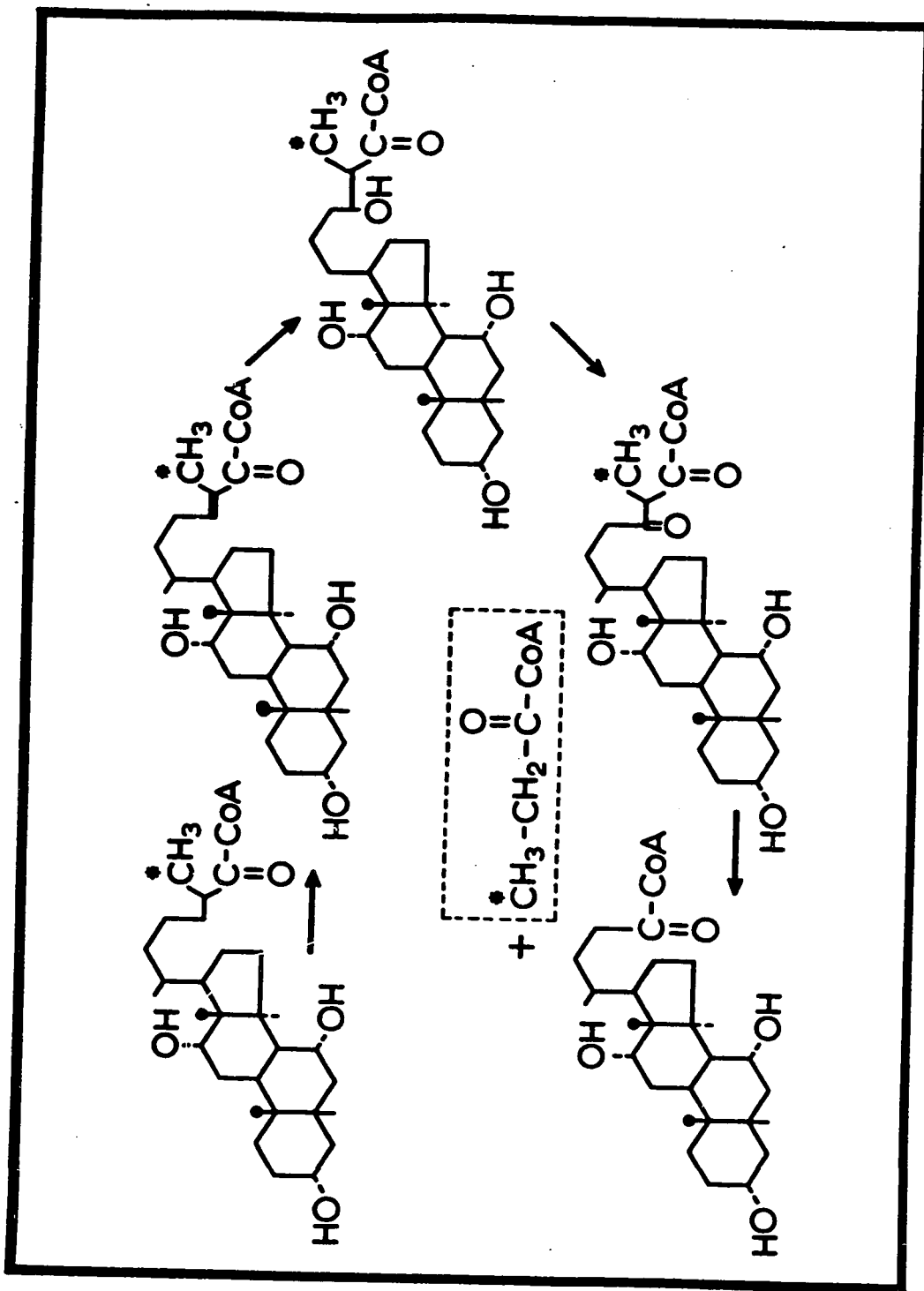
cholesterol to bile acids involves the removal of part of the sterol side chain (51). Chaikoff et al (37) and Meier et al (137) first showed that the 26 carbon atom from 26- ^{14}C -cholesterol is eliminated as CO_2 both in intact animals and by liver slices of rats. Zabin and Baker (211) later proved that cholesterol is converted into cholic acid by removal of no more than three carbon atoms, C-25, C-26, and C-27 and this was confirmed by Staple and Gurin (189). Anfinsen and Horning (3) described the enzymic oxidation of the 26- ^{14}C -cholesterol to $^{14}\text{CO}_2$ with a washed mitochondrial system of mouse liver fortified with microsome-free supernatant. These findings were later confirmed by Whitehouse et al (202) who showed that rat liver mitochondria in the presence of soluble factor also catalyzed the oxidation of the terminal methyl groups of cholesterol to $^{14}\text{CO}_2$ and that cholesterol oxidase activity was restricted solely to liver among several tissues studies.

Later, in 1962, Suld et al (191) isolated ^{14}C -propionic acid after incubating rat liver mitochondria with trihydroxy-26, 27- ^{14}C -coprostane and proposed a mechanism of side chain cleavage involving the formation of a CoA derivative, followed by splitting off of propionyl-CoA. Confirmatory evidence was presented by Mitropoulos and Myant (144) who showed the production of propionyl-CoA as the immediate product of cleavage of the side chain from 26- ^{14}C -cholesterol incubated with rat liver mitochondria. Dean and Whitehouse (56) recently demonstrated that liver mitochondria can rapidly degrade 3β -hydroxy-cholest-5-en-3-en-26-oic acid, one of the possible intermediates of bile acid formation,

to propionic acid or propionyl-CoA, whereas mitochondrial preparation from extrahepatic tissues cannot. They suggested that this must be one of the factors effectively restricting cholesterol degradation by loss of the terminal isopropyl group to liver tissue. The proposed scheme of the mechanism of the oxidative degradation of the terminal side chain is shown in Figure 1.

Production of $^{14}\text{CO}_2$ from 26- ^{14}C -cholesterol by intact animals has been used as a measure of the transformation of cholesterol to bile acids in the liver. Wostman et al (210) used 26- ^{14}C -cholesterol for the study of cholesterol catabolism in germ-free and conventional rats and demonstrated that after 72 hours in both groups, at least 75% of the ^{14}C not in the form of 3β -OH sterol can be accounted for as expired $^{14}\text{CO}_2$. Based on this, the amount of $^{14}\text{CO}_2$ expired by the rat after intravenous injection of 26- ^{14}C -cholesterol is a valid indicator of oxidative catabolism of cholesterol.

Myant and Lewis (149) estimated the rate of conversion of cholesterol to bile acid in man by measuring the rate at which $^{14}\text{CO}_2$ is excreted in the breath after intravenous injection of 26- ^{14}C -cholesterol. They evaluated the method by measuring the time course and completeness of excretion of $^{14}\text{CO}_2$ in the breath after the injection of ^{14}C -propionate, and by comparing the rate of bile acid formation estimated by the $^{14}\text{CO}_2$ method with that estimated from excretion of radioactive fecal bile acids after the injection of 4- ^{14}C -cholesterol. They indicated



PATHWAY OF THE SIDE CHAIN DEGRADATION

Figure 1.

that although the $^{14}\text{CO}_2$ method may underestimate the true rate of cholesterol degradation, it has an advantage over other methods for certain types of investigations, such as detecting rapid changes in the rate of cholesterol breakdown brought about by changing diet or by giving drugs or hormones.

On the other hand, Chevallier and Lutton (41) calculated the daily transformation of cholesterol into bile acids by measuring the daily CO_2 radioactivity in rats in a state of isotope equilibrium. They obtained a much higher value than those obtained by other methods based on isotopic dilution of radioactive cholic acid or measurement of the radioactivity in fecal bile acid, and concluded that this was a more physiological method of determining the quantity of cholesterol transformed into bile acids.

2. Regulation of Cholesterol Metabolism by Cholesterol and Bile Acids

The balance studies of cholesterol by Page and Menschick in rabbits (155) and cats (140) and by Schoenheimer and Breusch in mice (174) demonstrated that all these species are capable of destroying cholesterol when it is fed in large amounts. This conclusion was based on the finding that digitonin precipitable sterols disappeared from the system composed of animals, their food and their excreta. Since it is well established that cholesterol in all the tissues of the body except the central nervous system is in a state of dynamic equilibrium, i. e., is being continuously absorbed from the diet and synthesized in the body

on one hand, and metabolized to other compounds and excreted on the other hand, the apparent destruction observed in balance experiments could be due to either an actual increase in the rate of conversion to metabolic products or to a decrease in the rate of synthesis or both (82).

Despite the variation in dietary cholesterol, most animals are capable of maintaining their plasma cholesterol at a relatively constant level (183). Particularly in rats, the level of plasma cholesterol is not greatly influenced by the feeding of a diet rich in cholesterol. Therefore, it was suggested that the lack of a pronounced response of plasma cholesterol to exogenous cholesterol could result from the homeostatic regulation of cholesterol metabolism in the body.

A. By Cholesterol

(1) Effect of Dietary Cholesterol on Cholesterol Synthesis:

Insight into the means by which this homeostasis of plasma cholesterol is accomplished was first provided by Gould and associates (82, 88, 89) who demonstrated that the feeding of high cholesterol diets to rabbits and dogs resulted in a marked depression of hepatic cholesterol synthesis both in vitro by liver slices and in vivo in intact animals but had little effect on the in vitro synthesis by skin slices or intestinal mucosa.

Since this observation, only the question of hepatic cholesterol synthesis to exogenous cholesterol has been studied in great detail, in

different experimental animals such as rats (72, 196), monkeys (48) and chickens (169). The mechanism by which exogenous cholesterol is able to suppress cholesterol synthesis in liver and the biochemical site of this feedback reaction has been studied in many laboratories.

Gould and Popjak (87) in 1957 showed that the conversion of mevalonic acid into cholesterol in rat liver homogenates was not suppressed by cholesterol feeding although it is well known that cholesterol synthesis from ^{14}C -acetate is much suppressed in liver slices from cholesterol-fed animals. This provides evidence that the reaction step prior to mevalonic acid on the pathway of cholesterol synthesis is rate limiting. This was supported by the studies of Bucher et al (28, 30). Subsequently, the evidence that the specific location of the negative feedback regulation of cholesterol synthesis is at the conversion of β -hydroxy β -methyl-glutaryl CoA to mevalonic acid was indicated by Siperstein and Guest (182, 183), who demonstrated that the conversion of ^{14}C -labeled squalene to cholesterol was relatively unaffected and also the synthesis of squalene from mevalonic acid was not inhibited by cholesterol feeding. By using a gas-liquid chromatographic method for the separation and isolation of mevalonate and β -HMG, Siperstein and Fagan (182) confirmed that β -hydroxy β -methyl-glutaryl reductase is under very sensitive negative feedback control. Because of these results in vitro and in vivo, it has been inferred that cholesterol synthesis in the liver of intact animals is under homeostatic control.

Although it was established as early as 1950 that cholesterol is synthesized in virtually every tissue of the body (187), most attention has been given to the liver since it was suggested that only hepatic cholesterologenesis is under the homeostatic control of dietary cholesterol and that the liver is the main endogenous site or origin of serum cholesterol. Gould et al (85) demonstrated that in hepatectomized dogs very little isotopic cholesterol appeared in plasma four hours after ^{14}C -acetate administration compared to normal dogs. The finding that in functionally hepatectomized rats, the turnover of injected plasma ^{14}C -cholesterol practically stopped, led Hotta and Chaikoff (105) to the conclusion that the liver is an important donor of newly synthesized cholesterol to plasma. The indirect method for estimating relative contributions of dietary and endogenously synthesized cholesterol to plasma cholesterol showed that this was true as far as the experimental animals such as rats or dogs are concerned. Morris et al (146) found by feeding rats a diet containing 2% ^{14}C -cholesterol for a period of four to six weeks that synthesis contributed not more than 15 to 25% to the composition of serum cholesterol. On the other hand, 80% of serum cholesterol was of synthetic origin in rats that had been fed a diet containing 0.05% cholesterol. Comparable results were reported by Taylor et al (193) in dogs, demonstrating nearly complete replacement (90%) of the endogenous sources of plasma cholesterol by ring-labeled dietary cholesterol in dogs fed

4- ^{14}C -cholesterol. Therefore, the above authors concluded that the indirect studies were in good agreement with earlier studies demonstrating the dominant homeostatic role played by the liver in cholesterol metabolism of the rat and the dog and the liver appears to be the most important endogenous source of plasma cholesterol. However, the results obtained with the indirect method, which was based on the augmentation of ratio of specific radioactivity of plasma cholesterol to that of diet cholesterol with a high cholesterol diet, does not permit one to conclude that cholesterol synthesis is completely depressed by exogenous cholesterol in the intact animal (38). In this connection, Morris et al (146) pointed out that in the intact rat it was not possible to suppress completely the appearance of newly synthesized plasma cholesterol by feeding a 2% cholesterol diet which was known to lead to complete inhibition of biosynthesis of cholesterol from acetate- ^{14}C . Recently, Lindsey and Wilson (135) questioned the possible role of extrahepatic tissues in contributing synthesized cholesterol to blood and suggested that cholesterol synthesized in the intestine could contribute to plasma cholesterol, although the quantitative significance is not established. Further studies on the squirrel monkey by Wilson (206) demonstrated that cholesterol synthesized in the intestine does contribute to the circulating pool and that little endogenously synthesized cholesterol reaches the serum of the cholesterol-fed monkey when the intestine contribution is diverted from the circulation. Therefore, it was concluded

that the intestine is a biosynthetic site of serum cholesterol in this species and may represent a significant source of endogenous cholesterol in cholesterol-fed animals.

In contrast to animal species, the physiological significance of such a control mechanism in human beings has been questioned. Davis et al (55) in studies of the acetate uptake in humans fed high cholesterol diets showed no such striking suppression of acetate uptake as observed in the experimental animals (rats or dogs). Therefore, they questioned whether the liver in man is a minor source of endogenous synthesis or whether it may be an important source of endogenous synthesis of plasma cholesterol that is not suppressed by dietary cholesterol (49). Although Siperstein and Fagan (179) demonstrated that cholesterol synthesis by liver slices obtained from biopsies of human liver was suppressed by cholesterol feeding Taylor et al (193), using an isotopic equilibrium method in human subjects, showed that specific radioactivity of serum cholesterol never exceeded 40% of that of those fed radioactive cholesterol. The essential absence of a feedback mechanism in man for compensation for dietary cholesterol could be due to the fact that extrahepatic tissues of man are quantitatively more important as biosynthetic sites for circulating cholesterol than is the case in lower animals. In this regard, an interesting finding was reported by Chevallier (38), who used an isotopic balance method in which 4-¹⁴C-cholesterol was fed to rats for one month. He

found that the internal secretion of cholesterol in the intact animal was not suppressed by a high exogenous dietary cholesterol, suggesting that the role of the liver in the internal secretion of cholesterol may not be as large as has been hitherto believed. This supports the contribution of intestine which is evident as suggested by Lindsey and Wilson (135). Then he concluded that there is an absence of in vivo feedback regulation of cholesterol synthesis by exogenous cholesterol in rats as observed in humans by Taylor et al (193).

(ii) Effect of Dietary Cholesterol on Cholesterol Catabolism

While a good deal of information is available concerning the effect of dietary cholesterol on cholesterol synthesis, few data appear to have been published concerning the effects of cholesterol feeding on catabolism of cholesterol (51). It is well known that cholesterol is eliminated from the body almost entirely by excretion into the feces via two major pathways: one involves the degradation of cholesterol to bile acids and the second involves the excretion of cholesterol itself and neutral transformation products of cholesterol, notably coprostanol (203). As mentioned previously, the earlier balance experiments showed that a considerable amount of the cholesterol ingested could not be accounted for by sterol excreted in the feces or by that stored in the animals (155, 174). These findings were confirmed by Cook and his associates (46, 47) who also found that rats fed a diet containing 2% cholesterol with fat excreted an increased amount of acidic products

and that this increase was approximately equal in amount to the cholesterol unaccounted for in the balance studies.

Using 4-¹⁴C-cholesterol, Wilson (210) recently studied the regulatory role of dietary cholesterol in determining the pathway of cholesterol excretion in the normal rat, and demonstrated that the quantity of absorbed cholesterol plays a crucial role in determining both the relative and absolute importance of the two major routes of cholesterol excretion in rats. The addition of cholesterol to the diet of the rat under circumstances where significant cholesterol absorption is promoted by the addition of fatty acids to the diet results in an acceleration of the bile acid pathway, as measured indirectly both by the conversion of parenterally administered 4-¹⁴C-cholesterol to fecal bile acids and the degradation rate of cholesterol as determined by balance data. Furthermore, this effect was observed by the above author even in the presence of conditions which are known to result in a diminished rate of bile acid formation, such as the suppression of the bowel flora (94) and the administration of large amounts of exogenous bile acids (16). Therefore, Wilson (204) suggested that the magnitude of bile acid excretion in the rat is linked to the amount of cholesterol absorbed which in turn influences the amount of cholesterol available for catabolism. Applying the isotopic "steady state" technique (which has been attained by subcutaneous implantation of a capsule containing 4-¹⁴C-cholesterol) to the intact rats, Wilson (204) later clearly showed

that in animals fed 0.5% cholesterol in a synthetic diet, excretion of labeled neutral sterol did not increase but bile acid excretion rose from an average value of 3.5 mg. per day (cholesterol-free diet) to 9.8 mg. per day. When balance techniques were combined with isotopic measurement in the steady state, Wilson was able to demonstrate directly that bile acid excretion was sufficient to account for the entire positive balance of cholesterol, once equilibrium had again been attained. Therefore, it was suggested that acceleration of bile acid production in rats is the major compensatory response to cholesterol feeding. This response may explain the relative stability of rat blood cholesterol level despite the absorption of large amounts of dietary cholesterol. Chevallier (40) used the isotopic state method and also observed that in rats fed a synthetic diet containing increasing amounts of cholesterol from 0.015% to 2%, the internal secretion rate did not vary but the amount of bile acid formation was increased (7 fold in the 2% cholesterol diet). He also showed that the plasma cholesterol level was proportional to the sum of absorption and internal secretion and that the transformation rate of cholesterol to bile acids was proportional to the plasma cholesterol concentration. He therefore concluded that the major factor in regulation appeared to be in an increase in bile acid formation. This result is actually in agreement with those obtained by Wilson (205).

Therefore, the original question which arose from the earlier balance method whether the prime regulatory mechanism of cholesterol

metabolism is accomplished by way of depression of hepatic cholesterol synthesis or by increased bile formation or both still remains to be elucidated in both humans and experimental animals.

B. By Bile Acids

(i) Effect of Bile Acids on Cholesterol Catabolism:

It has been known for a long time that bile salts discharged into the intestinal lumen in the bile are very rapidly and efficiently absorbed from the intestine and transported via the portal vein to the liver and reutilized. This has been called the enterohepatic circulation of bile acids (51). It was suggested early in 1938 by Sobotka and endorsed by Josephson (111) that "equilibrium is maintained by synthesis of bile acids paralleling the rate of fecal loss." There is the concept that rate of bile acid formation is homeostatically regulated by bile acids that reach the liver via portal circulation. Experimental evidence for this concept was first provided by Bergstrom and Danielsson (16) in 1958 who demonstrated that the infusion of taurochenodeoxycholic acid into the duodenum of bile fistula rats depressed the rate of endogenous formation of taurocholic acid from about 30 mg. per day to 1-2 mg. per day, which is the normal range. On the other hand, the interruption of the enterohepatic circulation of bile salts was shown to cause an increase of bile acids synthesis. Eriksson (67) calculated that there was a 20 to 30-fold increase of normal bile acid production in bile fistula rats. The increased rate of bile acid excretion was also demonstrated in the perfused liver of bile duct cannulated rats

by Kay and Entenman (112) who suggested that this observed increase was due to an enhanced capacity of the liver to synthesize the bile acids.

In in vitro experiments by Whitehouse and Staple (201) it was shown that the addition of cholic acid conjugates (tauro- and glycocholic acids) depressed the oxidation of cholesterol but not that of sodium pyruvate or octanoate or 3α , 7α , 12α -trihydroxycoprostanone by fortified mitochondrial preparations. Cholesterol oxidation was selectively inhibited by cholanic acid conjugates and the degree of the inhibition increased as the number of oxygen functions on the steroid nucleus was decreased (124). It was shown that preparations from livers of bile fistula rats oxidized cholesterol to a greater extent than did controls (201). The intraduodenal infusion of taurochenodeoxycholic acid was found to inhibit strongly hepatic oxidation of cholesterol- U - H^3 to radioactive bile acids (123).

Recently the localization of the site of feedback regulation of bile acids synthesis from cholesterol by bile acids themselves has been studied in many laboratories. Originally Bergstrom in 1955(15) postulated the hypothesis that the rate-determining step in the conversion of cholesterol to bile acids formation was probably the 7α -hydroxylation reaction. It is this step which is sensitive to the conjugated bile acids in the enterohepatic circulation and which is under unique control by a feedback mechanism. Danielsson and co-workers (52) recently reported that in homogenates of liver taken from bile fistula rats 48 hours after the operation, the extent of 7α -hydroxylation of cholesterol

was about eight times greater than in homogenates of livers from control rats. The other reactions studied, the conversion of cholest-5-ene-3 β , 7 α -diol to 7 α -hydroxy cholest-4-en -3-one; the 12 α -hydroxylation of 7 α -hydroxy-cholest-4-en -3-one, and the 7 α -hydroxylation of taurodeoxycholic acid, were influenced much less by biliary drainage. They suggested that the hydroxylation of cholesterol to 7 α -hydroxy-cholesterol might be a rate determining step in the conversion of cholesterol to bile acids.

Similar observations were made by Boyd and associates (26) who reported that the activity of the microsomal enzyme system for 7 α -hydroxylation of cholesterol was greatly enhanced by subjecting rats to interruption of the enterohepatic circulation of bile salts either by biliary drainage, or by feeding resins such as cholestyramine. It was also shown by Schefer et al (177) that the microsomal 7 α -hydroxylase was inhibited 50% to 84% by conjugated di- and monohydroxy-5 β -cholenoic acids. The degree of inhibition produced by taurocholate was significant only at high taurocholate concentration. However, in vivo taurocholate produced an 80% to 90% inhibition of endogenous bile acid formation when administered at a rate of 33 mg. per kg. rat per hour in bile fistula rats.

In contrast to the above observations which appear to suggest a single site for feedback control of cholesterol catabolism by bile acids, multiple sites of control were recently proposed by Dean and

Whitehouse (57) who presented evidence that conjugated bile acids inhibited at least three steps: (1) transformation of cholesterol to trihydroxycoprostone, (2) oxidation of 26-hydroxycholesterol to the corresponding C₂₅-carboxylic acid, and (3) catabolism of 3 α -hydroxychol-5-en-26-oic to 3 α -hydroxychol-5-en-24-oic acid and propionate, in the overall sequence of sterol oxidation and bile acid formation, at least in vitro.

The significance of results in relation to the effects of bile salts on cholesterol oxidation in vitro may be difficult to evaluate (58) since, among different bile salts, taurodeoxycholate is not only the most potent inhibitor of cholesterol oxidation in vitro but also is most effective in uncoupling oxidative phosphorylation. This correlation could be explained by the hypothesis that bile salts diminish the supply of ATP which provides the driving energy for cholesterol catabolism by uncoupling oxidative phosphorylation or by activating ATPase in mitochondria (126).

In addition, both conjugated and unconjugated bile salts are effective detergents capable of disrupting the lipoprotein structure of biological membrane (58). Pope, Parkinson and Olson (158) found that commercial conjugated bile salts commonly contain contaminants which themselves affect the rate of many metabolic processes; thus, they pointed out that many rate-limiting controlling phenomena ascribed to conjugated bile salts may have to be re-evaluated with highly purified preparations.

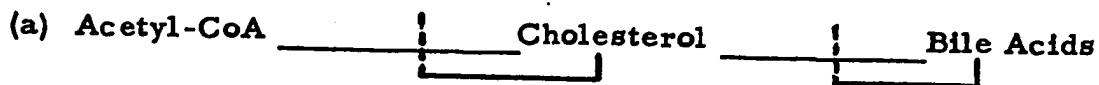
(ii) Effect of Bile Acids on Cholesterol Synthesis:

If the conversion of cholesterol to bile acids is regulated by a feedback mechanism, the accumulation of bile acids in the body would result in a decreased catabolism and an increased level of cholesterol in liver and blood. This in turn may inhibit the biosynthesis of cholesterol from acetate (103). Experimental evidence for such a regulatory mechanism has been provided by numerous investigators.

When bile acids are fed to animals together with cholesterol in the diet, higher levels of cholesterol are found in the blood and liver than when cholesterol is given alone. This was first demonstrated by Schonheimer and his pupils (172) and was later confirmed by numerous authors in experiments on different animal species (157). The effect of bile acids on deposition of cholesterol in the liver was attributed to a stimulatory effect of bile acids on cholesterol absorption by Schonheimer (172) and also subsequently by many other authors (139, 192, 167). In a balance experiment, Kim and Ivy (116) in 1952 found no stimulatory effect of deoxycholate on cholesterol absorption in rats. The possibility that bile acid ingestion might affect the processes of synthesis and degradation of cholesterol was actually suggested early in 1929 by Hummel (107) who observed that feeding different bile acids to normal mice led to a definite increase in the hepatic cholesterol content even in the absence of dietary cholesterol.

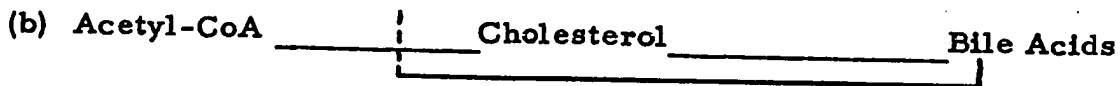
Furthermore, when mice with cholesterol deposited in the liver were fed certain bile acids on a cholesterol-free diet, the normal disappearance of cholesterol was prevented. In view of these facts, Pihl (157) carried out experiments for studying the effect of bile acids on absorption, deposition and synthesis of cholesterol in rats. He observed that dietary bile acids markedly increased the deposition of cholesterol when the absorption of cholesterol was not increased and when in liver slices of rats fed cholic acid without cholesterol, the incorporation of acetate into cholesterol was lower than in control rats. Therefore, he concluded that the increased deposition of cholesterol in rats fed bile acids could only be explained by a reduced catabolism of cholesterol in the liver. Beher and associates (9, 10, 11) also studied the effect of bile acid feeding on cholesterol metabolism in intact mice and found that cholic acid and its conjugates inhibited cholesterol synthesis and reduced the amount of labeled cholesterol converted to bile acid. Portman (161) indicated that in order to influence cholesterol synthesis and bile acid formation, a bile acid must meet certain structural requirements. Thus, in contrast to cholic acid, trifor-moxycholic acid had no effect on cholesterol synthesis in the intact rat. Further work of Beher and collaborators (12, 13) indicated the existence of a double feedback mechanism controlling bile acid formation. This is based on the observations that cholic acid brings about equal decreases in the rate of both hepatic cholesterol and bile

acid biosynthesis and inhibits bile acid synthesis in mouse liver independently of its effect on cholesterol synthesis.



Thus, in this set of reactions bile acids, the major end products of cholesterol metabolism, which are present in the enterohepatic circulation, reduce the rate of their own synthesis causing an increase in hepatic cholesterol level which in turn inhibits hepatic cholesterol synthesis. This formulation of the mechanisms operating in regulation of bile acid formation is in accord with the observations of Siperstein and Guest (183) who found that the direct addition of taurocholic acid to liver slices of rats had no effect on cholesterol synthesis.

On the other hand, Myant and Eder (148) have obtained a result that indicates the possible existence of an alternative mechanism for this feedback system in which bile acids might act primarily on the rate of cholesterol synthesis as expressed schematically below:



In this scheme, the bile acids directly reduce the rate of cholesterol synthesis from acetyl CoA, thus reducing the amount of substrate available for conversion to bile acids.

The above authors examined the synthesis of cholesterol in vitro in liver from bile fistula rats after varying periods of bile drainage and found increased cholesterol synthesis from acetate but not from mevalonate. They also found that the rise in cholesterol synthesis begins before the rise of bile acid excretion. Huff et al (106) showed that administration of cholestyramine, a bile acid binding polymer (194), to rats on a normal diet produced a rise in fecal bile acid excretion which was accompanied by an increase in hepatic de novo cholesterol synthesis. It was shown that removal of bile acids by bile fistula or use of a binding agent resulted in acceleration of cholesterol biosynthesis in rats although concentration of cholesterol in the liver remains constant (148, 106).

The view that removal of bile acid feedback inhibition takes precedence over simple cholesterol feedback inhibition was further suggested by Dupont et al (64) who carried out experiments to see whether acetate incorporation into cholesterol and half-life of the sterol nucleus are correlated in rats. She found that even in rats treated with triiodothyropropionic acid, an inhibitor of cholesterol synthesis, cholestyramine caused an increase in acetate incorporation into cholesterol as well as an increase in fecal bile acid excretion. Therefore, she concluded that control of cholesterol metabolism is related to turnover rate, rather than biosynthesis only.

Recently, Figmonari and Rodwell (69) demonstrated that certain bile salts, but not Triton X-100, inhibit the conversion of β -hydroxy- β -methyl glutaryl CoA into mevalonic acid, suggesting that bile salts thus serve as end product inhibitors of this early reaction. This was further supported by Miller and Gaylor (143) who studied the effect of bile salts on the terminal reaction step of cholesterol synthesis in vitro. They demonstrated that rather high concentrations of bile salts were required to obtain significant inhibition of lanosterol demethylation by microsomal enzyme. Thus, they ascribed the inhibitory effect to an enhanced rate of irreversible time dependent denaturation of the microsomal enzyme.

The concept that it is bile salts per se which regulate intracellular sterol synthesis was further supported by Dietschy and Siperstein (60) and Dietschy (59) in studies of the effects of bile salts on intestinal cholesterologenesis. Early investigation by Gould and associates in 1953 (89) demonstrated in dogs that the feeding of cholesterol diet suppressed hepatic but not intestinal cholesterologenesis. This finding was confirmed by many other workers in various experimental animals (48, 59, 60, 61).

In striking contrast to the apparent insensitivity of the intestinal sterol synthetic pathway to dietary cholesterol, the presence or absence of bile within the gut lumen was shown to have a marked influence on the rate of cholesterologenesis by small bowel (58, 59).

Dietschy (58) demonstrated that biliary diversion either by bile fistula, bile duct ligation or by cholestyramine feeding resulted in a marked enhancement of the rate of acetate incorporation into sterol by intestinal slices taken from every level of the small bowel, without affecting the rate of acetate incorporation into fatty acids or CO_2 whereas the infusion of whole rat bile or taurocholate caused a pronounced suppression in sterol synthesis. The results obtained from this kind of experiment deserve some comments. Wilson and Reinke (209) recently demonstrated that in the only lymph-cannulated rat most of ^{14}C -cholesterol synthesized by intestine was in the intestinal wall or intestinal lymph whereas in the lymph-cannulated rat with bile fistula most of them were in the intestinal lumen and only small portions were found in the intestinal wall, suggesting that bile acid is required for the transport of cholesterol synthesized by the wall into the lymph. Therefore, it is not clear whether the magnitude of contribution of the synthesized cholesterol by intestinal wall in the bile fistula rat to the body pool is increased or not.

3. Involvement of Intestinal Micro-Organisms in Cholesterol and Bile Acid Metabolism

A. Transformation of Bile Acids

The bile acids excreted in the feces are known to be transformed by micro-organisms in the intestinal tract into a complex mixture of metabolites.

Early in 1942, Schmidt et al, in a series of papers (104, 175, 176) reported that cholic acid injected intravenously into guinea pigs disappeared from the body and was not eliminated as such in excreta. They suggested that the disappearance of this bile acid was due to the action of bacteria, particularly Alcaligenes fecalis. They also found that cholic acid administered per os was quantitatively recovered in the feces as keto acids.

Bergstrom and Norman (17) found that after administration of 4-¹⁴C-cholesterol intraperitoneally into rats, most of the conjugates in the feces had been split and that no unchanged cholic acids seemed to be left in the feces, suggesting the transformation of bile acids in the intestine by intestinal microflora.

Lindstedt and Norman (131) gave labeled cholic acid and chenodeoxycholic acid intraperitoneally to rats and recovered all of the radioactivity in the feces within 10 days, mainly in the form of unidentified transformation products which were different from the bile acids injected. Other investigators also administered labeled cholic acid and observed a complicated pattern of excretion (17, 129, 153).

The microbial transformation seems to begin in the cecum and includes hydrolysis of the conjugates, dehydroxylation at C-7 and oxidation of hydroxyl groups at C-3, C-7 and C-12 to keto groups which can be reduced to both α - and β -hydroxy groups. Portman (160) studied the chromatographic distribution of radioactivity in

hydrolyzed samples of portal sera, bile and gastro-intestinal contents of rats injected previously with cholic acid-24- ^{14}C and showed that the modification of bile salts in the G-I tract seemed to take place mainly in the cecum, since there was very little alteration of the steroid portion of the conjugated bile salts proximal to the cecum. Norman and Sjoval (152) also demonstrated that in the bile and in the small intestinal contents, the bile acids occurred in a conjugated form, whereas in the large intestine free bile acids predominated. This suggests that the first reaction is the splitting of the amide bond and this splitting was demonstrated by several workers to be due to the action of microorganisms. Conjugated bile acids are almost entirely split by microbial enzymes prior to their excretion in feces (93). The hydrolysis of taurine and glycine conjugates can be performed in vitro by several species of Clostridia and Enterococcus (151). It has been shown that in the rabbit and in man, deoxycholic acid is formed from cholic acid during enterohepatic circulation probably through the action of intestinal organisms. Lindstedt and Sjoval (134) administered cholic acid -24- ^{14}C intraperitoneally to a bile fistula rabbit and found that all the radioactivity was confined to the cholic acid peak whereas none was present in the deoxycholic acid which is the major bile acid in the rabbit. However, when they administered it to the whole animal and collected bile from a fistula three days later, they found that only traces of radioactivity

remained at the position of the cholic acid whereas practically all of it was found in the deoxycholic acid band. This suggested that deoxycholic acid was not formed primarily in the liver but arose from the attack of microorganisms on the cholic acid.

The fact that transformed products were not found in germ-free rats or rats treated with chemotherapeutic agents further strengthened the idea that these products are formed through the action of intestinal microorganisms. Thus, Gustafsson et al (93) observed that taurocholic acid-24- ^{14}C was the only metabolite found in feces of germ-free rats fed cholic acid-24- ^{14}C . They also showed that infection of germ-free rats with Clostridium perfringens alone resulted in free cholic acid in feces whereas infection with Aspergillus niger produced no change. Norman (150) also found that in rats treated with oxytetracycline or phthalyl sulfathiazolum, tauro- and glyco-cholic acids comprised the major portion of bile acids in the feces with only 1% to 5% of free cholic acid.

Gustafsson et al (93) showed that the half-life of cholic acid in germ-free rats was 11.4 days compared to two days in conventional rats. In both germ-free rats and antibiotic-treated rats the rate of fecal bile acid excretion was found to be greatly decreased (94, 133, 150). These findings were further supported by Wostman and co-workers (210) who observed that germ-free rats released much less $^{14}\text{CO}_2$ from injected 26- ^{14}C -cholesterol than conventional rats and

that the presence of a "normal intestinal microflora" increased the rate of oxidative conversion by at least 40% over the values found in the germ-free animals.

It is apparent from the composition of bile that, excluding deoxycholic acid, a number of the microbially formed metabolites of bile acids escape reabsorption. The synthesis of bile acids appears to be regulated by the concentration of bile acids in the portal blood. Hence a decreased reabsorption of bile acids might influence the turnover of bile acids. Therefore, it was suggested that changes in diet might induce changes in intestinal flora in such a manner that more or less of the bile acids which appear not to be absorbed under physiological conditions are formed. However, no detailed data is available concerning the type of bile acids or microflora in the intestine on different diets (51).

B. Transformation of Cholesterol

Another pathway for elimination of cholesterol is by excretion as neutral sterol in the feces and coprostanol is generally the most important fecal sterol, quantitatively (51). The site of coprostanol formation is considered to be the large intestine (45). The process of conversion appears to be due to microbial action in the large intestine because it is prevented by the administration of bacteriostatics (such as succinylsulfathiazole or compounds such as carbasone which inhibit protozoal growth)(168, 199). Snog-Kjaer et al (185) demonstrated the conversion of cholesterol to coprostanol in a suspension of

feces. Coleman and Baumann (43, 44) showed that administration of antibiotics abolished coprostanol formation in vivo. Danielsson and Gustafsson (53) found that the neutral sterol fraction of feces from germ-free rats was at least 80% cholesterol and that no coprostanol was present.

The amount of fecal neutral sterols excreted was found to vary to some extent with diet. Although it was suggested that the effect of diets is probably mediated by the intestinal microflora (51), there is no evidence to support this. Graber et al (90) studied the effect of high fat diets on intestinal microflora and on serum cholesterol levels in rats, and found that fecal flora remained relatively stable irrespective of diet, although cholesterol levels rose in animals fed butter and sodium cholate. They found that Clostridium perfringens was the only organism that could be consistently isolated from feces of animals given butter diets, particularly those containing sodium cholate, and suggested that if Clostridium perfringens had not been present in the gut, converting exogenous cholesterol to unabsorbable coprostanol, it might have been possible to obtain even more elevated plasma cholesterol levels. However, they could not ascertain from their experimental results the effect of changes in bacterial flora on plasma cholesterol levels.

4. Effect of Dietary Factors on Cholesterol Metabolism

The mechanisms by which saturated and unsaturated fats influence

serum cholesterol has been vigorously investigated since Kinsell and his associates (118) and Groen and associates (91) observed that the feeding of saturated fats resulted in an elevation whereas the feeding of unsaturated fats caused a lowering of serum cholesterol. Much of the work has been concerned with the influence of feeding of various amounts and different kinds of fats on cholesterol synthesis, catabolism and excretion and especially excretion of bile acids and neutral sterols. (50).

A. On Cholesterol Synthesis

In 1952, Alfin-Slater et al (1), using deuterium water, reported that the amount of newly formed cholesterol present in the liver and plasma of rats prefed a low fat (2.2%) was unchanged when the animals were placed on a high fat diet (30%) although the cholesterol concentration in liver was higher when the animals were placed on the high fat diet.

In contrast, Avigan et al (8), using ^{14}C -acetate found that rats fed diets containing fat synthesized more cholesterol than those fed diets containing little or no fat.

The amount of fat in the diet was also shown to influence hepatic sterol synthesis. Thus, Diller et al (63) demonstrated that hepatic sterol biosynthesis was lowest in liver slices from either a fat-free diet or a 5% sunflower oil supplemented diet whereas it was greatly enhanced in rats fed a 20% fat diet. An intermediate level of acetate

incorporation into sterol was found with 10% fat supplemented diet. Dupont (65) also reported a similar finding that the incorporation of ^{14}C -acetate into hepatic sterol was elevated as the corn oil content of the diet was increased. Jensen et al (110) recently reported that the presence of 20% corn oil in the diet decreased hepatic synthesis of fatty acids from (U)- ^{14}C -glucose by 85% as compared to a diet containing 1% corn oil whereas the incorporation into cholesterol was not found to be significantly affected by the amount of corn oil in the diet.

Evidence that the type of fat also alters the rate of acetate incorporation into hepatic cholesterol was provided by Avigan and Steinberg (6) in studies of the effect of corn oil and coconut oil in Purina chow diet on cholesterol metabolism in rats. They showed that the rate of in vivo hepatic synthesis of cholesterol from 1- ^{14}C -acetate was increased significantly by feeding corn oil but not by coconut oil. The similar observation was also made by Kritchevsky et al (119). A marked increase in liver cholesterol concentration was found in rats fed a diet containing 20% corn oil but no significant changes in rats fed coconut oil compared to controls. Linzasoro et al (128) obtained somewhat different results in short term feeding experiments. The fats used were lard, corn oil, Wesson oil, and hydrogenated vegetable oil in synthetic diets. It was found that feeding of the fat containing diets for three days increased the liver's capacity

for incorporating acetate carbon into cholesterol by rat liver slices and the fats tested were of about equal value in stimulating hepatic cholesterogenesis. Reiser et al (162) also reported that the ingestion of 30% lard in a synthetic diet stimulated the synthesis of cholesterol in the same magnitude as in that of trilinolein and safflower oil.

Wilson and Siperstein (208) also could not demonstrate in rats and rabbits a difference in the effects of saturated and unsaturated fats on cholesterol synthesis in the intact animals and in liver.

Carroll (32, 33) observed that acetate incorporation into hepatic cholesterol was influenced by the type of fat fed and also by the kind of basic diet used. He reported that fats fed in a commercial diet have much greater stimulation than fats fed in a semisynthetic diet. The above author suggested that the effect of diets seemed to be specific for acetate incorporation into cholesterol since corresponding changes were not seen for the incorporation into fatty acids measured in the same experiments. Thus, liver slices from rats fed commercial diets incorporated 5 to 10 times as much labeled acetate into cholesterol as did slices from rats fed semisynthetic diets. This finding was also observed in intact rats from the same laboratory (35).

B. On Cholesterol Catabolism

In recent years, several attempts have been made to study the effect of fats and other dietary components on fecal excretion of bile acids and neutral sterols in man and experimental animals. However,

the literature is rather confusing and inconclusive.

Gordon and co-workers (80, 81) first reported that the fecal excretion of bile acids in man was considerably increased by inclusion of sunflower oil in the diet but not by inclusion of coconut oil. This suggested that unsaturated fat reduces the serum cholesterol level by promoting catabolism and excretion of cholesterol. Similar results were also reported by Lewis (126). Haust and Beveridge (99) noticed a greater increase of fecal elimination of bile acids, primarily of the dihydroxycholanic acid type, when human subjects were transferred from butter fat diet to corn oil diet and suggested that decreases in serum cholesterol level produced by feeding unsaturated fat were associated with increased output of fecal bile acids.

Other groups of investigators have also supported the hypothesis that the effect of unsaturated fat is predominantly due to an increased formation of bile acids from cholesterol. Thus, Goldsmith et al (78) observed an increase of approximately 20 to 25% in fecal excretion of bile acids on changing from saturated to unsaturated fat. There was also an increase in total sterol excretion amounting to about 300 to 400 mg. daily. Similar evidence was presented by Antonis and Bersohn (5) who demonstrated that fecal excretion of bile acids and sterols was higher on diets containing unsaturated fat than on diets containing saturated fat. Besides, they found that increasing the fiber content of the diet produced bulkier stools which contained appreciably greater amounts of bile acids and sterols, while decreasing the dietary

fiber content produced the opposite effect.

Roles and Hashim (165) found a marked reduction in the daily excretion of fecal bile acids when coconut oil was substituted for corn oil in amounts sufficient to induce a rise in serum cholesterol. By means of a single injection of 4- ^{14}C -cholesterol, Moore et al (145) found a significant increase in the fecal excretion of the unsaponifiable fraction and bile acids on a safflower oil diet compared with those on butter diets.

On the other hand, studies in which fecal excretion of isotope was followed after administration of 4- ^{14}C -cholesterol to human subjects on solid or formula diets, showed no such effect of unsaturated fat on the total bile acids output. Hellman and Rosenfeld (100) found that the decrease in serum cholesterol level produced by feeding unsaturated fat was accompanied by an increase in fecal excretion of neutral sterols while no change in excretion of bile acids was observed.

Avigan and Steinberg (7) using an isotopic balance method found no apparent correlation between the effects of changes of the type of dietary fat and changes in bile acid or sterol excretion. Further study by Spritz et al (188) also supported the finding that the degree of unsaturation of fat does not significantly alter the rate of excretion of fecal bile acids and neutral sterols. They found that dietary plant sterols significantly increased bile acid excretion whether the dietary fats were saturated or unsaturated. In determining the turnover and

pool size of cholic acid with a tracer technique, Lindstedt et al (130) and Hellstrom and Lindstedt (101) found that the pool size and turnover of cholic acid were not affected consistently by substituting coconut oil for corn oil and vice versa.

Several reports have also appeared dealing with the influence of diet on fecal excretion of bile acids and neutral sterols in animals. Byers and Friedman (31) studied the effect of fats on bile acid production in bile-duct cannulated rats and found that the total amount of cholate excreted by rats given walnut oil was twice as great as that observed in rats ingesting a sterol-free diet and 25% greater than that excreted by rats given coconut oil. Merrill (138) reported in a preliminary note that the addition of 10% linoleic acid to the diet increased the rate of incorporation of radiocarbon into liver as well as fecal excretion of the bile acids and sterols while a saturated fat did not. When labeled cholesterol bound to lipoprotein from animals fed soybean oil or butter was injected into recipient animals fed stock diet, Lewis et al (127) found a pronounced difference in bile acid and neutral sterol output. The output of both bile acids and neutral sterols was greater in animals receiving the lipoprotein from animals fed soybean oil than those from animals fed butter fat. The difference in bile acid excretion was found to be more pronounced than that of neutral sterols.

On the other hand, several groups of workers presented evidence that dietary fats had no significant effect either on degradation or excretion of cholesterol following the feeding of diet containing saturated

or unsaturated fats. Thus, Wilson and Siperstein (208) demonstrated that the rate of cholesterol degradation, as measured by the excretion of total 4- ^{14}C -cholesterol end products in bile, was not influenced by the feeding of semisynthetic diets containing no fats, lard or corn oil to rats. Wilson (207) later showed by means of gas-liquid chromatographic analysis that linoleic acid accelerated coprostanol formation and excretion while palmitic acid and oleic acid inhibited it.

Lambiotte (122) studied the metabolism of endogenous cholesterol in adult rats kept for a prolonged time on cholesterol-free diets containing either maize oil, total ethyl esters of this oil or ethyl esters of karite butter freed from linoleate and found no difference in the fecal excretion of bile acids in the three groups. Similarly, Bieberdorf and Wilson (18) reported that rabbits in isotopic equilibrium showed no change in excretion of bile acids or neutral sterols following a shift from coconut oil to corn oil in the diet.

Kritchevsky (120) observed that liver mitochondria from rats fed saturated fat oxidized 26- ^{14}C -cholesterol to $^{14}\text{CO}_2$ to a much greater extent than did liver mitochondria from rats fed unsaturated fat. The oxidation of sodium pyruvate was found not to vary with dietary fats.

Using 4- and 26- ^{14}C -cholesterol, Coniglio and collaborators (Anderson et al (2); Ferguson et al (68)) studied ^{14}C excretion in feces and expired air of rats fed saturated and unsaturated fat and found no significant influence by the type of fat ingested on the amount of ^{14}C

excreted in feces and converted $^{14}\text{CO}_2$.

Many workers found that bile acid formation and excretion was influenced by the type of basic diet rather than by the type of fat in the diet. Thus, Portman and Murphy (162) demonstrated that rats fed Purina laboratory chow excreted more cholate and degradation products of cholate per day compared to rats fed purified diets. They also showed that the half-life of cholate was shorter in rats fed Purina chow diet than those on purified diets. A similar finding was observed in Cebus monkeys by Portman (161).

In studies of the turnover of deoxycholic acid in rabbits, Hellstrom et al (102) clearly showed that the type of diet is a factor influencing bile acid elimination. Feeding semisynthetic diets caused a marked reduction in daily production of deoxycholic acid as compared with the commercial diet, both containing coconut oil and corn oil, respectively. The decrease in daily production was due to prolongation of half-life of deoxycholic acid. It was also shown that fecal excretion of deoxycholic acid decreased pronouncedly when the diet was changed from a commercial pellet diet to a semisynthetic diet.

In a balance study in rats, Bloomfield (23) observed that bile acids and neutral sterols in feces were greater in rats fed commercial diets than in those fed synthetic diets, suggesting that fecal steroid excretion (including bile acids and neutral sterols) was proportional to the fecal residue or bulk which in turn increases cholesterol

biosynthesis, but results in a decrease in the carcass cholesterol.

In most of the above studies, bile acid excretion in feces was determined by titrimetric or colorimetric methods, with or without previous purification of the bile acid fraction by chromatographic means (101). Since the bile acid and neutral sterol fractions of feces represent a complex mixture of microbially formed metabolites, it is difficult to isolate and determine quantitatively the different fecal bile acids and sterols, and the possible inadequacy of some of the methods used so far must be taken into account in the evaluation of the results (51). Similarly, after a single administration of a labeled bile acid, the kinetic study of its specific radioactivity permits the determination of the rate of its turnover, however, this kind of method must be applied to each bile acid or at least to the major ones among them; for example, cholic and chenodeoxycholic acids in the rat.

5. Other Factors Influencing Cholesterol Metabolism

A. Suckling Rats

Cholesterol metabolism in suckling animals has been found to be different from that in weaned or adult animals. It was observed that serum cholesterol levels are higher in suckling rats than in weaned rats (19, 35, 96). Carroll (19) found that liver from suckling rats incorporated very little labeled acetate into cholesterol in vitro while liver from fetal rats or from weaned rats gave good incorporation.

Since cholesterol concentration in rat milk was only 15.5 mg./100 ml. he questioned whether this amount of cholesterol in the diet could be solely responsible for the slow rate of incorporation. In this regard, Harris et al (97) observed in suckling rats a four to five fold increase in the half-life of cholic acid over normal rats on stock diets. Cholesterol synthesis from acetate- ^{14}C by livers of suckling rats was increased two to three fold within 24 hours after the cannulation of the bile duct and inhibited when cholic acid was infused into the cannulated animal. A slow rate of fecal excretion of cholic acid was observed in suckling rats and rats fed synthetic diets (simulated milk), suggesting that under these dietary conditions bile salt retention either directly or indirectly influences hepatic synthesis of cholesterol.

B. Fasting, Triton-Treatment and X-Irradiation

Caloric restriction also has an inhibitory effect on the liver's ability to form cholesterol. Tomkins and Chaikoff (195), using rat liver slices, observed a decrease in synthetic rate to about 10% of the control value after 24 hours of fasting and to even lower values after 72 hours. A similar result was observed with intact animals but the magnitude of decrease was far less than that found with liver slices (108). On the other hand, the biosynthesis of cholesterol from acetate in rat liver is greatly augmented by treatment with Triton WR-1339 (71) or by exposure of the animal to X-irradiation (28, 83, 86).

Intravenous injection of detergent Triton WR-1339 (into rats) has been shown to produce a rise in serum cholesterol (73). Franz and Hinkelman (71) demonstrated in rats by both in vivo and in vitro methods that there was about a three fold increase in the rate of synthesis at 24 hours after the injection of Triton. They also found that the depressant effect of cholesterol feeding on cholesterol synthesis was completely overcome by Triton. Gould et al (84) and Bucher et al (28) found that whole body X-irradiation resulted in a pronounced increase in the rate of hepatic cholesterol synthesis in rats, estimated from incorporation of 1-¹⁴C-acetate both in vivo and in vitro. The diminished activity in fasting controls was absent in X-irradiated rats which actually exhibited activity several times that of normally fed animals. In fasting and X-irradiated animals, only hepatic cholesterol synthesis was found to be greatly influenced and that in extrahepatic tissues was either much less or not significantly influenced.

The biochemical site of inhibition and stimulation of cholesterol synthesis by fasting, Triton treatment and X-irradiation was demonstrated to be between acetate and mevalonic acid, specifically the reduction of β -hydroxy- β -methyl-glutaryl-CoA to mevalonic acid (27, 29). Using ¹⁴C-labeled β -HMG-CoA, Linn (136) recently reported a drastic decrease in reductase activity in microsomes and solubilized enzymes of fasted rat liver.

During the course of steroid balance studies in rats, Bloomfield (23) observed that total animal sterol biosynthesis was nearly proportional to caloric intake and that animals which lost weight and failed to grow had actually a higher concentration of sterols in the carcass than controls. Van Bruggen et al (198) also found a relative constancy of cholesterol mass in the presence of decreased incorporation of ^{14}C -acetate into cholesterol in fasted rats. This maintenance of cholesterol concentration in tissues in the presence of decreased incorporation might suggest an associated decrease in cholesterol catabolism. However, no report is available to support the suggestion as to whether there is any decreased catabolism in fasting conditions or not. In X-irradiated rats, Gould et al (83, 84) found that liver cholesterol concentration was lower than that of fasted and fed controls. They studied the turnover rate of tritium-labeled cholesterol in X-irradiated rats and found an increased turnover rate. Since the increased synthesis in X-irradiated rats was associated with decreased hepatic concentration, it was suggested that the effect was due to increased utilization or excretion of cholesterol which depleted the liver cholesterol and stimulated synthesis (45). However, no evidence to support this suggestion is available.

PRELIMINARY STUDIES

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1. Studies on Intestinal Bacteria in Relation to Cholesterol Metabolism

Most cholesterol is eliminated from the body as bile acids or cholesterol, both of which are excreted by way of the intestinal lumen in which they are modified by intestinal bacteria. Most of these microbially-modified metabolites of cholesterol are believed to be absorbed poorly or not at all from the intestinal lumen so that re-utilization is decreased or prevented (46, 52). It has been suggested for a long time that changes in cholesterol metabolism observed in animals fed on different diets may be mediated through changes, both quantitative and qualitative, in intestinal microflora.

Peterson et al (157) reported that several bacteria isolated from soils in Houston, Texas, could utilize cholesterol as a sole source of carbon and grew well on a mineral salt plate enriched with cholesterol. After incubation at 30°C for four days, good growth with two patterns of sterol utilization was noted on the agar plate containing blended cholesterol. Some cultures caused a clearing of the cholesterol opaqueness surrounding the colonies while others did not.

The present studies were carried out to investigate the possibility that some species of intestinal bacteria might utilize cholesterol as a sole source of carbon and that they might be quantitatively changed by

means of different diets, which in turn influence cholesterol metabolism.

The purpose of this work was (i) to investigate whether any intestinal bacteria utilize cholesterol as a sole source of carbon; (ii) to isolate such bacteria if any by using mineral salt media containing cholesterol as a sole source of carbon; and (iii) further to see whether there is any difference in cholesterol utilization by intestinal bacteria in relation to experimental diets which had been shown to affect acetate incorporation into cholesterol by liver slices.

Experimental

In an attempt to isolate intestinal bacteria which utilize cholesterol as a sole source of carbon the following experimental procedures were followed:

Test No. 1

The contents of small and large intestines taken from rats (200 g.) fed on laboratory chow diet were separately suspended in sterile saline. Various dilutions were streaked on mineral salt plates enriched with cholesterol and incubated at 35°C for four days. The composition of the mineral salt-cholesterol (Ms-C) media was as follows:

<u>Basal Media:</u>	K ₂ HPO ₄	-----	0.2500 g.
	MgSO ₄	-----	0.2500 g.
	NaCl	-----	0.0050 g.
	Fe ₂ SO ₄	-----	0.0001 g.
	NH ₄ NO ₃	-----	1.0000 g.
	Dist. H ₂ O	-----	1000.0 ml.
	pH	-----	6.8 with N/10 HCl

Cholesterol ----- 1.0000 g.
Agar ----- 10,000 g.

Basal media with agar were autoclaved at 121°C for 20 minutes blended in a Waring blender with sterilized cholesterol for 2 minutes and poured into sterile Petri dishes (about 15 ml. per dish). This homogenization gave an even distribution of cholesterol in the agar media as reported by Peterson et al (155).

No bacterial growth was observed on the plate after four days of incubation at 35°C or 37°C.

In order to determine whether the lack of growth on Ms-C plates was due to a deficiency of nutrients, the suspensions were inoculated on Ms-C plates enriched with 0.1% tryptose or 0.5% glucose. Some growth was observed on the plates enriched with tryptose but the colonies were diffuse and rough. No colonies were found on the plates enriched with glucose.

Test No. 2

Since no growth was observed on the Ms-C plates, a second experiment was carried out in which the feces were first inoculated into liquid mineral salt-cholesterol media and then streaked on plates.

In preparing the liquid media, 0.5 mg. of cholesterol dissolved in acetone was added to each 25 ml. Erlenmeyer flask and the solvent was evaporated in a stream of nitrogen. Then 5 ml. of mineral salt solution was added and the flasks were plugged with cotton and auto-

claved for 15 minutes at 121°C. The basal composition of the mineral salt solution was the same as that used for the plates.

Feces were collected in sterile saline. Several loopfuls of the suspension were inoculated into 5 ml. of Ms-C media and incubated in a shaking incubator at 35°C for four days. Then several loopfuls of the culture were inoculated on the following plates:

- (i) Mineral salt-cholesterol plate: very poor growth and no clearance around colonies.
- (ii) Mineral salt-cholesterol plate enriched with 0.1% tryptose: several colonies appeared but no clearance around them.
- (iii) Mineral salt-cholesterol plate enriched with 0.1% yeast extract: Same as (ii).

Since no growth was observed on Ms-C plates in Test No. 1 and a single diffuse rough colony appeared on those in Test No. 2, the following subcultures were carried out in order to increase the adaptability of bacteria to utilize cholesterol and to eliminate by successive transfers those which are not able to grow in the cholesterol media.

Saline fecal suspension was inoculated into liquid Ms-C media and incubated for four days at 35°C. Several loopfuls of this culture were inoculated into fresh media. This subculturing was repeated three times at two-day intervals. After the successive transfers, the

last culture was streaked on the following plates:

- (i) Tryptose* plate: flourishing growth. All the colonies were Gram-negative Bacilli.
- (ii) Mineral salt-cholesterol plate: There was a single diffuse, rough colony. It contained several different kinds of bacteria and it was not possible to pick a single bacterial species from the plate. Therefore, this colony was inoculated on a tryptose agar plate in order to identify the species of bacteria present on the Ms-C plate. These bacteria were identified by morphology and biochemical reactions as presented in Appendix II(a). Whenever the mineral salt-cholesterol cultures were streaked on a tryptose plate, there was always flourishing growth consisting of mostly Gram-negative Bacilli.
- (iii) Mineral salt-cholesterol plate enriched with 0.1% tryptose: a better growth than (ii) but no clearance around the colonies and poorer growth compared with those on the tryptose plate.
- (iv) Mineral salt-cholesterol plate enriched with 0.2% yeast extract: a better growth than (ii) but no clearance around the colonies.

* Tryptose agar plate containing 4.1% tryptose.

All these tests showed that mineral salt media enriched with cholesterol as a sole source of carbon does not provide favorable conditions for growth of the intestinal bacteria.

Test No. 3

These experiments were carried out to study whether intestinal bacteria isolated from rat feces and grown on the mineral salt-cholesterol media or the known bacteria, E. coli, oxidize cholesterol as an energy source. A Warburg respirometer was used for this purpose.

Suspension of rat feces or of E. coli were inoculated into 5 ml. of mineral salt-cholesterol media and incubated in a shaking incubator at 35°C for four days. These were successively transferred into a fresh liquid medium every two days and the transfer was repeated three to four times. The last culture was inoculated on the tryptose agar plate in order to identify the bacterial species which are presented in Appendix II (b). At the same time, this last culture was transferred into 500 ml. of mineral salt-cholesterol media enriched with 0.2% tryptose or mineral salt media without cholesterol with 0.5% glucose, and aerated for 18 hours. The E. coli subculture was also inoculated into mineral salt-cholesterol enriched with 0.2% yeast extract.

After 18 hours growth, the cultures were harvested and washed three times with 0.05 M phosphate buffer, pH 6.5 or 6.7. The cells were suspended in the same buffer to give a turbidity corresponding to a given dry weight of cells (9 mg.). An accurate measurement of dry weight was not possible because of the interference by colloidal cholesterol in the culture media.

Two ml. of cell suspensions were placed in the main compartments of Warburg vessels in a water bath at 37°C. After equilibration, the colloidal cholesterol or glucose or buffer solutions were tipped in from the side arm and the rate of oxygen consumption was measured for one hour, using the interval method. The summarized results of respiration are presented in Table i.

This Table indicates that intestinal bacteria do not oxidize cholesterol and even bacteria grown in mineral salt-cholesterol media enriched with tryptose or yeast extract lost the ability to oxidize glucose. The rate of O₂ uptake was virtually the same in both cases for glucose or for cholesterol. On the other hand, bacteria grown in the presence of glucose and in the absence of cholesterol showed a much greater rate of O₂ uptake for glucose.

Test No. 4

In these experiments, attempts were made to isolate bacteria utilizing cholesterol from feces of rats fed on semisynthetic diet containing butter or corn oil (see Appendix I(a)).

Table i

OXIDATION OF CHOLESTEROL BY BACTERIA ISOLATED FROM RAT FECES USING A RESPIROMETER

Cell Samples*	Flask No.	Cholesterol Concentration M	Glucose M	Phosphate Buffer 0.05M at pH 6.6	Q02
Cells grown in MS-C** enriched with 0.2% Tryptose	D-19	8.7x10 ⁻⁴			5.18
	I-18		6.7x10 ⁻³		5.73
	3-N			only	3.12
Aged-Cells. Cells grown in MS-C media enriched with 0.2% Tryptose and kept overnight in buffer in refrigerator	13-G	8.7x10 ⁻⁴			2.55
	5-R		6.7x10 ⁻³		2.80
Cells grown in MS*** media enriched with 0.2% glucose (no cholesterol added into the final culture media)	D-19			only	2.37
	E-18	8.7x10 ⁻⁴			7.04
	L-21		6.7x10 ⁻³		30.80
<u>E. Coli</u> cultured in MS-C media enriched with 0.2% yeast extract	3-N			only	6.81
	13-G	8.7x10 ⁻⁴			5.10
	F-11		6.7x10 ⁻³	only	5.21
					2.74

Each flask contains cells suspended in 2 ml. of 0.05M phosphate buffer, pH 6.5. Final volume in main compartment is 3.0 ml. The center well contained 0.2 ml. of 20% (w/v) KOH and a fluted filter paper. Incubation carried out in air at 37°C for 1 hour.

* Dry weight = 9 mg. per flask
O.D. = 1

** MS-C = Mineral salt media containing cholesterol.

*** MS = Mineral salt media.

Feces of rats fed on the special diets were inoculated in mineral salt-cholesterol liquid media and incubated at 35°C for four days in a shaking incubator. These cultures were transferred to the fresh mineral salt-cholesterol media several times. The final cultures were inoculated on the following plates:

- (i) Tryptose plate: flourishing growth.
- (ii) Mineral salt-cholesterol plate: a single diffuse rough and dry colony. This colony was re-inoculated on a tryptose plate to identify the bacterial species which were identical to those presented in Appendix II(b).

Since the overall picture of these results was very similar to that obtained with feces from rats on the stock diet, no attempts were made to carry out studies of cholesterol oxidation using the respirometer.

Discussion

It has been observed from the above tests that intestinal bacteria fail to grow on the mineral salt media containing cholesterol as a sole source of carbon. The growth of bacteria was always poorer on the mineral salt-cholesterol media enriched with 0.1% tryptose or yeast extract than the growth on the tryptose plate (4.1%).

Graber *et al* (90) reported that the species of intestinal bacteria in rats fed stock laboratory diet and a semisynthetic diet containing butter or cholesterol were not qualitatively different.

Intestinal microflora reported by the above authors are as follows:

Gram-negative bacteria: Proteus species, Escherichia coli, Pseudomonas aeruginosa, Klebsiella Aerobacter, Bacteroides species; and Gram positive bacteria: Enterococci, Micrococci, Lactobacilli, Staphylococcus aureus, Corynebacterium species, Streptococcus.

Among the intestinal microflora reported above, Bacteroides and Clostridium species are strict anaerobes. Since mineral salt-cholesterol plates or fluid media were incubated in an aerobic condition, the strict anaerobes would not grow on the present culture media.

As presented in Appendix II(a) and (b), the bacteria that survived through successive subcultures in mineral salt media were all Gram negative bacilli except one case.

Even when intestinal bacteria were cultured solely in mineral salt-cholesterol liquid media for two weeks, the ability to oxidize cholesterol as an energy source was not observed and furthermore, they lost the ability to oxidize glucose.

Therefore, feces of rats raised on the laboratory diet or on the semisynthetic diet containing butter or corn oil do not contain bacteria which have the ability to oxidize cholesterol for their energy source like some strains of soil bacteria such as Streptomyces, Nocardia, etc.

2. Studies on the Distribution of 4-¹⁴C-Cholesterol Administered Orally to Rats Fed on Different Diets

Since the previous experiments failed to show that rat intestinal bacteria utilize cholesterol as a sole source of carbon, another study was carried out in order to investigate the distribution of 4-¹⁴C-cholesterol administered orally to rats fed on the different types of diet. This approach is indirect and based on the hypothesis that feeding different diets might bring changes, quantitatively or qualitatively, which might in turn influence modification of cholesterol, leading to a difference in absorption of ingested cholesterol.

Experimental

1. Male rats of the Sprague-Dawley strain, weighing around 80 to 90 g. and raised in our laboratory, were fed the commercial or semisynthetic diets containing 15% butter or corn oil for two or three weeks (see Appendix I (a) and I (b)). The average weight at the end of the feeding period varied from 170 to 200 g.

2. 1 μ c. of 4-¹⁴C-cholesterol dissolved in 0.1 ml. of tri-octanoin was administered orally, using a 0.1 ml. volumetric pipette. The pipette was inserted and supported by the corner of the mouth while the solution was discharged well to the back of the mouth.

3. The rats were not fasted nor restricted in food intake but the time of oral administration of the radioactive cholesterol was kept relatively constant at between 9.00 and 10.00 o'clock in the morning.

4. The animals were sacrificed at different time intervals varying from 0.5 to 12.0 hours after the oral administration.

5. Lipids were extracted from stomach contents, small intestinal contents, small intestinal wall, large intestinal contents and feces and liver with chloroform:methanol (2:1, v/v). The small intestine was flushed twice with 10 ml. of saline, using a syringe, and the washings were transferred to a separatory funnel and the lipids extracted twice with chloroform:methanol (2:1, v/v). The small intestinal wall was cut into small pieces, ground in a mortar and pestle and the lipid extracted with 20 volumes of chloroform:methanol (2:1). The liver was rinsed in saline, blotted dry, and homogenized in chloroform:methanol to extract the lipid.

All lipid extracts were made to known volumes in a graduated cylinder and aliquots were taken for measurement of the radioactivity by a Geiger-Muller Gas Flow Counter with micromil window (Nuclear Chicago, Model 2001).

6. Total radioactivity recovered from all of the above extracts at a time interval of 30 min. after administration of the radioactive cholesterol was taken as 100% and all results were expressed as a percentage based on this figure.

Results and Discussion

The levels of radioactivity from stomach contents, small intestinal wall, liver, and large intestinal contents and feces at various

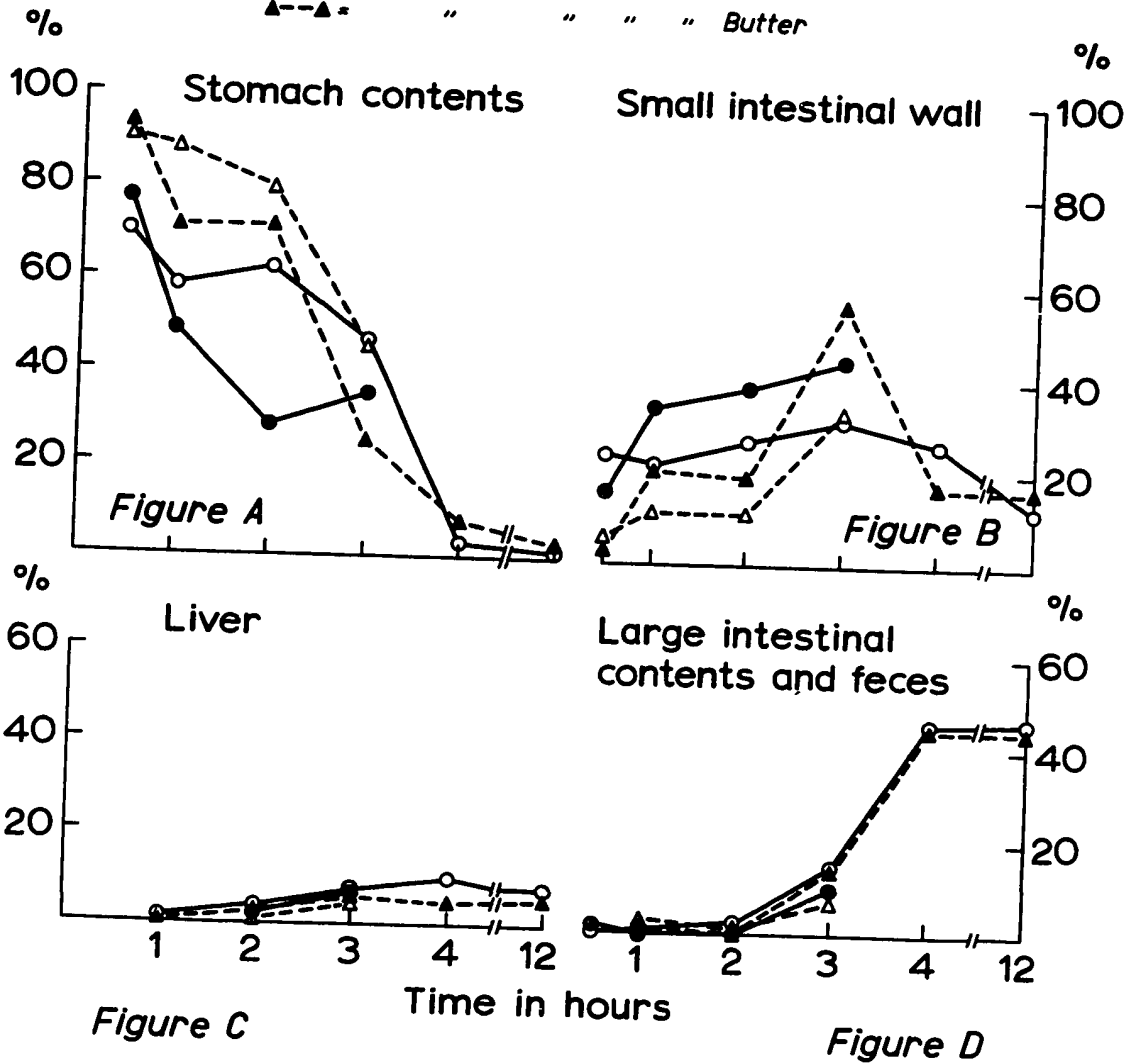
time intervals in rats fed different diets are shown in Figures A, B, C, and D.

In general, the rate of disappearance of the label from stomach contents is slower during the first two hours after administration in rats fed the semisynthetic diets compared to those on the commercial diets. This difference may be due to the fact that there was always more food in the stomach during this time interval when semisynthetic diets were fed. The rate of appearance in the small intestinal wall during this time interval was just the reverse of that in the stomach contents and levels of radioactivity in liver, large intestinal contents and feces were almost negligible.

After the first two hours, the rate of passage from the stomach was quite rapid and most of the radioactivity was evacuated by four hours after administration. Not much difference between different dietary groups was found. In intestinal wall, the level seemed to increase more rapidly to the peak at three hours in the semisynthetic dietary groups compared to the groups fed commercial diets and the rapid appearance may be due to the abrupt fall in activity of the stomach contents between two and three hours after the feeding. The level also appeared to fall more rapidly after three hours in the semisynthetic dietary groups but this was not accompanied by an increased uptake of radioactivity by liver compared to the groups fed commercial diets.

Recovery of radioactivity following oral administration of 4-¹⁴C-Cholesterol

○—○ = Commercial diet with 15% Corn oil
 ●—● = " " " " Butter
 △--△ = Semisynthetic diet with 15% Corn oil
 ▲--▲ = " " " " Butter



In large intestinal contents and feces, the maximum radioactivity was found at four hours after the feeding and the activity was not changed much even after 12 hours. This suggests that recirculated labeled cholesterol does not contribute to excretion during 12 hours. In the two dietary groups studied over the 12 hour period, around 45% of the administered dose was excreted without absorption.

The intention was to carry out these experiments with rats in physiological condition rather than in those fasted or restricted in time or amount of food intake, and the rate of absorption of the administered labeled cholesterol from the small intestinal wall in rats fed the different diets was found to be a function of the rate of passage from the stomach. This is also why there was so much variation in individual values as presented in Tables ii and iii. These tables indicate average as well as extreme values. If one looks at the tables carefully, it can be noticed that the lowest values in recovery of radioactivity from small intestinal wall, large intestinal contents and feces and liver always correspond to the maximal values from stomach contents. Therefore, it was concluded that studies of the distribution of radioactivity after a single administration of labeled material to naturally-fed animals do not give much information on relative rates of absorption in animals on different diets.

The experiments were not continued because of the variation in the individual values due to the difficulty of controlling the amount of

Table II

DISTRIBUTION OF RADIOACTIVITY AT DIFFERENT TIME INTERVALS AFTER FEEDING 4-14C-
CHOLESTEROL IN RATS FED ON COMMERCIAL DIETS*

Hour After Feeding	Stomach Contents	Small In-testinal Contents	Small In-testinal Wall	Large Intestinal Contents and Feces	Liver	No. of Rats
0.5	77.1** (63.5-87.1)***	7.0 (1.0-11.7)	15.3 (20.9-51.6)	0.4 (0.01-1.4)	0.05 (0.01-0.14)	3
1.0	49.2 (28.8-61.8)	15.8 (11.4-19.0)	34.5 (20.9-51.6)	0.21 (0.0-0.21)	0.33 (0.3-0.4)	3
2.0	28.1 (6.8-73.4)	15.9 (2.3-28.8)	38.0 (22.6-39.1)	13.3 (0.91-33.6)	3.0 (1.6-4.1)	4
3.0	35.6 (3.9-81.7)	4.3 (2.2-8.3)	43.9 (14.0-68.0)	9.7 (0.5-14.5)	6.5 (1.6-11.4)	3
<hr/>						
0.5	70.0 (54.5-94.5)	5.5 (0.2-10.1)	23.6 (3.8-38.6)	0.6 (0.05-1.6)	0.4 (0.2-0.7)	3
1.0	53.0 (43.8-77.4)	5.7 (2.3-11.6)	21.3 (2.3-42.9)	0.06 (0.06-0.06)	1.0 (0.4-1.8)	3
2.0	62.2 (38.0-88.0)	3.9 (2.1-6.9)	26.5 (7.5-48.0)	2.0 (0.1-5.6)	3.8 (1.8-7.7)	3
3.0	46.3 (12.3-80.3)	0.9 (0.4-1.4)	30.7 (9.2-52.2)	14.6 (5.8-19.3)	6.7 (3.3-15.8)	2
4.0	3.3 (2.3-4.4)	5.9 (1.9-12.8)	15.6 (12.8-19.0)	45.6 (25.6-70.0)	10.0 (7.0-18.0)	4
12.0	1.6 (1.3-1.9)	0.3 (0.2-0.4)	12.1 (4.9-19.3)	46.4 (20.8-54.4)	7.9 (4.7-11.1)	2

** Average values

*** Expressed as percent of total radioactivity recovered at the time interval after 30 minutes of administration of 4-14C-Cholesterol.

*** Extreme Values

Table III

DISTRIBUTION OF RADIOACTIVITY AT DIFFERENT TIME INTERVALS AFTER FEEDING 4-14C-CHOLESTEROL IN RATS FED ON SEMISYNTHETIC DIETS*

Hour After Feeding	Stomach Contents	Small Intestinal Contents	Small Intestinal Wall	Large Intestinal Contents and Feces	Liver	No. of Rats
0.5	93.0** (90.2-94.7)***	2.5 (0.7-4.2)	4.4 (1.0-7.0)	0.03 (0.01-0.07)	0.07 (0.06-0.09)	3
1.0	71.3 (55.2-94.3)	7.2 (2.3-16.1)	20.0 (3.3-29.7)	0.42 (0.01-1.2)	0.9 (0.09-0.7)	3
2.0	71.2 (42.8-96.4)	2.0 (0.5-4.4)	19.0 (2.0-37.5)	3.7 (0.1-9.3)	3.6 (1.0-7.6)	3
3.0	25.1 (21.7-28.5)	3.4 (0.7-6.1)	51.2 (48.9-53.4)	14.4 (11.0-17.8)	6.2 (6.0-6.4)	2
4.0	7.6 (2.3-21.3)	6.1 (0.2-13.2)	17.0 (12.8-21.2)	44.5 (6.0-79.8)	4.5 (1.9-7.8)	4
12.0	3.0 (2.6-3.5)	2.2 (0.3-4.1)	16.9 (10.7-23.1)	44.2 (39.9-48.5)	5.8 (4.9-6.9)	2
0.5	91.1 (83.7-97.0)	3.1 (0.6-4.6)	5.8 (2.4-11.7)	0	0.02 (0.01-0.03)	3
1.0	87.7 (76.9-91.6)	2.8 (1.4-3.5)	11.0 (4.8-17.7)	0.5 (0.04-1.6)	0.14 (0.03-0.22)	3
2.0	79.3 (67.5-86.7)	6.5 (0.8-15.0)	10.5 (8.1-12.0)	0.8 (0.06-5.2)	0.54 (0.14-0.9)	3
3.0	45.2 (32.9-57.9)	6.8 (3.0-10.7)	32.9 (30.7-35.1)	7.2 (1.6-19.9)	4.2 (2.7-5.8)	2

** Average Values

*** Extreme Values

* Expressed as percent of total radioactivity recovered at the time interval after 30 minutes of administration of 4-14C-Cholesterol.

food in the stomach when the labeled cholesterol was given, without fasting the animals or limiting the time during which food is available.

MAIN STUDIES:

OXIDATIVE CATABOLISM OF CHOLESTEROL

MATERIALS AND EXPERIMENTAL PROCEDURES

MAIN STUDIES: OXIDATIVE CATABOLISM OF CHOLESTEROL

Materials and Experimental Procedures

1. Materials

A. Instruments

(a) Metabolism Cage: For some experiments, a metal metabolism cage with a plastic top was used for the collection of expired CO₂. However, most of the experiments were carried out with glass cages purchased from Delmar Scientific Laboratories, Maywood, Illinois.

(b) Liquid Scintillation Spectrometer: In some early experiments, the radioactivity was measured by a Nuclear Chicago Spectrometer, Model 724-725, through the courtesy of Dr. I. G. Walker, Cancer Research Laboratory. In later experiments, the determinations were carried out with a Packard Tri-Carb Spectrometer, Model 3375, Packard Instrument Company Inc., Downers Grove, Illinois.

B. Chemicals

(a) ²⁶-¹⁴C-cholesterol in benzene, specific activity 62.2 mc/mg., obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England.

(b) Scintillators: PPO; 2, 5-diphenyloxazole (Scintillation Grade), Fluor Max. 3800 A.

POPOP; 1, 4-bis-2-(5-phenyloxazolyl) Benzene (Scintillation Grade), Fluor Max. 4200 A.
purchased from the Packard Instrument Company, Inc., Downers Grove, Illinois.

(c) Toluene (Scintillation and Fluorometric Grade), obtained from Matheson Coleman and Bell Company, Inc., Norwood, Ohio, East Rutherford, New Jersey.

(d) Monoethanolamine and Methylcellosolve (Ethylene Glycol Monomethylether), obtained from Fisher Scientific Company, Ltd., Toronto, Ontario.

(e) Tween 20: Polyoxyethylene Sorbitan Monolaurate, purchased from Fisher Scientific Company Ltd.

(f) Drierite, indicating (anhydrous Ca_2SO_4), 8 mesh, purchased from W.A. Hammond Drierite Company, Xenia, Ohio.

(g) Ascarite, indicating, 20 to 30 mesh, obtained from Arthur H. Thomas Company, Philadelphia, Pa.

(h) Triton WR-1339, obtained from Rohm and Hass, Philadelphia, Pa.

C. Animals

Inbred male rats of the Sprague-Dawley strain, raised in Collip Laboratory.

D. Diets

(a) Commercial Diet: Master Fox Breeder Starter Ration, purchased from Toronto Elevators Ltd., Toronto, Ontario.

(b) Semisynthetic Diet:

1. Casein, Phillips-Hart salt mixture, water-soluble and fat-soluble vitamins, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

2. Hydrous dextrose U.S.P., purchased from Ingram and Bell Ltd., Toronto, Ontario.

3. CellufLOUR, obtained from the Chicago Dietary Supply House Inc., Chicago, Illinois.

(c) Fats: Butter and corn oil (Mazola), purchased from local grocery store.

2. Experimental Procedures

A. Treatment of Animals

(a) Dietary Treatment

Young rats of the Sprague-Dawley strain, weighing 80 to 90 g. were fed commercial or semisynthetic diets containing no fat, 15% butter or 15% corn oil by weight. The composition of the two types of diets is presented in Appendix I. The diets were fed for 14 to 16 days and after this period the animals attained a weight of 150 to 170 g.

(b) Normal Controls

Young adult male rats weighing around 170 g. which had been maintained on the commercial Master Fox Ration from weanling age until they were used for the experiments served as a control group for other experiments.

(c) Suckling and Weanling Rats

Some experiments were carried out with male suckling rats weighing around 30 g. (15 days old). Other young male rats were weaned directly to the commercial Master Fox Ration Meal at the age of 21 days and were maintained on the same diet until they were used for the experiments at the age of 36 days and an average weight of around 90 g.

(d) Fasted Rats

Male adult rats weighing an average of 183 g. were fasted for 40 to 48 hours. Water only was given ad libitum to the animals during this period.

(e) Triton-treated Rats

Young adult male rats weighing around 160 g. were injected intravenously with 1 ml. of saline containing 100 mg. of Triton WR-1339, 24 hours before the experiments.

(f) X-Irradiated and Fasted Rats

A microtron, built by the Physics Department of this University, was used for X-irradiation of rats. Young rats weighing around

180 g. were kept in restraining cages individually and irradiated at approximately 40 roentgen per minute for about one hour. Total dose was 2400 roentgen.

The irradiated animals were deprived of food from the time of irradiation for 40 hours until they were used. Water only was given ad libitum to the animals during the fasting period.

B. Preparation of Aqueous Solution of 26-¹⁴C-Cholesterol for Injection

An aqueous solution of cholesterol was prepared according to the method of Meier, Siperstein and Chaikoff (136).

Ten microcuries (160 μ g.) of cholesterol in benzene was transferred to a 15 ml. test tube and dried with a stream of nitrogen on a warm water bath. Two mg. of Tween 20 were added to the dry cholesterol and 2 ml. of ethanol was added to bring the cholesterol into solution with the Tween 20. The ethanol was then evaporated on a steam bath with nitrogen and 10 ml. of saline were added to make an aqueous solution in which 1 ml. contained 1 μ c. (16 μ g.) of 26-¹⁴C-cholesterol.

C. Experimental Procedures for the Metabolism Study After Injection of Labeled Cholesterol

(a) For Collection of Respiratory CO₂ and Analysis of Radioactivity in Expired Air

Each animal was injected by tail vein, under light ether anesthesia, with an appropriate volume of the solution of 26-¹⁴C-cholesterol,

according to the age of the rat used. One ml. of the solution containing 1 μ c. was used for the young adult rats. In recently-weaned and suckling rats, 0.5 ml. containing 0.5 μ c. and 0.2 ml. containing 0.25 μ c. were used respectively. Immediately after the injection, individual animals were placed in a metabolism cage and kept for 10 hours in most cases and for 48 hours in two experiments. For the first hour, CO₂ was collected every 20 minutes (15 minute-collection with a 5 minute-interval) and after the first hour every 30 minutes (15 minute-collection and 15 minute-interval). During the entire period of CO₂ collection, the animals had access to food and water and feces were collected.

Respiratory CO₂ was collected as shown in Figure 2. Air from a compressor (1) was passed through Drierite and Ascarite (2) to remove moisture and CO₂ respectively, and then through the animal chamber (3) at a constant rate which was measured by a flow meter. The air coming out of the chamber was re-dried by passing through the moisture-condensing cylinder (4), surrounded by dry ice in acetone. Finally, the dry air was passed through an alkaline solution of ethan-amine:methylcellosolve (1:2, v/v) to trap respiratory CO₂ (108).

Six ml. of the alkaline solution were found to be sufficient for the collection of respiratory CO₂ for a 15 minute period. The collection efficiency was tested by trapping expired air in a tube with a side arm which was connected to another tube. The efficiency was found to be 99.6%. One ml. aliquots of the ethan-amine-carbonate solution were

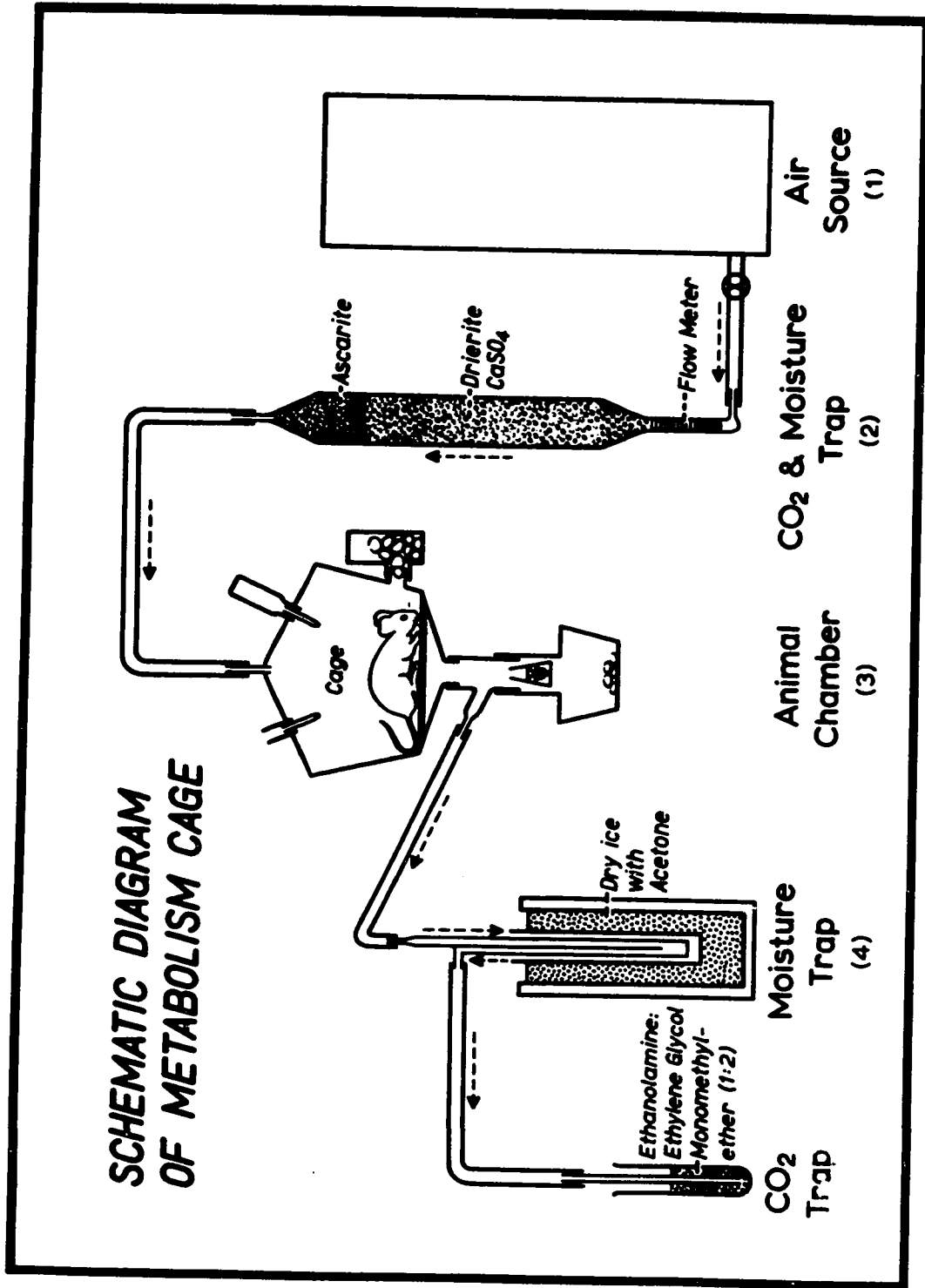


Figure 2.

diluted with 15 ml. of toluene:methylcellosolve (2:1, v/v), containing 0.55% PPO and 0.022% of POPOP (w/v). The radioactivity was assayed with a Liquid Scintillation Spectrometer. The counting efficiency was 70% according to the method of Automatic External Standard Ratio.

The results were expressed as a percentage of the injected dose expired per hour as $^{14}\text{CO}_2$ and also the percentage expired over the entire experimental period.

(b) For Determination of Radioactivity Distribution Among Blood, Liver and Whole Intestine and Feces

At the end of the experimental period, the animals were anesthetized with ether and 1 to 2 ml. (for small rats) or 3 to 5 ml. (for adult rats) of blood were removed from the heart with a syringe rinsed with heparin and the radioactivity in the whole blood was determined. The blood was mixed with 10 volumes of ethanol:ether (3:1, v/v). The mixture was heated to boiling, allowed to cool, the additional solvent was added to make 25 volumes in total, and the extracts were filtered. The filter papers and residues were rinsed three times with the same solvent mixture and these lipid extracts were made to 50 ml. in volumetric flasks.

The excised livers were rinsed in distilled water or saline, blotted dry, weighed, and homogenized in chloroform:methanol (2:1, v/v). The whole intestine (small and large intestinal tracts and their contents) was removed, rinsed in saline, combined with the

feces, and ground in a mortar and pestle. The ground tissues, contents and feces were washed into 250 ml. Erlenmeyer flasks with chloroform:methanol. Total lipids were extracted with 20 volumes of the solvent mixture at room temperature. The mixtures were then filtered into 250 ml. Erlenmeyer flasks and the filter papers rinsed at least three times. The extracts of liver and of whole intestine and feces were washed with 0.2 volumes of water in a separatory funnel according to the method of Folch et al (69) and the lower chloroform phase was collected. After being taken to dryness, total lipids were weighed and redissolved in chloroform and made up to 100 ml. Aliquots from the extracts of the three tissues were transferred to glass scintillation counting vials. The chloroform was evaporated under nitrogen, the lipids were dissolved in 15 ml. of the scintillator solution and 1 ml. of the CO₂ trap mixture was added. The samples were counted with Liquid Scintillation Spectrometer. The counting efficiency was the same as that obtained in CO₂ experiments. One ml. aliquots from the solution which was given to each individual animal was assayed to determine the total counts given to an individual animal and this count was used as a standard. The data were expressed as a percentage of ¹⁴C in blood of whole body, in whole liver and in intestine and feces, based on the total counts injected.

(c) Liver Uptake and Disappearance of Injected 26-¹⁴C-Cholesterol from Plasma

Under light ether anesthesia, the animals were injected by tail vein with a

solution containing an appropriate amount of the labeled cholesterol according to the age of the animals used. After various time intervals from five minutes to eight hours, blood was obtained by heart puncture using a heparin-rinsed syringe and the liver was removed at the same time. Plasma was obtained from the removed blood after centrifugation at 1,500 rpm for 30 minutes and 1 ml. of plasma was mixed with 10 volumes of ethanol:acetone (1:1, v/v). The mixture was heated to boiling, allowed to cool, solvent was added to make 25 volumes, and the extract was filtered. The filter paper and the residue were rinsed three times with the same solvent mixture and the combined lipid extract was evaporated in a flash rotary evaporator and was made to 10 ml. in a volumetric flask. For extraction of lipid from liver the procedures described in the previous section were followed. For the assay of radioactivity 1 ml. of aliquots from both the plasma and liver lipid extracts were transferred to glass counting vials. The solvent was evaporated with nitrogen and the lipids dissolved in 15 ml. of the scintillator solution as used in the CO₂ collection experiments for counting with a scintillation counter. The counting efficiency was 86% according to the method of Automatic External Standard Ratio. The results were plotted as a percentage of ¹⁴C recovered from lipid extracts against time intervals based on the total counts injected into an individual rat.

Free and total cholesterol in plasma and liver were determined by the Sperry-Webb method (185).

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

1. Studies on Effect of Diets

The oxidative degradation of 26- ^{14}C -cholesterol was studied by measuring expired $^{14}\text{CO}_2$ in rats fed commercial and semisynthetic diets containing no fat, 15% butter or 15% corn oil. Since the commercial diet contained more bulk than the semisynthetic diet used here, some experiments were also carried out with rats fed a fat-free semisynthetic diet supplemented by 25% cellulose as bulk (a high-cellulose diet) in order to make the semisynthetic diet comparable to the commercial diet (see Appendix I(a) and I(b) for the composition of the diets).

Table I shows the distribution of radioactivity 10 hours after the administration of the labeled cholesterol. Two results obtained 48 hours after administration are also included. The values are expressed as a percentage of the injected dose. The percentage of radioactivity expired as $^{14}\text{CO}_2$, as shown in this table, gives a general picture of the degree of oxidation of cholesterol for animals on different diets. However, since CO_2 was collected from intact animals after a single injection of radioactive cholesterol and since liver is known to be the site of oxidation, a number of factors must be taken into consideration in interpreting the results.

TABLE I
 DISTRIBUTION OF ¹⁴C IN EXPIRED AIR AND TOTAL LIPID EXTRACTS OF TISSUES AFTER INJECTION
 OF 26-¹⁴C-CHOLESTEROL IN RATS FED DIFFERENT DIETS

DIETS	EXPIRED		BLOOD $\frac{(\%)*}{+}$	LIVER $\frac{(\%)}{+}$	INTESTINE AND FECES $\frac{(\%)}{+}$	BODY WEIGHT $\frac{(g.)}{+}$	EXPERI- MENTAL PERIOD $\frac{(hr.)}{+}$
	¹⁴ CO ₂ $\frac{(\%)}{+}$						
A. No fat							
Commercial	11.7 ± 0.80	12.8 ± 0.83	18.2 ± 0.64	9.9 ± 0.47	150 ± 4.4	10	
Semisynthetic	5.6 ± 0.13	13.8 ± 0.99	21.1 ± 0.66	8.0 ± 0.39	160 ± 3.8	10	
High Cellulflour	6.7 ± 0.28	12.4 ± 0.10	18.3 ± 0.14	7.2 ± 0.07	166 ± 3.8	10	
B. 15% Butter							
Commercial	11.2 ± 0.91	14.6 ± 2.70	18.0 ± 0.90	11.0 ± 0.65	155 ± 7.9	10	
Semisynthetic	4.8 ± 0.40	16.1 ± 4.10	22.0 ± 1.15	7.8 ± 0.55	164 ± 3.4	10	
C. 15% Corn Oil							
Commercial	9.8 ± 0.41	11.7 ± 1.92	19.8 ± 1.24	15.2 ± 0.94	155 ± 2.8	10	
Semisynthetic	5.4 ± 0.62	12.8 ± 2.72	20.8 ± 0.54	12.2 ± 1.86	161 ± 3.7	10	
15% Butter*							
Commercial	27.3	4.3	7.2		167	48	
Semisynthetic	16.4	4.2	10.5		172	48	

MEAN ± S. E., 6 determinations for A (High Cellulflour = 4 determinations) and C; 5 determinations for B.
 + = Expressed as percent of the administered dose.
 ** = Blood volume calculated on the basis of 6% of the body weight.
 * = Single result for 48 hr. experiments.

It is first of all important to know the level of 26-¹⁴C-cholesterol in the liver of the rats during the experimental period. The second factor is the liver cholesterol pool. Third is the liver weight and body weight of the animals used. The last factor is particularly influential in comparing results obtained from suckling, weaned and young adult rats. This kind of problem is also encountered in explaining the observed differences in the percentage of radioactivity recovered from the whole intestine and feces. In this case, the level of radioactivity present in the circulatory system throughout a ten-hour period will be one of the factors. A second factor will be the plasma cholesterol concentration, and, lastly, the size of the animals used.

In order to obtain the information on the level of radioactivity in the liver and plasma, animals were injected with 26-¹⁴C-cholesterol and sacrificed at various time intervals. In all of these experiments, cholesterol concentration of liver and plasma was also determined.

Figures 3, 4 and 5 show the levels of radioactivity in liver and plasma at various time intervals after the intravenous injection of 26-¹⁴C-cholesterol. Each point represents an average value of two or three determinations. These Figures indicate that the injected cholesterol disappeared rapidly from the circulatory system and was removed mostly by the liver. In the basically different dietary groups, the same

**LEVELS OF RADIOACTIVITY IN LIVER AND PLASMA
FOLLOWING INJECTION OF 26-¹⁴C-CHOLESTEROL.**

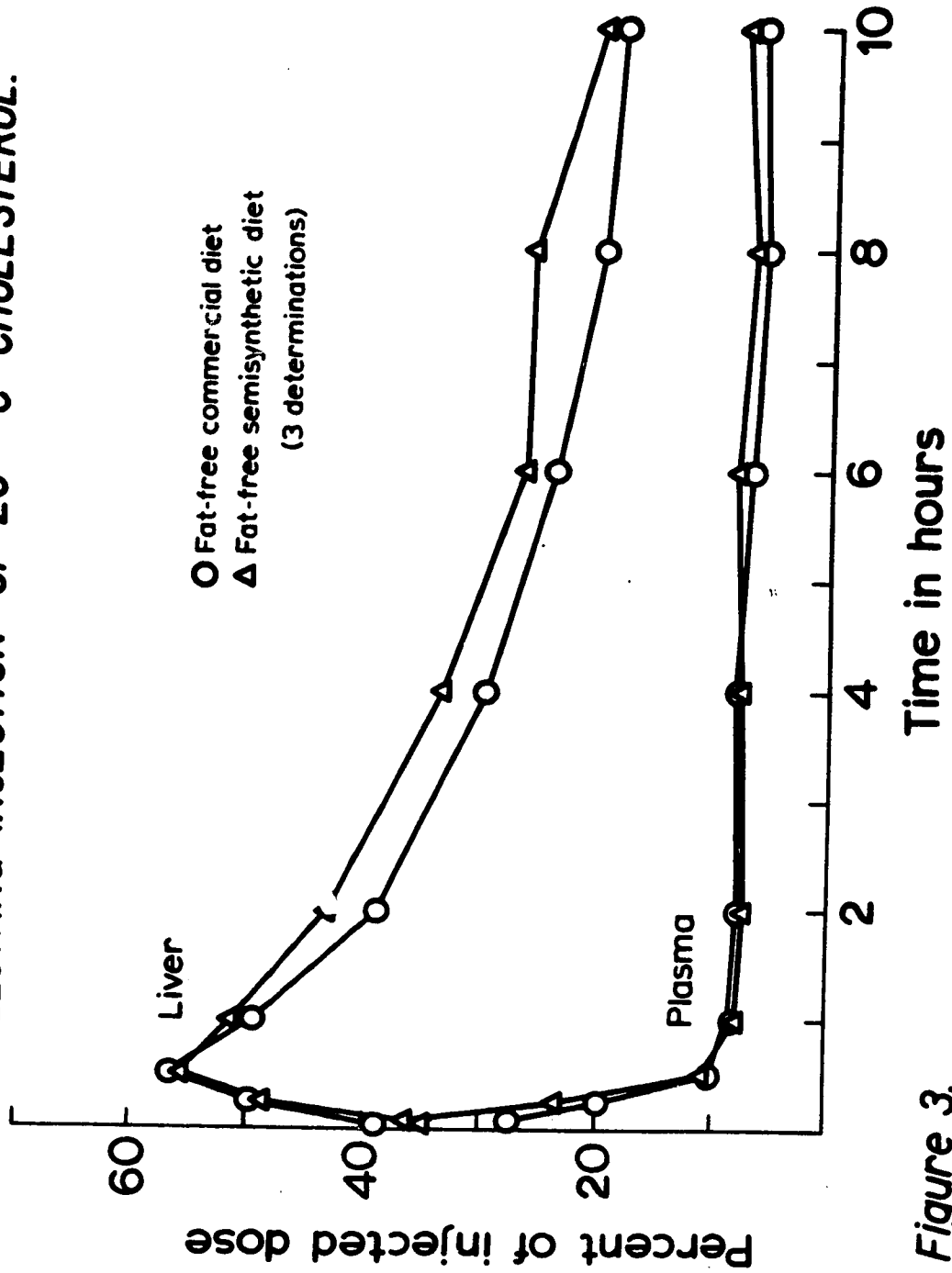


Figure 3.

**LEVELS OF RADIOACTIVITY IN LIVER AND PLASMA
FOLLOWING INJECTION OF 26-¹⁴C-CHOLESTEROL.**

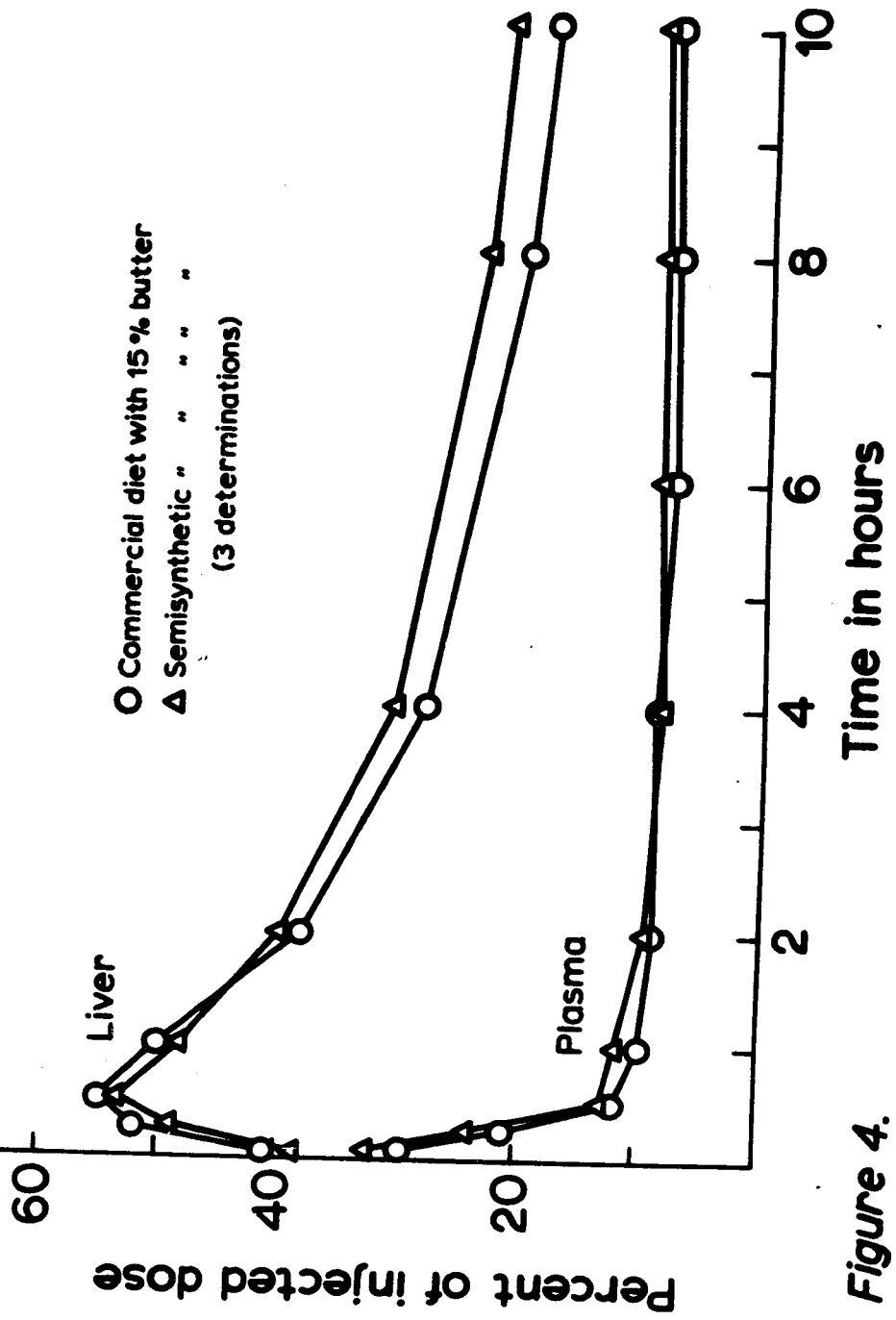


Figure 4.

**LEVELS OF RADIOACTIVITY IN LIVER AND PLASMA
FOLLOWING INJECTION OF 26-¹⁴C-CHOLESTEROL.**

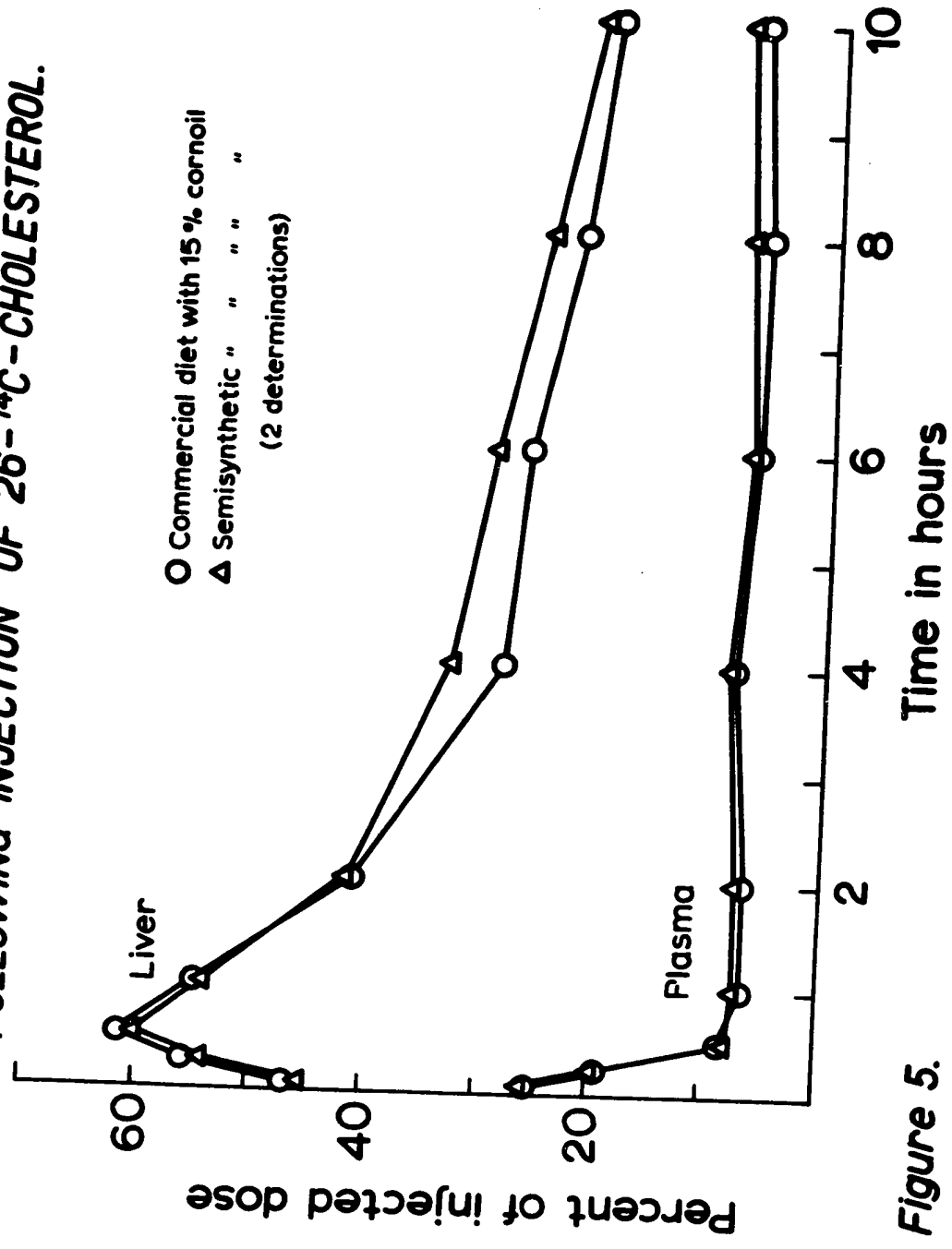


Figure 5.

pattern of fall of the label in plasma with simultaneous rapid rise of radioactivity in the liver occurred within 30 minutes. This was followed by a relatively constant level in plasma and a gradual decline of the liver level. The comparisons between rats fed the two basic diets do not show particular differences at the early time intervals, but later the liver levels in animals fed commercial diets are consistently lower than in those fed semisynthetic diets.

The rate of $^{14}\text{CO}_2$ production in rats fed different diets were plotted against time intervals in Figures 6, 7, and 8. Since CO_2 was collected for 15 minute intervals, the rates per hour were calculated according to the following formula:

$$\frac{(\text{Total cpm recovered from 15 min. CO}_2 \text{ collection X 4) X 100}}{\text{Total dose injected in cpm}}$$

These values were expressed as percent of the injected dose. Each point represents an average of five to six determinations. It was interesting to see here that the rates of $^{14}\text{CO}_2$ production showed the same pattern as the liver level at different time intervals.

Figure 6 shows the rates in groups given either commercial or semisynthetic diets containing no fat. The patterns have three characteristic phases: a rapid increase up to one or one and one-half hours, and relatively constant rate throughout the remaining period. The maximum rate of $^{14}\text{CO}_2$ production in the commercial diet and the semisynthetic diets occurs between one and two hours after injection and is

**RATE OF $^{14}\text{CO}_2$ PRODUCTION
FROM INJECTED ^{14}C -CHOLESTEROL**

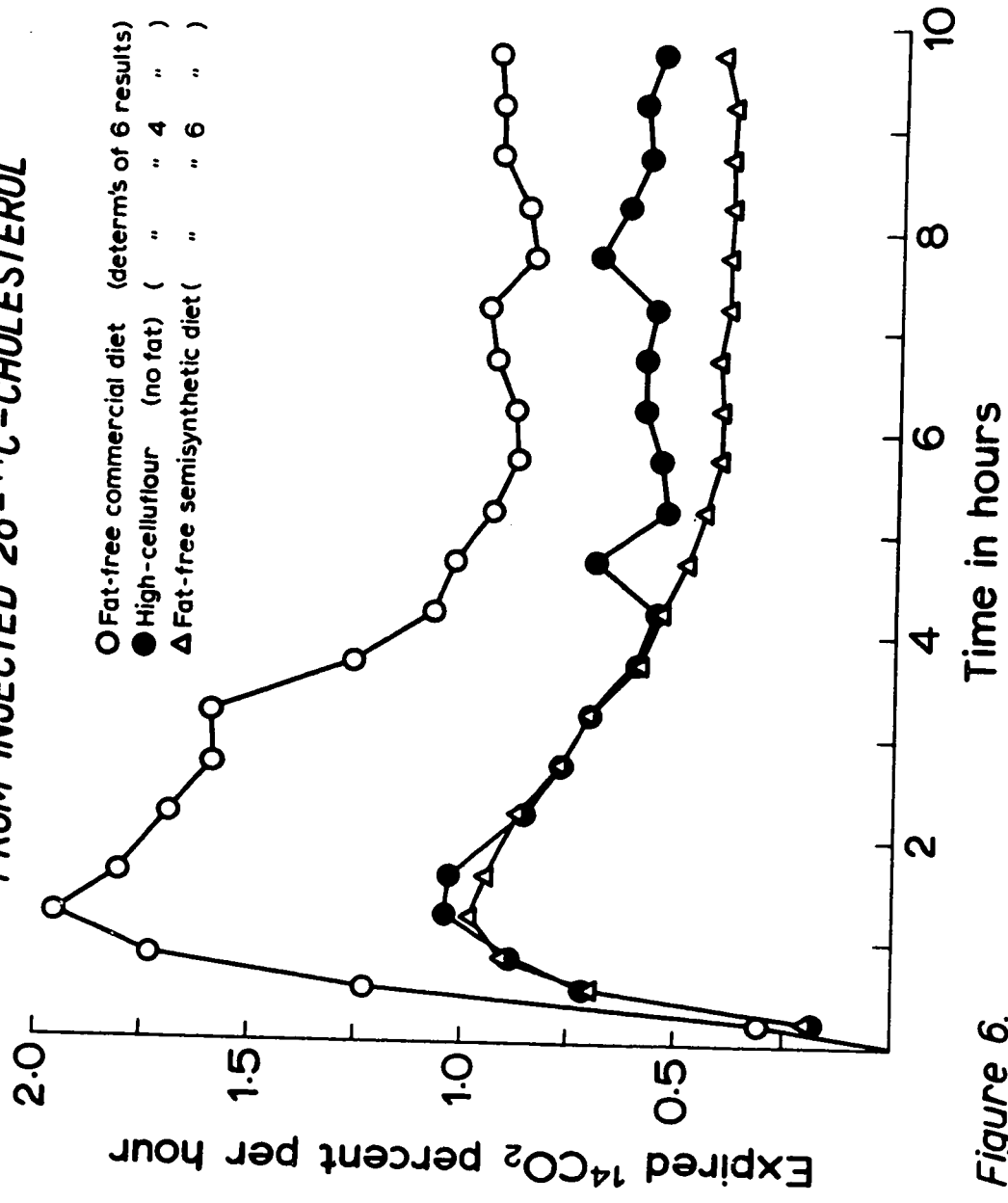


Figure 6.

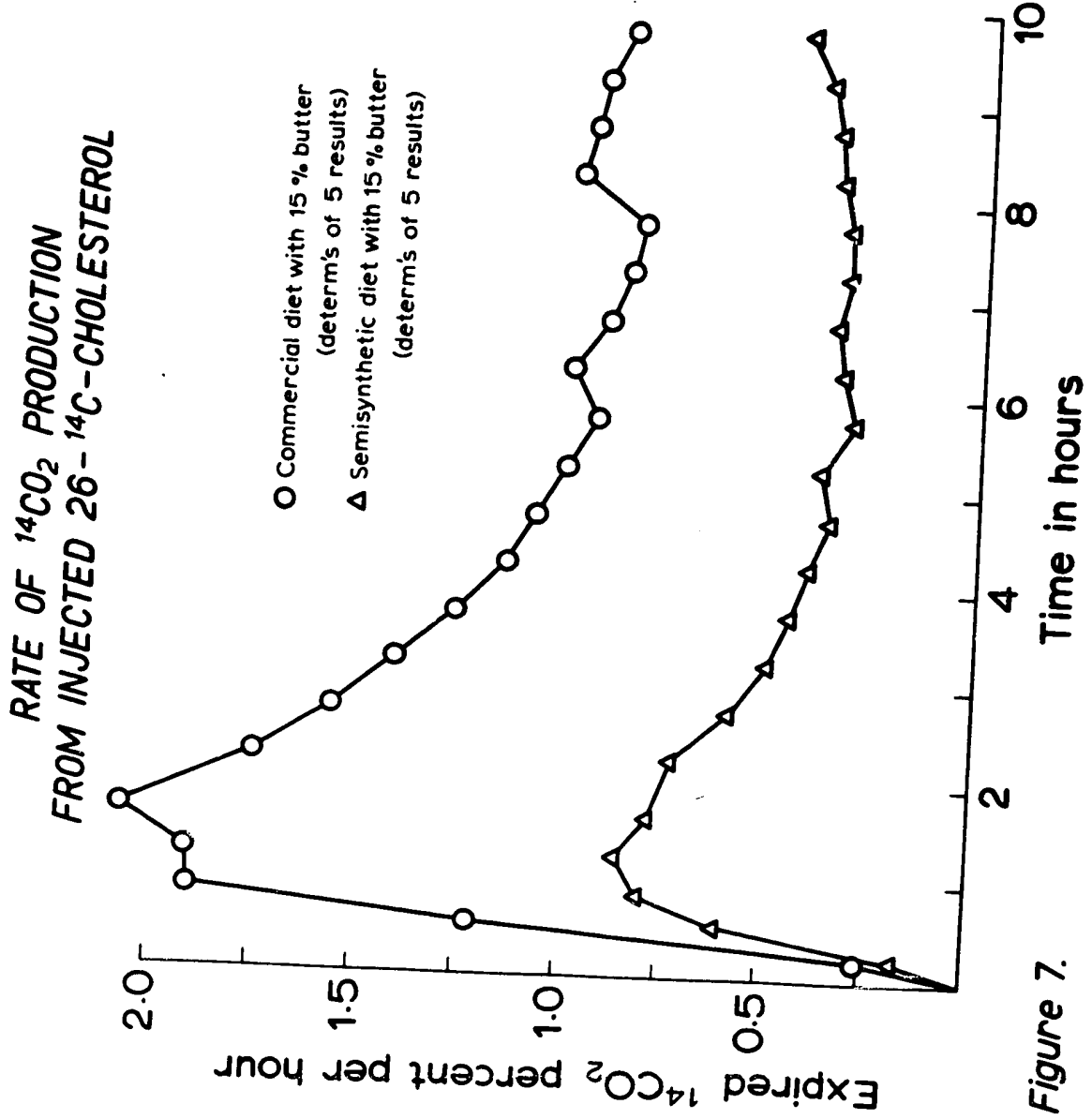


Figure 7.

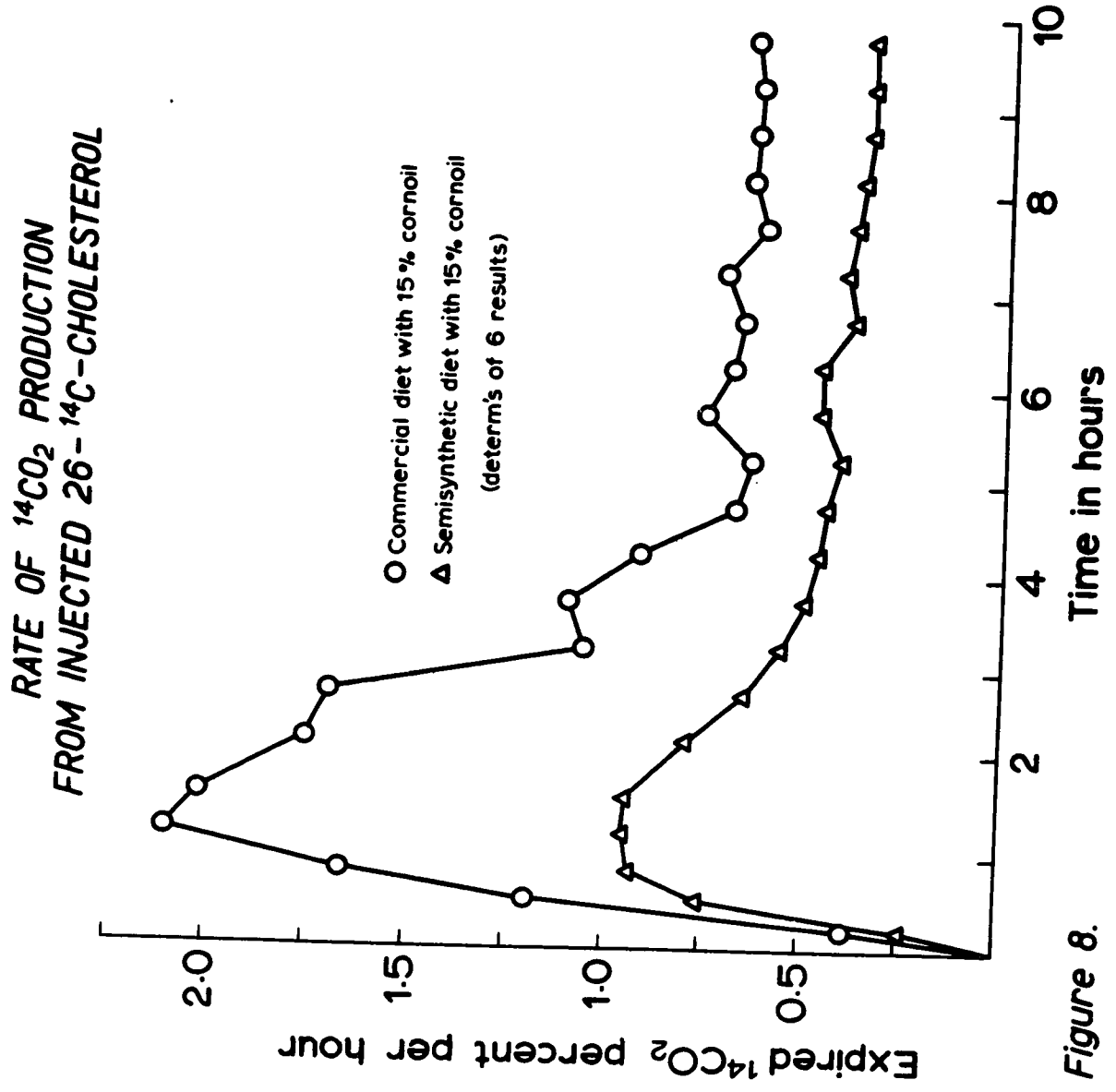


Figure 8.

around 2% and 1% per hour of the injected dose, respectively. Thus, the rats fed the commercial diet excreted $^{14}\text{CO}_2$ at a much higher rate than those fed the semisynthetic diets through the ten-hour experimental period. Rats fed a high-cellulose diet excreted $^{14}\text{CO}_2$ at a slightly higher rate than those fed a low cellulose diet from five hours after injection. The rates of $^{14}\text{CO}_2$ production in the groups fed two types of diets containing 15% butter are compared in Figure 7. They are not significantly different from those in Figure 6. Figure 8 shows the comparison of the rates between two groups given the 15% corn oil diets. The semisynthetic dietary group shows a similar pattern to those in Figures 6 and 7, but the commercial dietary group shows a more rapid fall in level between two and six hours and a lower level at later time intervals. However, here again it is noticeable that rats given the commercial diet excreted $^{14}\text{CO}_2$ at a higher rate than those fed the semisynthetic diet throughout the ten-hour period.

In order to test the possibility that a longer term experiment would show a different picture of $^{14}\text{CO}_2$ production, one rat from those fed the commercial and semisynthetic diets containing 15% butter was chosen and the expired CO_2 was collected for 48 hours after injection of the labeled cholesterol. The results are shown in Figure 9. This demonstrates that even beyond ten hours there is still a definite difference in the rate of $^{14}\text{CO}_2$ production in rats fed the different diets, although this difference is not marked in much later time intervals.

**RATE OF $^{14}\text{CO}_2$ PRODUCTION
FROM INJECTED $^{26}\text{-}^{14}\text{C}$ -CHOLESTEROL FOR 48 HOURS.**

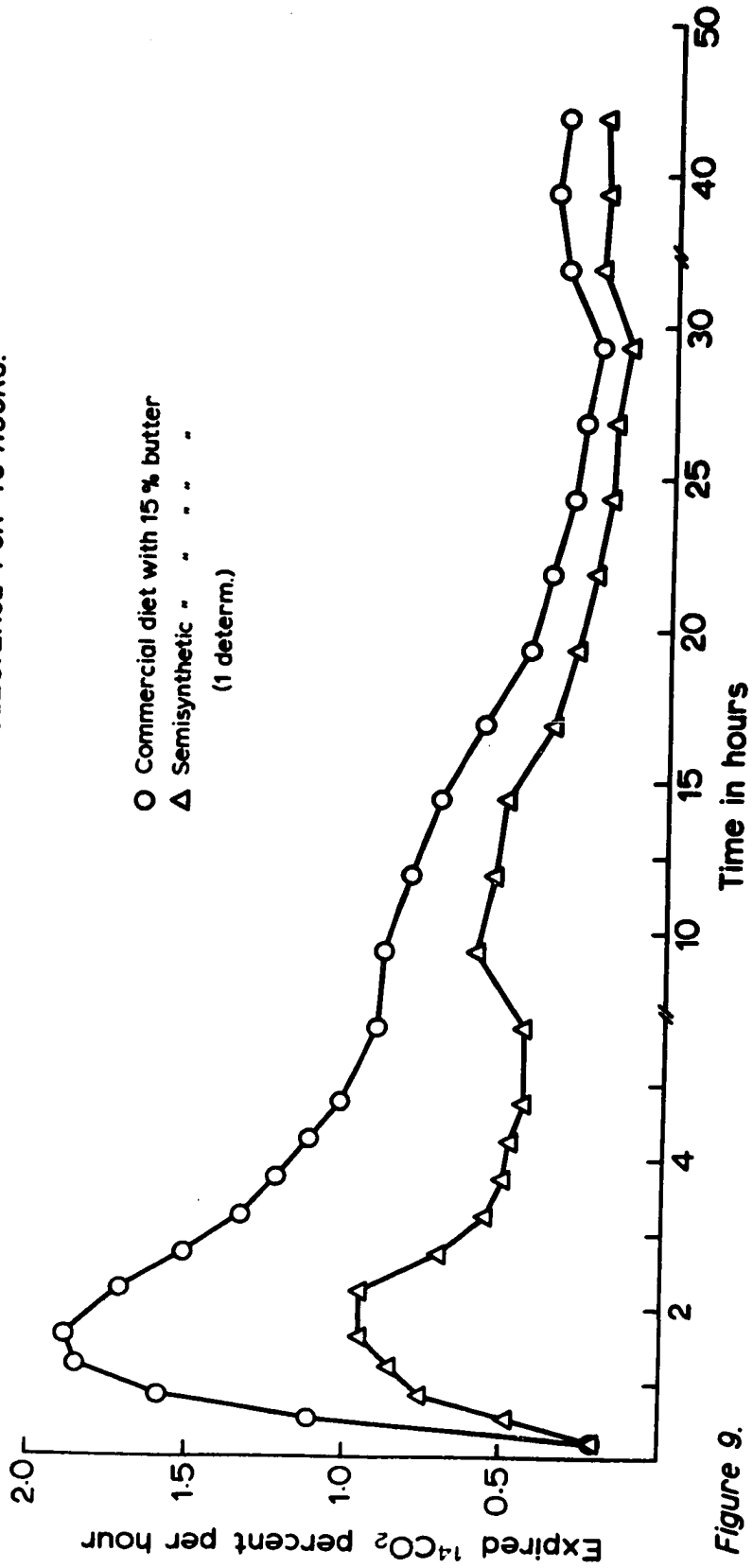


Figure 9.

This latter phenomenon appears to be related to the amounts of radioactive cholesterol available for catabolism in the liver in this time interval.

Since the patterns of rates of $^{14}\text{CO}_2$ production shown in the above figures were basically similar to those of liver levels at various time intervals, an attempt was made to express the rates on the basis of liver levels at given time intervals. However, as shown in Figure 10 as an example, a direct comparison of ratio of the rate of $^{14}\text{CO}_2$ production to liver level at the corresponding time intervals was not possible because there is a distinct time lag in $^{14}\text{CO}_2$ production. In other words, the maximum value occurred between one and two hours in $^{14}\text{CO}_2$ experiments but the maximum was attained at 30 minutes in liver level experiments.

Therefore, a simple and reasonable comparison in the amount of $^{14}\text{CO}_2$ expired for ten hours can be made by the ratio of percent of $^{14}\text{CO}_2$ expired for ten hours to the average percent of level of the radioactivity present in liver throughout the experimental period. For these latter values the sum of the liver levels which were read every 30 minutes from the liver level curves (Figures 3, 4 and 5) were divided by the corresponding number of the readings. The method of calculation is explained in Figure 11.

An interesting finding observed in the process of calculation was that the average liver level values were consistently in each case 1.6

COMPARISON OF RATE OF $^{14}\text{CO}_2$ EXPIRATION WITH RADIOACTIVITY LEVEL OF LIVER.

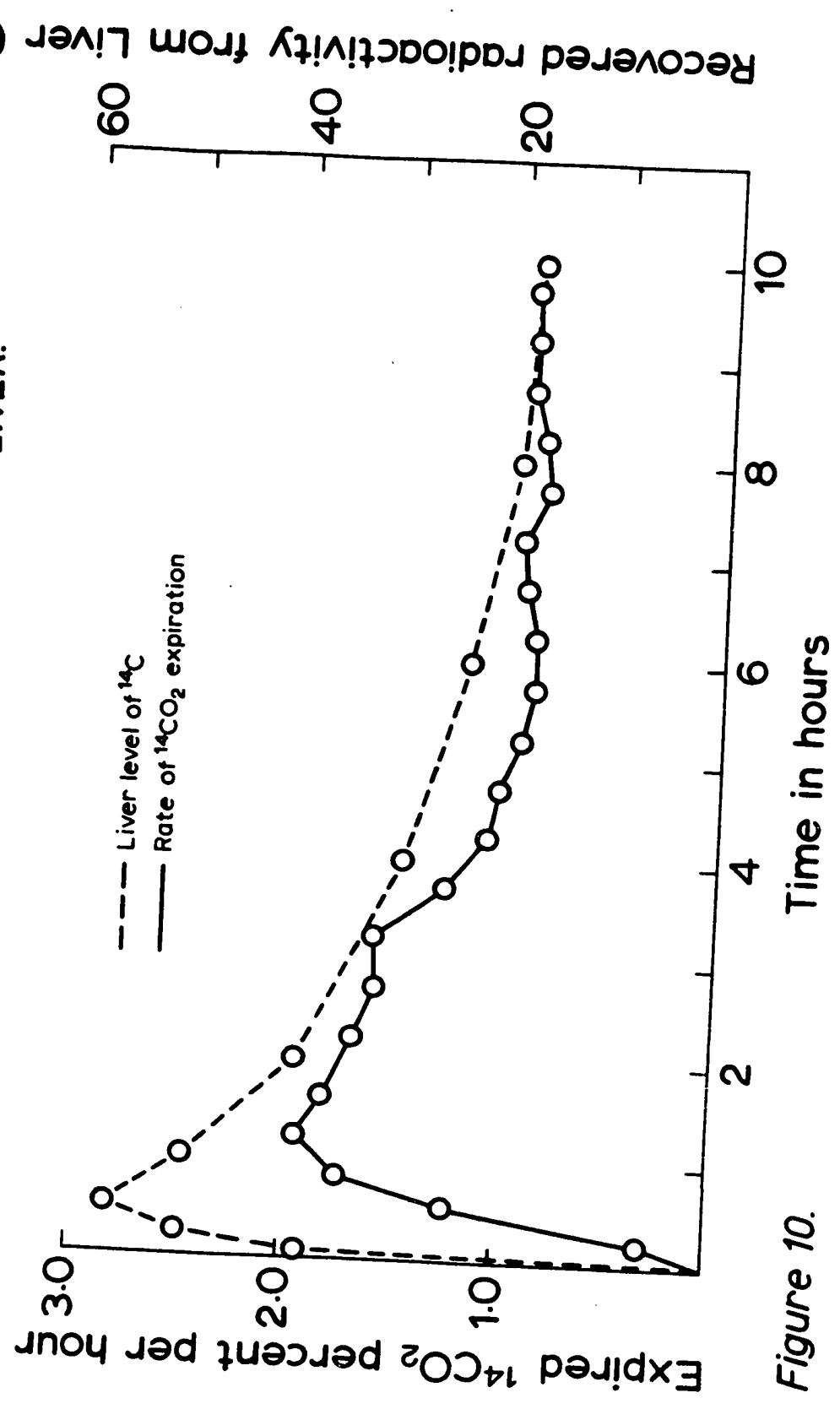
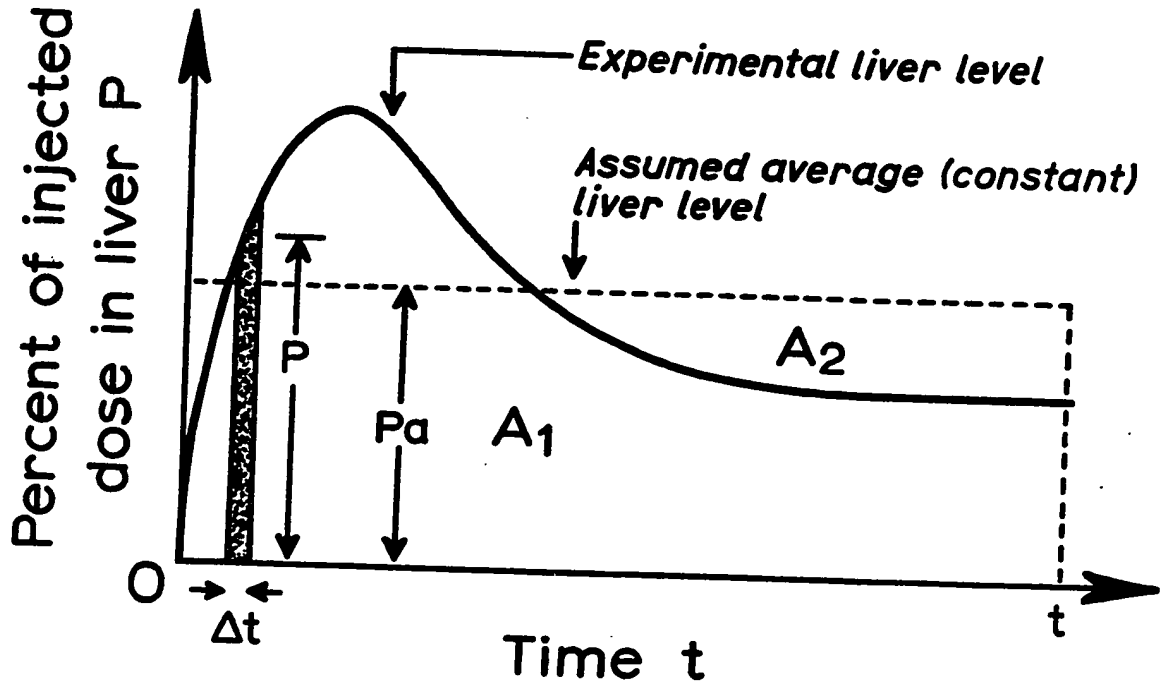


Figure 10.

CALCULATION METHOD FOR AVERAGE LIVER LEVEL



$t = n \cdot \Delta t$ where n = number of time interval

$$A_1 = \Delta t (P_1 + P_2 + P_3 + \dots + P_n)$$

$$A_2 = P_a \cdot t = P_a \cdot n \cdot \Delta t$$

For $A_1 = A_2$ Equivalent average liver level

$$P_a = \frac{1}{n} (P_1 + P_2 + P_3 + \dots + P_n)$$

P = percent of radioactivity in liver

Figure 11.

times greater than the values of the residual radioactivity in livers of rats after a ten-hour collection period regardless of the type of diets used. Based on this finding, the average liver level for the high-cellulose dietary group was obtained by multiplying the percent of the residual radioactivity in the liver after CO₂ collection period by 1.6 ($18.3 \times 1.6 = 29.3$) since liver level was not measured in this group.

These average values in each dietary group are equivalent to liver level of the radioactivity throughout ten hours. Both the percent of ¹⁴CO₂ and of liver levels were expressed as percent per gram of liver for further correction of the values. These are shown in Table II. These ratios of percent of ¹⁴CO₂ production per gram of liver were obtained without consideration of liver cholesterol concentration.

Table III shows cholesterol concentration in liver and plasma in different dietary groups. Total liver cholesterol concentration was increased in rats by feeding corn oil in either diet as compared to those by feeding butter or no fat in the respective diets. Therefore, it is necessary to consider the dilution effect of the injected radioactive cholesterol per gram of liver.

Thus, the ratios in the third column in Table II were corrected for cholesterol pool equivalent to 2 mg. per gram of liver since liver cholesterol concentration in most cases is around 2 mg per gram of liver. In other words, these values were multiplied by correction factors which are

TABLE II

QUANTITATIVE ESTIMATION OF CONVERSION OF CHOLESTEROL TO BILE ACID IN RATS FED DIFFERENT DIETS

Diets	(a)	(b)	(c)		Cholesterol converted		Liver Weight (g.)
	Expired $^{14}\text{CO}_2$ per g. of liver for 10 hr. (%)	Average level of ^{14}C per g. of liver for 10 hr. (%)	Ratio a/b	Corrected Ratio	to Bile Acid	Per 6 g. of liver 24 hr. (mg.)	
No Fat	2.0	5.0	0.39	0.45	0.90	13.0	5.70±0.17
	0.9	5.2	0.17	0.20	0.40	5.8	6.25±0.16
15% Butter	1.9	4.8	0.39	0.39	0.78	11.3	5.96±0.32
	0.8	5.2	0.16	0.16	0.32	4.6	6.21±0.13
15% Corn Oil	1.7	5.4	0.31	0.51	1.02	14.6	5.74±0.11
	0.9	5.6	0.16	0.24	0.48	7.0	5.95±0.15
No Fat	1.1	4.9*	0.22	0.26	0.52	7.5	6.00±0.15

MEAN ± S. E.

* See the text.

(c) Corrected for liver cholesterol pool equivalent to 2 mg. per g. of liver weight.

TABLE III
CONCENTRATION OF CHOLESTEROL IN PLASMA AND LIVER OF RATS FED DIFFERENT DIETS

Diets	Plasma Cholesterol mg. / 100 ml.			Liver Cholesterol mg. / 100 g.		
	Free	Ester	Total	Free	Ester	Total
A. No Fat						
Commercial	17.3±0.95	49.0±1.36	66.3±1.53	204.2±6.08	26.4±0.97	230.6±5.93
Semisynthetic	18.4±0.22	50.8±1.70	69.2±1.77	205.8±7.70	31.2±5.20	236.9±7.22
B. 15% Butter						
Commercial	14.0±1.05	40.5±3.92	54.5±4.80	172.0±5.90	30.0±4.30	202.0±15.3
Semisynthetic	17.3±1.13	42.9±4.80	60.2±5.00	179.0±6.15	31.0±5.62	210.0±16.7
C. 15% Corn Oil						
Commercial	20.0±1.35	56.4±2.37	76.4±2.72	226.3±9.14	102.8±8.55	329.1±13.05
Semisynthetic	22.7±0.78	60.8±2.32	83.5±3.02	219.0±5.83	81.5±5.22	300.5±9.46
D. No Fat						
High-Celluloflour	21.6±0.46	45.7±0.46	67.3±0.51	228.2±9.04	17.5±0.34	245.7±7.19

MEAN ± S. E.

A = 24 determinations

B = 6 determinations

C = 16 determinations

D = 6 determinations

the ratio of cholesterol mg. per gram of liver in each group to 2 mg. of cholesterol. These final values show how much of the radioactivity available for each gram of liver was excreted as $^{14}\text{CO}_2$ over ten hours. It is clear from this table that rats fed the commercial diets produced more than twice as much as $^{14}\text{CO}_2$ as those fed the semi-synthetic diets.

An attempt was made to quantitate the cholesterol in mg. per gram of liver converted to bile acids using these corrected ratios. Since these ratios were already corrected for 2 mg. of cholesterol per gram, it was possible to obtain the quantity of cholesterol in mg. converted to bile acid as shown in the fifth column of the table. For 150 g. rats, with livers weighing approximately 6 g., about 11 to 14 mg. of cholesterol was transformed into bile acid during 24 hours in animals given the commercial diets, whereas only about 5 to 7 mg. was converted to bile acid during the same period of time in rats given the semisynthetic diets. In addition, feeding corn oil in either diet slightly increased the transformation of cholesterol to bile acid as compared to those fed 15% butter or no fat in the corresponding basic diets. On the other hand, the addition of 15% butter to either diet appears to inhibit the formation of bile acid slightly as compared with the addition of no fat to the respective diets.

The addition of cellulflour as a bulk slightly increased the formation of bile acid as compared with a low-cellulflour diet (fat-free

semisynthetic diet). However, the magnitude of this increase was far less than that in rats fed the fat-free commercial diet.

Therefore, it is concluded that oxidative degradation of cholesterol is mainly influenced by the basic diet, commercial or semisynthetic, and only slightly influenced by the type of fats or by the addition of cellulose as bulk.

For reasons explained previously, the amounts of radioactivity recovered from the whole intestine and feces were expressed relative to the average level of radioactivity present in the plasma during the experimental period. For the latter values, as in the calculation for the average liver level, the sum of the plasma levels of radioactivity, which were taken at 15 minute intervals and including the values for 5 minutes after injection from plasma level curves in Figures 3, 4 and 5, was divided by the corresponding number of readings. They are expressed as percent of the injected dose and equivalent plasma level present throughout the experimental period.

The calculated ratios are present in the third column of Table IV. These ratios, as in the case of calculation for $^{14}\text{CO}_2$ were corrected for plasma cholesterol concentration (see Table III) of 69 mg. per 100 ml., since the average cholesterol concentration was 69 mg. per 100 ml. In these cases, the correction factor for cholesterol concentration was also made by dividing the original cholesterol mg. in each group by 69 mg. and the ratios presented in

TABLE IV

DISTRIBUTION OF 26-14C-CHOLESTEROL TO THE WHOLE INTESTINE IN RATS FED DIFFERENT DIETS

Diets	(a)*	(b)	(c)		(d)	
	Total 14C Recovered from Whole Intestine (%)	Average Plasma Level of 14C for 10 hr. (%)	Ratio a/b	Corrected Ratio	Cholesterol Distributed to Intestine for 10 hr. (mg.)	Cholesterol Distributed to Intestine for 24 hr. (mg.)
No Fat	9.9	8.0	1.24	1.19	4.07	9.77
Commercial Semisynthetic	8.0	8.6	0.93	0.93	3.18	7.63
15% Butter	11.0	9.1	1.21	0.96	3.28	7.87
Commercial Semisynthetic	7.8	10.2	0.76	0.72	2.46	5.90
15% Corn Oil	15.2	7.3	2.08	2.31	7.90	18.96
Commercial Semisynthetic	12.2	8.0	1.52	1.84	6.29	15.20
No Fat	7.2	8.0	0.9	0.88	3.01	7.22
High-Celluloflour						

(c) Corrected for plasma cholesterol equivalent to 69 mg. per 100 ml.

(d) Based on plasma cholesterol in whole body (3.42 mg.).

* Data presented in Table I.

the third column were multiplied by these correction factors.

Only these values indicate how much of the radioactivity present in the plasma in the whole body was distributed to the whole intestine during ten hours. Assuming that the animal body weight was 150 g. and the blood volume was 6 ml. per 100 g. with a hematocrit of 0.45, the amount of plasma cholesterol distributed to the whole intestine for ten hours or for 24 hours, in extrapolating for a longer period, can be derived from these ratios.

From these last two columns, one can notice two points: one is the effect of the commercial diet versus the semisynthetic diet and the second is the effect of corn oil versus butter or fat-free diets. The former effect is only slight but the second is very marked. Thus the amount of cholesterol contributed to the whole intestine from circulating plasma cholesterol is increased more than twice in rats fed corn oil in either diet compared to those fed butter in either diet. On the other hand, there were slight decreases in rats fed butter in commercial and semisynthetic diets as compared to those fed the respective fat-free diets.

2. Studies on Suckling and Recently-Weaned Rats

The oxidative degradation of cholesterol was also studied in suckling and weaned rats. The presentation of results obtained from these groups, the calculation of the average values of liver level of radioactivity and the expression of the amounts of $^{14}\text{CO}_2$ production

and of the recovered radioactivity from whole intestine and feces will be followed as in the previous dietary experiments.

Table V shows the distribution of radioactivity after a ten-hour experimental period. Results with young adult rats on standard commercial diet are included for comparison. These values are expressed as a percentage of the injected dose of 26- ^{14}C -cholesterol. The comparison of results obtained from these different groups is particularly difficult because of the different ranges of body weight. The suckling and weaned rats weighed one-sixth and one-half, respectively, as much as the young adult rats. In addition, plasma cholesterol concentration in the suckling rats was markedly different from that in weaned and young adult rats. Therefore, the expression of results obtained from these groups of rats based on total injected dose as a whole will give no information without normalization of the results obtained.

In order to determine the amount of $^{14}\text{CO}_2$ production for ten hours and the amount of cholesterol converted to bile acid, the level of injected 26- ^{14}C -cholesterol in liver with time was also studied and is shown in Figure 12. The general pattern of appearance and disappearance of the injected radioactive cholesterol in liver with time is very similar in the different groups and is also similar to those in the dietary groups of rats: the initial rapid uptake, reaching a maximum level within 30 minutes and the gradual decline of the radioactivity after

TABLE V
 DISTRIBUTION OF ^{14}C IN EXPIRED AIR AND TOTAL LIPID EXTRACTS OF TISSUES AFTER
 INJECTION OF 26- ^{14}C -CHOLESTEROL IN RATS OF DIFFERENT
 AGES

<u>Rats</u>	<u>Expired $^{14}\text{CO}_2$ (%) *</u>	<u>Blood (%)*+</u>	<u>Liver (%)*</u>	<u>Intestine and Feces (%) *</u>	<u>Body Weight (g.)</u>
A. Young Adult	11.8 ± 0.90	13.3 ± 1.60	17.2 ± 0.88	9.0 ± 0.55	168 ± 3.3
B. Weaned	8.8 ± 1.16	12.7 ± 0.06	16.0 ± 0.07	10.4 ± 0.10	88 ± 2.6
C. Suckling	4.8 ± 0.51	10.4 ± 0.48	12.3 ± 0.60	5.5 ± 0.24	28 ± 1.8

MEAN ± S.E., 5 Determinations for A and C; 4 determinations for B.

*Expressed as a percent of the administered dose.

+ Blood volume calculated on the basis of 6 percent of body weight.

LIVER LEVELS OF RADIOACTIVITY FOLLOWING THE INJECTION OF 26-¹⁴C-CHOLESTEROL.

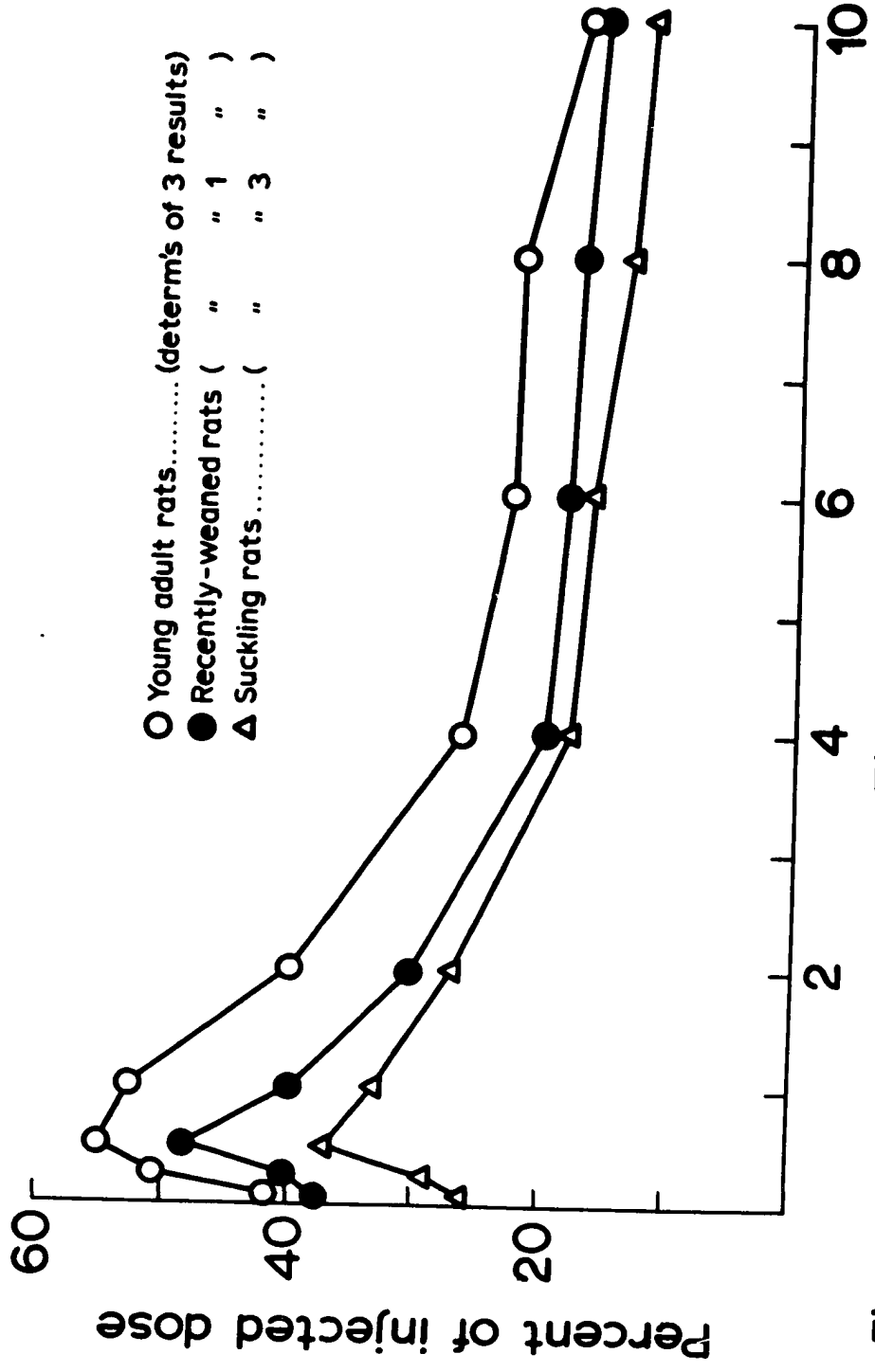


Figure 12.

this time interval. As in the case of the previous experiments, the rate patterns of $^{14}\text{CO}_2$ production with time in these groups are also similar to those of liver level curves. They are shown in Figure 13.

Since a direct comparison by using ratios of rate of $^{14}\text{CO}_2$ production to liver level with time is not possible due to the presence of a time lag as explained previously, the amounts of $^{14}\text{CO}_2$ expired for ten hours are also expressed as ratios. These are presented in Table VI. These ratios are also corrected for liver cholesterol concentration equivalent to 2 mg. per g. of liver (see Table VII for cholesterol concentration in plasma and liver). Only these corrected ratios or the calculated amount of cholesterol converted to bile acid by each gram of liver allows a comparison of the ability of the liver to oxidize cholesterol in each group of rats. Thus, in suckling rats the rate of conversion of cholesterol to bile acid is only half of that in young adult rats and the rate in weaned rats is intermediate to those of suckling and young adult rats.

It is possible to quantitate from these ratios the amount of bile acid formed from cholesterol in suckling and weaned rats: 1.2 mg. per rat per day in the former and 5.8 mg. per rat per day in the latter.

Total radioactivity recovered from the whole intestine and feces during ten hours in each group shown in Table V was also normalized for body weight and plasma cholesterol concentration. They are expressed relative to the levels of injected ^{14}C -cholesterol present in

**RATE OF $^{14}\text{CO}_2$ PRODUCTION
FROM INJECTED $26\text{-}^{14}\text{C}$ -CHOLESTEROL**

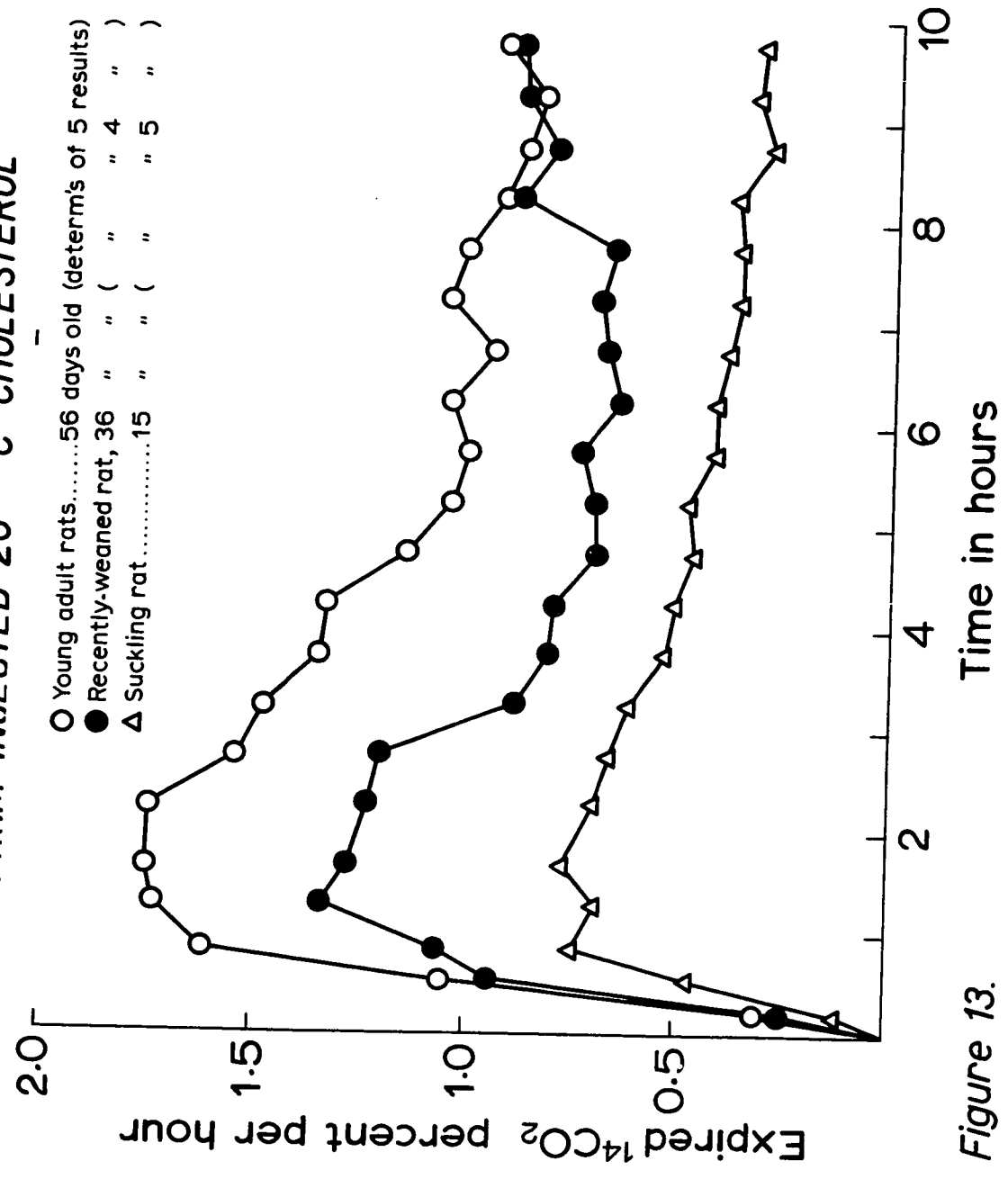


Figure 13.

TABLE VI
 QUANTITATIVE ESTIMATION OF CONVERSION OF CHOLESTEROL TO BILE ACID IN RATS
 OF DIFFERENT AGES

Rats	(a)	(b)	(c)				
	Expired ^{14}C per g. of Liver for 10 hr. (%)	Average Level of ^{14}C per g. of liver for 10 hr. (%)	Ratio a/b	Corrected Ratio	Cholesterol converted to Bile Acid	Liver* Weight (g.)	
					Per g. of Liver for 10 hr. (mg.)	Per 6 g. of Liver for 24 hr. (mg.)	
Young Adult	1.7	4.1	0.41	0.47	0.94	13.5	6.95±0.13
Weaned	2.5	6.7	0.37	0.35	0.70	10.1	3.46±0.10
Suckling	4.8	19.5	0.25	0.25	0.50	7.2	1.00±0.06

* MEAN ± S.E.

(c) Corrected for liver cholesterol pool equivalent to 2 mg. per g. of liver weight.

TABLE VII
CHOLESTEROL CONCENTRATIONS OF PLASMA AND LIVER OF RATS OF DIFFERENT AGES

<u>Rats</u>	<u>Plasma Cholesterol mg./100 ml.</u>			<u>Liver Cholesterol mg./100 g.</u>		
	<u>Free</u>	<u>Ester</u>	<u>Total</u>	<u>Free</u>	<u>Ester</u>	<u>Total</u>
* Young Adult	23.0 ± 0.92	59.5 ± 1.23	82.5 ± 3.95	198.0 ± 3.85	27.7 ± 2.60	225.7 ± 2.70
** Weaned	22.5	48.0	70.5	170.2	21.8	192.0
*** Suckling	38.0	69.8	107.8	180.0	30.0	210.0

* MEAN ± S.E., 16 determinations.

** Pooled data of three animals.

*** Data from K.K. Carroll, Published in *Canad. J. Biochem.* Vol. 42: 79-86, 1964.

blood, measured after a ten-hour experimental period and corrected for plasma cholesterol concentration equivalent to 69 mg. per 100 ml. These corrected values are presented in Table VIII. On all the other experiments, it was observed that the level of radioactivity in plasma was relatively constant from one hour after the injection up to ten hours and that the average level was very near to that obtained after a ten-hour experimental period. Therefore, the radioactivity levels of blood measured at the end of the experiment can be considered to be representative during ten hours.

Total radioactivity recovered from the whole intestine and feces presented in the column (a) in Table VIII is lower in suckling rats than in weaned or young adult rats. However, the normalized values (the column (c)) show that the magnitude of the distribution of cholesterol from the circulatory system to the intestine is similar in every group. The lower radioactivity recovered from the intestine of suckling rats is therefore due to the dilution effect of the injected labeled cholesterol by a higher plasma cholesterol concentration.

3. Studies on the Effects of Fasting, X-Irradiation and Triton-Treatment

The oxidative degradation of $26\text{-}^{14}\text{C}$ -cholesterol was also studied in fasted, X-irradiated-fasted and Triton-treated rats and the results were compared with those obtained from normal young adult rats as a control. The presentation and expression of the results and

TABLE VIII
DISTRIBUTION OF 26-¹⁴C-CHOLESTEROL TO THE WHOLE INTESTINE IN RATS OF DIFFERENT AGES

<u>Rats</u>	(b)		Corrected Ratio
	(a)	(c)	
	Total ¹⁴ C Recovered from Whole Intestine and Feces (%)	Blood Level of ¹⁴ C After 10 Hr. (%)	Ratio a/b
Young Adult	9.0	13.3	0.68
Weaned	10.4	12.7	0.82
Suckling	5.5	10.4	0.53

(a) Data presented in Table V.

(b) Data presented in Table V.

(c) Corrected for plasma cholesterol pool equivalent to 69 mg. per 100 ml.

the calculations are the same as in the previous experiments.

Table IX shows the distribution of the radioactivity after ten hours. In X-irradiated-fasted rats there was a marked increase in liver weight compared to the fasted groups. Fasting reduced liver weight proportionately more than total body weight as shown by the decrease in liver weight expressed as a percentage of whole body weight (see the last column of the table). Liver weight decreased from 4.1 to 3.6. Radiation, on the other hand, not only counteracted the effect of fasting but also caused an actual increase in liver weight in irradiated-fasted rats as compared to fed controls.

Figure 14 shows the level of injected cholesterol in liver at different time intervals in three experimental groups and the control. The liver level patterns of radioactivity in fasted and X-irradiated-fasted rats are similar to those of the control group. However, Triton-treated rats showed a marked difference. In the latter group, the levels are relatively constant from five minutes after injection throughout the remaining period. The lower liver level in the Triton-treated group is accompanied by a higher level in plasma as shown in Figure 15. In the control group, the plasma level pattern is almost the same as those seen in the previous dietary experiments, but in the Triton-treated group the injected labeled cholesterol disappeared gradually and continuously from the circulatory system throughout the ten-hour period. The lower liver levels in Triton-treated groups are

TABLE IX

DISTRIBUTION OF ^{14}C IN EXPIRED AIR AND TOTAL LIPID EXTRACTS OF TISSUES AFTER INJECTION
OF 26- ^{14}C -CHOLESTEROL IN RATS UNDER DIFFERENT TREATMENT

Rats	Expired $^{14}\text{CO}_2$ (%)*	Blood (%)*+	Liver (%)*	Intestine and Feces (%)*	Body Weight (g.)	Liver Weight (g.)	Liver Weight as Percent of Body Weight
A. Control	11.8 ±	13.3 ±	17.2 ±	9.0 ± 0.55	168 ±	6.95 ±	4.1
	0.90	1.60	0.88		3.3	0.11	
B. Fasted	6.3 ±	15.3 ±	18.0 ±	9.3 ± 0.67	Original	5.38 ±	3.6
	0.31	0.63	0.35		183±2.0 Actual 150±3.1	0.11	
C. X-irradiated - Fasted	10.4 ±	15.7 ±	19.0 ±	8.8 ± 0.79	Original	7.40 ±	4.6
	0.80	0.60	1.02		180±4.4 Actual 160±6.1	0.20	
D. Triton-treated	6.4 ±	44.6 ±	16.6 ±	6.7 ± 0.32	159 ±	6.34 ±	4.0
	0.42	1.90	0.35		5.0	0.20	

MEAN ± S.E., 5 determinations for A, B and D; 4 determinations for C.

* Expressed as percent of the administered dose.

+ Blood volume calculated on the basis of 6% of body weight.
Control means young adult rats fed on laboratory chow diet.

LIVER LEVELS OF RADIOACTIVITY FOLLOWING THE INJECTION OF 26-¹⁴C-CHOLESTEROL.

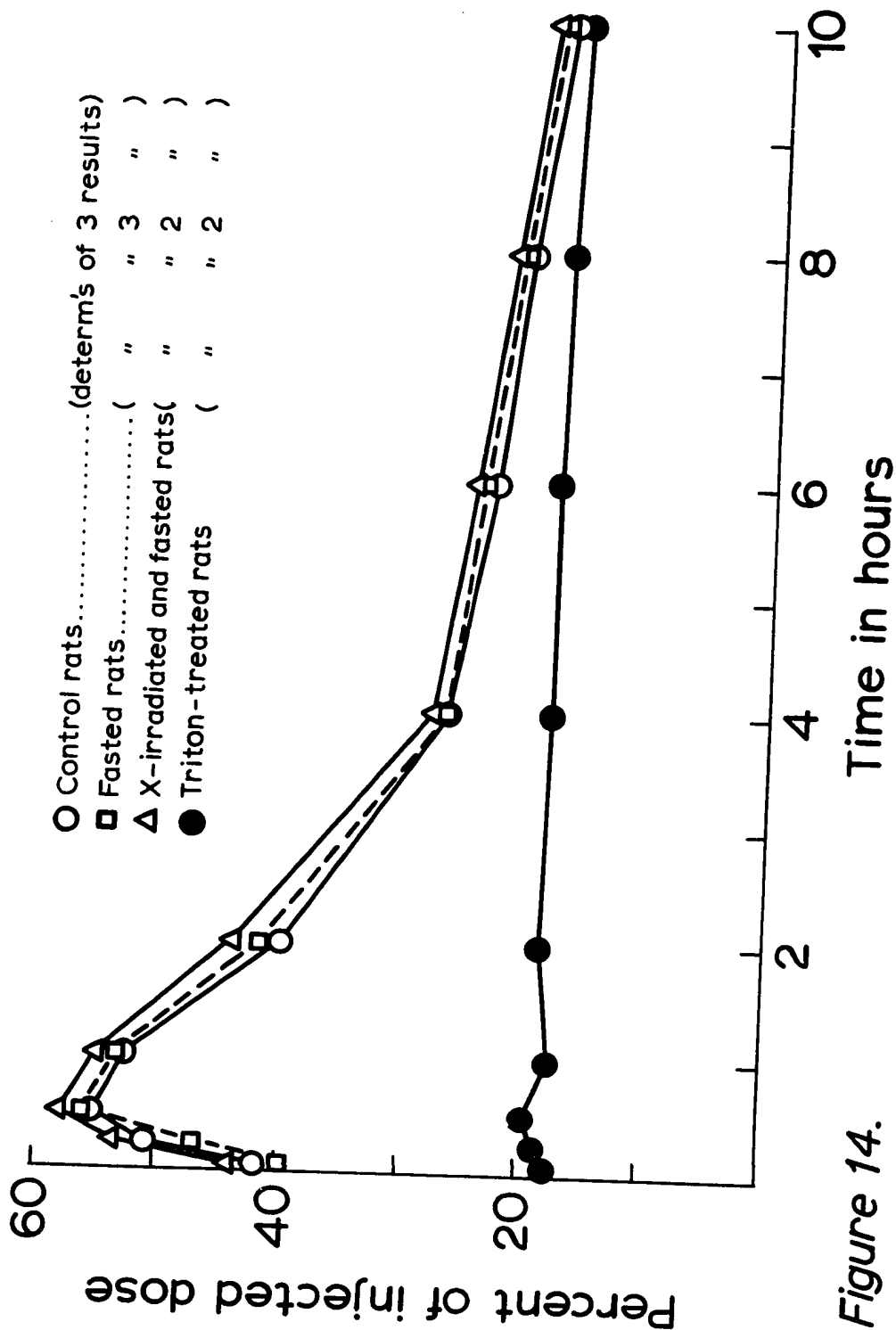


Figure 14.

104

169

PLASMA LEVELS OF RADIOACTIVITY
FOLLOWING THE INJECTION OF 26-¹⁴C-CHOLESTEROL.

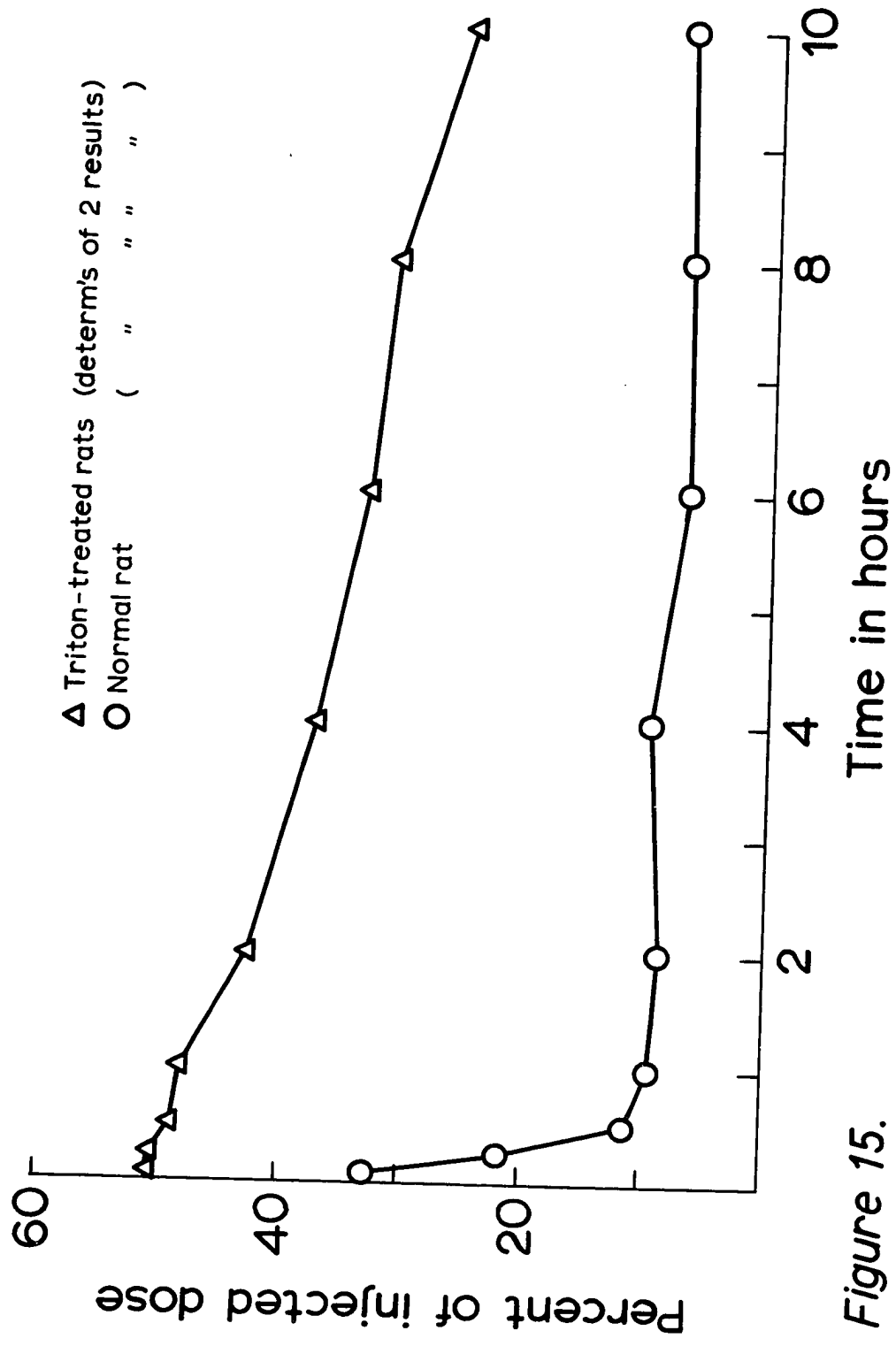


Figure 15.

probably due to a dilution effect of the injected labeled cholesterol by a nine-fold increase in plasma total cholesterol concentration as shown in Table X.

The rate of $^{14}\text{CO}_2$ excretion is also different in Triton-treated rats from all the other experimental groups. These results are shown in Figure 16. It is interesting to see in Triton-treated rats that the pattern of $^{14}\text{CO}_2$ excretion rate is similar to that of the liver level of radioactivity. This similarity indicates that $^{14}\text{CO}_2$ production from injected $^{26}\text{-}^{14}\text{C}$ -cholesterol is a function of radioactivity level in the liver. On the other hand, in X-irradiated-fasted rats, the rates up to four hours after injection are not as high as the controls although the liver levels of radioactivity were very similar to those of controls during these time intervals. However, the rates throughout the remaining period in this group came close to that in controls.

A lower amount of injected cholesterol was present in livers of Triton-treated rats throughout the experimental period and the liver weight was increased in X-irradiated-fasted rats. In addition, cholesterol concentration in liver and in plasma is different in different conditions as shown in Table X. Liver cholesterol concentration is slightly increased in fasted and Triton-treated rats and slightly decreased in X-irradiated-fasted rats as compared to controls. Therefore, the amounts of $^{14}\text{CO}_2$ excretion in different groups are also expressed as

TABLE X
 CHOLESTEROL CONCENTRATIONS IN PLASMA AND LIVER IN RATS UNDER DIFFERENT TREATMENTS

<u>Rats</u>	<u>Plasma Cholesterol mg./100 ml.</u>			<u>Liver Cholesterol mg./100 g.</u>		
	<u>Free</u>	<u>Ester</u>	<u>Total</u>	<u>Free</u>	<u>Ester</u>	<u>Total</u>
Control	23.0 ± 0.92	59.5 ± 1.23	82.5 ± 3.95	198.0 ± 3.85	33.3 ± 2.60	231.3 ± 2.70
Fasted*	22.4	64.0	86.4	250.0	50.0	300.0
X-Irradiated-Fasted*	42.0	75.0	117.0	187.0	25.3	212.3
Triton-treated	363.6 ± 9.38	259.5 ± 8.14	623.1 ± 20.18	256.7 ± 3.12	14.4 ± 1.76	271.1 ± 2.47

111

MEAN ± S. E., 16 determinations.

* Pooled data of three rats.

Control means young adult rats fed on laboratory chow diet.

RATE OF ¹⁴CO₂ PRODUCTION FROM INJECTED 26 - ¹⁴C-CHOLESTEROL

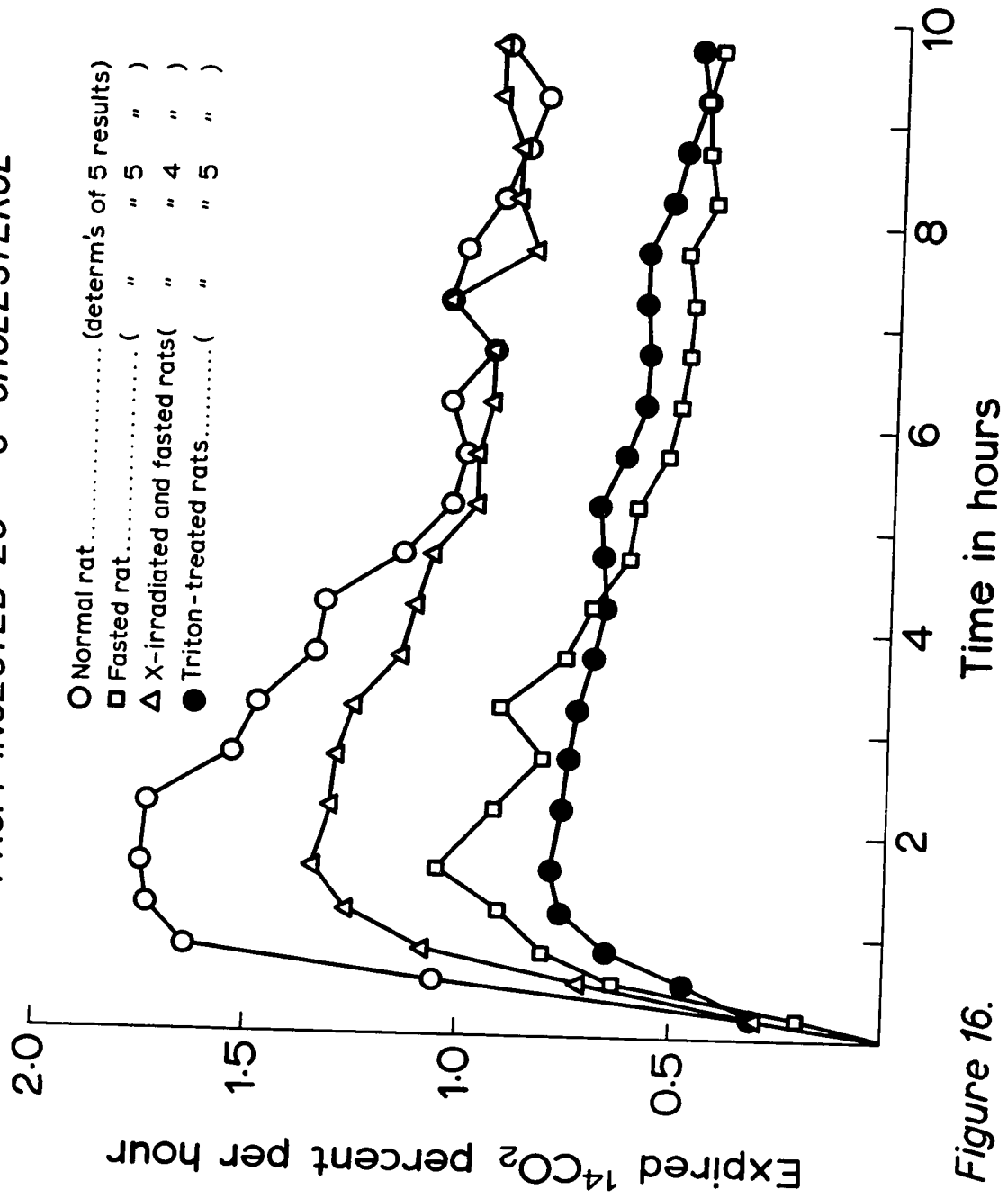


Figure 16.

corrected ratios, which are presented in Table XI. It is clear from this Table that fasting inhibits the production of $^{14}\text{CO}_2$ from injected 26- ^{14}C -cholesterol and X-irradiation treatment does not increase oxidation as much as suggested by the values in Table IX. The rate of oxidation is actually of the same magnitude as in the fasted group. On the other hand, Triton-treatment does not appear to inhibit the oxidation compared to the control as suggested by the values in Table IX. For rats weighing 150 g., about 13 mg. and 14 mg. of cholesterol is transformed into bile acid in control and Triton-treated rats, respectively, whereas 9 mg. and 10 mg. in fasted rats and X-irradiated-fasted rats, respectively.

Table XII shows the recovered radioactivity of injected cholesterol from the whole intestine and feces, expressed relative to the average plasma levels of radioactivity which were obtained from Figure 15 or relative to the blood levels of radioactivity measured after the ten-hour experimental period. These ratios are also corrected for plasma cholesterol concentration equivalent to 69 mg. per 100 ml. These indicate that the magnitude of the distribution of injected cholesterol is not much different in any groups from controls.

As shown in Table XI, free cholesterol represents only one-third of the total plasma cholesterol in controls, fasted and X-irradiated-fasted rats. This was also found in the previous diet experimental groups. On the other hand, Triton-treated rats had a higher proportion of free cholesterol in both plasma and liver compared to other groups.

TABLE XI

QUANTITATIVE ESTIMATION OF CONVERSION OF CHOLESTEROL TO BILE ACID IN RATS UNDER DIFFERENT TREATMENTS

Rats	(a)	(b)	(c)		Cholesterol Converted to Bile Acid	
	Expired $^{14}\text{CO}_2$ per g. of liver for 10 hr. (%)	Average level of ^{14}C per g. of liver for 10 hr. (%)	Ratio a/b	Corrected Ratio	Per g. of liver 10 hr. (mg.)	Per 6 g. of liver 24 hr. (mg.)
Control	1.7	4.1	0.41	0.47	0.94	13.5
Fasted	1.2	5.5	0.21	0.32	0.64	9.2
X-Irradiated-Fasted	1.4	4.0	0.35	0.37	0.74	10.6
Triton-treated	1.0	2.8	0.36	0.50	1.00	14.4

(c) Corrected for liver cholesterol pool equivalent to 2 mg. per g. of liver weight.
Control means young adult rats fed on laboratory chow diet.

TABLE XII
DISTRIBUTION OF 26-¹⁴C-CHOLESTEROL TO THE WHOLE INTESTINE IN RATS UNDER DIFFERENT TREATMENT

Table XII - A Rats	(a)	(b)	(c)		(d)	
	Total ¹⁴ C Recovered from Whole Intestine for 10 hr. (%)	Average Plasma Level of ¹⁴ C for 10 hr. (%)	Ratio a/b	Corrected Ratio	For 10 hr. (mg.)	For 24 hr. (mg.)
Control	9.0	8.4	1.07	1.35	4.62	11.09
Triton-Treated	6.7	36.6	0.18	1.62	5.54	13.30

Table XII - B Rats	(a)	(b)*	(c)	
	Total ¹⁴ C Recovered from Whole Intestine for 10 hr. (%)	Blood Levels of ¹⁴ C After 10 hr.	Ratio a/b	Corrected Ratio
Control	9.0	13.3	0.68	0.82
Fasted	9.3	15.8	0.62	0.79
X-Irradiated-Fasted	8.8	15.7	0.56	0.95

(a) and (b)* - Data presented in Table IX.
(c) - Corrected for plasma cholesterol pool equivalent to 69 mg. per 100 ml.
(d) - Based on plasma cholesterol in whole body (3.42 mg.).

DISCUSSION

DISCUSSION

1. Validity of the Methods

Since the conversion of cholesterol into bile acid is quantitatively an important pathway for the elimination of cholesterol from the body in rats, many investigators have attempted to determine the rate of bile acid formation using different methods.

The quantity of bile acids excreted through the feces has been determined titrimetrically or spectrophotometrically with or without preliminary purification by column chromatography (78, 126). However, an accurate quantitative determination for different fecal bile acid fractions is difficult because they are a complex mixture of microbially-formed metabolites (51). Recently, Grundy and collaborators (92) have proposed a method using gas-liquid chromatography.

A second method is the determination of quantity of bile acids excreted by a bile-fistula rat. However, the disadvantage of this method is that the cannulation interrupts the enterohepatic circulation and thus increases the formation of bile acids (16, 67, 148).

A third method is determination of half-life and pool size of bile acid, using an isotopic dilution technique and calculating daily production of bile acid from these two parameters (93, 101, 102, 130, 160).

This method can be applied easily to a large animal such as a rabbit or to a human but cannot be easily applied to a small animal such as a rat. In addition, the rate of turnover must be determined for each different bile acid and there are at least two main bile acids, cholic and chenodeoxycholic acids.

The method used for estimation of bile acid formation from cholesterol in the present experiment is rather physiological and is based on three facts: first, during the transformation of administered 26-¹⁴C-cholesterol, the 26th carbon is eliminated as CO₂ in the expired air (37); secondly, the degradation of cholesterol into other steroids is quantitatively negligible compared to degradation to bile acids (45, 51); thirdly, conversion of cholesterol to bile acids is a function of the liver only. Tissues or organs other than liver do not contain the enzyme system required for the oxidation to form bile acids (56, 191).

Therefore, the estimation of the rate of bile acid formation was made by measuring the radioactivity in expired CO₂ and in liver. The expression of the amount of radioactivity in expired air based on the total count injected, instead of based on the radioactivity in liver, will not give a true picture of the oxidation in the present experiments. The oxidation is, as mentioned, a function of liver enzymes and also of the amount of labeled and cold cholesterol in the liver. The former effect can be seen from the results obtained from different dietary groups and

different conditions of the rats. This will be discussed later. The latter can be seen from the fact that the patterns of rate curve of $^{14}\text{CO}_2$ expiration are similar to those of the level of radioactive cholesterol in liver at the corresponding time intervals.

The level of radioactive cholesterol in liver seems to be influenced by two factors. One of them is plasma cholesterol concentration. This can be seen from Table XIII in which the average liver levels of radioactivity are expressed relative to the average levels of ^{14}C in plasma for ten hours or the radioactivity levels in blood measured after the ten-hour experimental period and are corrected for plasma cholesterol concentration equivalent to 69 mg. per 100 ml. The ratios a/b are for the normalization of body weight of animals used and the corrected ratios are for that of plasma cholesterol concentration.

In suckling rats, the average liver level of injected cholesterol looks inferior to those in young adult and weaned rats. However, the corrected ratio shows that it is not lower, but rather similar to that of young adult rats and higher than that of weaned rats. The extreme example is in the case of Triton-treated rats. The distributed amount of the injected cholesterol to liver of this group is, instead, very close to that of control, as indicated by corrected ratio, if nine-fold increase of plasma cholesterol concentration is considered. Therefore, the lower liver level in these cases was the consequence of dilution effect

TABLE XIII
EFFECTS OF DIET, AGE AND TREATMENT ON DISTRIBUTION OF INJECTED 26-14C-CHOLESTEROL TO LIVER IN RATS

Rats	(a)	(b)	(c)
	Average Levels of 14C in Whole Liver During 10 hr. (%)	Average Plasma Levels of 14C During 10 hr. (%)	Corrected Ratio
No Fat	29.3	8.0	3.66
Commercial Semi-synthetic High-Cellulflour	32.7	8.6	3.80
15% Butter	29.4	8.0	3.68
Commercial Semi-synthetic	28.8	9.1	3.16
15% Corn Oil	32.1	10.2	3.15
Commercial Semi-synthetic	31.3	7.3	4.29
Control	33.4	8.0	4.18
Triton-Treated	28.8	8.4	3.43
	17.6	36.6	0.48
	(a)	(b)*	(c)
	Average Levels of 14C in Whole Liver during 10 hr. (%)	Blood Levels of 14C After 10 hr. (%)	Corrected Ratio
Rats			
Young Adult (Control) Weaned	28.8	13.3	2.16
Suckling Fasted	23.5	12.7	1.85
X-Irradiated	19.5	10.4	1.87
	23.2	15.3	1.52
	29.6	15.7	1.88

(c) Corrected for plasma cholesterol pool equivalent to 69 mg. per 100 ml.
 (b)* Presented in Tables V and IX.

of the injected cholesterol rather than the lower ability of liver to take up plasma cholesterol.

Another factor seems to be the effect of diets or treatments given to rats (see Table XIII). In groups fed corn oil in either basic diet, the corrected ratios show that the liver level in this group is higher than in those fed butter diet and fat-free diet, respectively. Although X-irradiated rats have a higher plasma cholesterol concentration than controls, the dilution effect as seen in suckling or Triton-treated rats cannot be observed. Therefore, the uptake of radioactive cholesterol from the circulatory system by liver of this group is in fact slightly higher than those by livers of the control group.

The quantity of bile acid formed was estimated from the quotient of radioactivity in expired CO₂ to radioactivity in liver during the experimental period. Chevallier and Lutton (41) determined the quantity of bile acid formation by measuring daily radioactivity of CO₂ expired by rats in an isotopic equilibrium state which was attained by feeding 26-¹⁴C-cholesterol continuously in the diet. They based their calculation on the specific radioactivity of the blood cholesterol ($\mu\text{c./mg.}$) since the specific radioactivity of blood cholesterol was the same as that of liver cholesterol and since rats were all under the same dietary treatment.

In the present experiments, the rats were not in an isotopic equilibrium state throughout the experimental period. It was possible

to obtain the amount of bile acid formation from the value of quotient of radioactivity of expired CO₂ by the specific radioactivity of liver cholesterol calculated from average liver level of the radioactivity. However, the value obtained from the latter method is not an informative measure for the comparison of the rate of bile acid formation in different experimental conditions because this is still influenced by the absolute amount of cholesterol. By using the latter expression, only 1.2 mg. and 5.8 mg. of cholesterol are converted daily to bile acid in suckling and weaned rats in comparison with 15.6 mg. in young adult rats. It is natural that the former two groups of rats produced the smaller amount of bile acid than the latter group because the absolute body weight and total cholesterol amount in the body is smaller in the former groups than those in the latter group. In effect, the comparison in three groups is only possible when the assumption is made that young adult rats have the same condition as suckling stage or recently-weaned stage or vice versa.

In addition, 13.0 mg. and 8.2 mg., instead of 10.6 mg. and 9.2 mg. respectively, of cholesterol were converted daily to bile acid in X-irradiated-fasted rats and fasted rats, respectively, since liver weight is greater in the former group than in the latter although cholesterol concentration is smaller in the former. Similarly, the comparison is only possible in this case when the rats are supposed to have the same condition in both groups except treatment given. Therefore,

unless two parameters (body weight and cholesterol concentration) are close to one another among each experimental group, the expression of results which was calculated from the quotient of radioactivity of expired CO₂ by specific radioactivity of liver level was not valid or informative for the comparison of the results obtained in this study.

The values of quantities of cholesterol transformed into bile acid determined by the method used in this thesis are greater than those determined by other methods described at the beginning of the discussion but are close to those determined by Chevallier and Lutton (41). The values obtained with the present method in rats fed Purina chow are 2.5 to 4 times greater than those by cholic acid decay method (162, 190) and were about twice as great as the values obtained by titration method (166) and by the method using gas-liquid chromatography (92). When results for rats on semisynthetic diets are compared, our values again tend to be higher than those obtained by other methods (92, 162). It is difficult to explain whether this discrepancy only occurs from a technical problem or not. Cholic acid decay and titration methods, as indicated earlier, certainly have disadvantages since it is difficult to determine the pool size of different bile acids in rats by the former method and it is not possible to isolate bile acids completely by the latter method. However, the method using gas-liquid chromatography, as the authors insisted, is relatively reliable, but the value obtained by this is still lower than those obtained

in this thesis.

On the other hand, the value obtained by Chevallier and Lutton (41), who used the same basic method as here, is very close to ours. They fed rats only on a semisynthetic diet, the composition of which was quite comparable to that used in these experiments, except for the amount and type of fat (8% lard). Their value (37 mg./kg.) was similar to the average values (32.7 mg./kg.) obtained from groups fed the semisynthetic diets containing no fat (1.5% corn oil) and 15% butter. The fact that the amount of bile acid formed, calculated by the present method, is similar to that obtained by the above authors who are believed to use a relatively thorough and reliable method, at least among several methods hitherto known, is very encouraging.

From the experience obtained from this study, a simpler method of quantitative estimation of rate of bile acid formation for future studies is proposed. Six hours after a single injection of 26-¹⁴C-cholesterol, respiratory ¹⁴CO₂ is collected for 30 minutes at a time when the liver level of radioactivity is relatively constant and the animal is killed in order to measure the radioactivity level in liver at that time. Then the fraction of the radioactivity recovered from 30 minute-collection by the radioactivity level in the liver at that moment is corrected for cholesterol concentration. This procedure is repeated several times through 24 hours in order to obtain the whole picture of the oxidation since it was observed that the rate of the

oxidation varied with time during 24 hours (41).

One of the possible objections to the measurement of bile acid formation by collecting radioactive CO₂ after a single injection of radioactive cholesterol is that the injected labeled cholesterol may not behave in the same manner as that of endogenous plasma cholesterol. The observations presented in Table XIV show that the percentage of the radioactivity present as esterified cholesterol becomes nearly the same as the actual percentage of esterified cholesterol a few hours after the injection, suggesting that the behavior of the injected radioactive cholesterol is similar to that of endogenous plasma cholesterol. Wheratt and Staple (200) also claim that cholesterol dissolved in Tween 20 coupled to serum in vitro and injected intravenously into rats disappeared from the circulation at the same rate as cholesterol labeled endogenously. It is well known that cholesterol exists in plasma as one of the constituents of lipoproteins which transport cholesterol from tissues to the circulatory system and vice versa. Eckles et al (66) demonstrated that isotopically labeled free cholesterol rapidly exchanges between plasma, liver and red blood cells. Porte and Havel (159) demonstrated that 4-¹⁴C-cholesterol incubated with serum for one hour was equally distributed in low and high density lipoproteins which contain most of the plasma cholesterol (170). Based on the above findings and on the fact that the amount of labeled cholesterol injected (16 μg.) is negligible compared with that of plasma

TABLE XIV
PERCENTAGE OF ¹⁴C IN ESTERIFIED CHOLESTEROL OF PLASMA AFTER INJECTION OF 26-¹⁴C-CHOLESTEROL

Rats' Diets	(a) Relative Percent of Injected 26- ¹⁴ C-Cholesterol as Esterified Form in Plasma at Times								(b) Esterified Cholesterol in Plasma (%)
	5 min.	15 min.	30 min.	1.0 hr.	2.0 hr.	4.0 hr.	6.0 hr.	8.0 hr.	
No Fat	29.4	31.3	42.6	50.0	65.5	70.8	70.7	73.3	74.0
Commercial Fat	15.8	17.4	44.4	55.9	67.5	68.3	71.0	71.9	74.0
15% Butter	20.2	----	32.2	50.0	63.7	68.2	70.0	73.6	74.0
Commercial Butter	23.5	----	25.2	41.3	59.8	67.8	69.0	69.2	71.0
15% Corn Oil	15.1	29.2	43.4	55.7	64.8	66.9	68.7	69.0	74.0
Commercial Corn Oil	17.0	23.5	43.4	48.6	59.6	69.5	70.0	71.0	73.0
Control	18.2	24.7	39.3	42.6	58.9	62.4	68.9	71.2	72.0
Triton-Treatment	0	0	0	0.7	2.3	3.9	11.6	25.9	42.0

125

(a) Percentage of ¹⁴C in esterified cholesterol based on ¹⁴C of total cholesterol.
(b) Percentage of endogenous esterified cholesterol in plasma.

cholesterol in the whole body, it may be reasonable to assume that the labeled cholesterol taken up by the liver within 30 minutes represents that which was rapidly exchanged with free cholesterol in plasma lipoproteins.

Another possible objection is that the products formed during transformation may not be converted to radioactive expired CO₂ because they are reincorporated into other different constituents, such as fatty acids. However, Chevallier and Lutton (41) found that the radioactivity of the saponifiable fraction represents only 0.3 to 0.5% of the radioactivity in the whole animal in isotopic equilibrium established by a long period of feeding 26-¹⁴C-cholesterol in the diet. In the present study, it was found that the radioactivity recovered from liver lipid extract after the ten-hour experimental period was almost all in the cholesterol fraction. Thus, the radioactivity loss through reincorporation is negligible.

If propionic acid is the main immediate product of oxidation of the isopropyl side chain of cholesterol and is oxidized through the TCA cycle as proposed by Suld et al (191) and Mitropolous et al (144), then the pool of propionate and TCA cycle intermediates in animals on the different diets and under different conditions might influence the amount of ¹⁴CO₂ expiration from the injected 26-¹⁴C-cholesterol. In this study, this problem was not considered since correction of the results for this kind of problem is not practically possible.

As already explained in the "Result" section, the calculation was based on the average radioactivity level of liver during the experimental period because there was a distinct time lag between the maximum rate of uptake of the injected radioactive cholesterol in liver and that of $^{14}\text{CO}_2$ production in the corresponding time intervals. Two possible explanations may be considered: one is that the radioactivity measured at a given time when the maximum uptake was shown may not necessarily be in contact with the enzyme system for the conversion of the cholesterol into bile acids, but simply in the capillaries or sinuses of liver; second is that the maximum amount of radioactivity may be present in the site where the enzyme system is present, but simply $^{14}\text{CO}_2$ expiration may be delayed.

The facts that the injected radioactive cholesterol very rapidly appeared in liver (see liver level curves) and that the extreme permeability of hepatic sinuses brings the fluids of the hepatic blood into extremely close contact with the liver parenchymal cell, thus facilitating rapid exchange of nutrient material between the blood and the liver cells (36), provide some arguments against the first possibility.

In the present experiments, the calculation of daily production of bile acids was made on the basis of liver cholesterol concentration, since the liver is the only organ which is known to convert cholesterol into bile acids and since a large proportion of the single dose of labeled cholesterol injected was taken up by the liver within a short time period. This does not necessarily mean that the amount of cholesterol converted

daily to bile acids obtained in this thesis is the amount of cholesterol synthesized only by liver in situ. The cholesterol could originate from various sources. It is well known that the cholesterol pool of liver is in a complete dynamic equilibrium with that of plasma. Its origins are diverse and it may arise from absorbed dietary cholesterol or the cholesterol synthesized in various tissues or organs including liver. These are finally mixed with plasma and other body pools of cholesterol, and excreted into the intestinal tract as acidic or neutral sterols. Chevallier (40) also demonstrated that liver cholesterol does not turn over by synthesis in situ. In other words, cholesterol synthesized by liver is completely equilibrated with plasma (transfer space) cholesterol which is finally metabolized to other products for excretion.

2. Studies on Effect of Diets

The results presented in this thesis show clearly that the daily production of bile acid in rats is markedly influenced by the type of basic diet ingested, commercial or semisynthetic. The production is more than double in the former groups as compared to the latter (see Table II). This finding is in good agreement with that reported by Portman and Murphy (162) who observed that rats fed Purina chow excreted more cholic acid than those fed a purified diet. Hellstrom et al (102) demonstrated a similar finding in rabbits. The former authors also

reported that feeding 20% cellulflour in a semisynthetic diet doubled the daily production of bile acid compared with the 5% cellulflour diet and that there was only a further 60% increase in rats fed Purina chow in comparison with those fed the high-cellulflour diet. In the present experiments, addition of 25% cellulflour to a semisynthetic diet somewhat different from that used by the above authors, did not cause an increase in daily production of bile acid so much as to suggest that the difference in the effect of two basic diets results only from the amount of roughage contained in the respective diets. However, the possibility that cellulose used as roughage may not have the same effect as that in the commercial diet can not be ruled out.

It is believed that cholesterol disappears from the body through two main processes (51): transformation into bile acids excreted by the intestinal tract and excretion of cholesterol by the same route. Although the quantitative importance of the two pathways for elimination of endogenous cholesterol (absorbed dietary and synthesized cholesterol) is not well established, the former pathway, at least in rats, outweighs the latter. Siperstein et al (180, 184) noted that 80% of endogenous cholesterol is excreted as bile acid and 20% as fecal sterols. A similar result was also obtained by Chevallier (40).

Since one of the major pathways of elimination of cholesterol is excretion as bile acids, the quantity of bile acid production presented here represents, at least in the experiments carried out with young

adult rats, the greater part of daily turnover rate (mg./day) of body cholesterol. Unlike adult rats, those used in the diet experiments were still in the growing stage and therefore some of the cholesterol synthesized or absorbed does deposit in the body. Thus, the disappearance of cholesterol represents only part of the appearance.

It has been shown that there is a very close relationship between input of cholesterol (from diet and synthesis) and output of cholesterol as fecal bile acids. Thus, feeding large amounts of exogenous cholesterol to animals in the diet brings about an increased bile acid excretion in order to maintain the constant cholesterol concentration in the body (40, 204). On the other hand, feeding cholestyramine to rats, which binds bile acids and thus prevents their reabsorption (64) or bile evacuation through cannulation (148) never depletes the body cholesterol pool in intact animals. This means that increased cholesterol synthesis proportionately fulfills the increased demands for bile acid formation. It has also been demonstrated that feeding bile acids to animals causes a deposition of cholesterol in the body, suggesting that the relationship between supply and demand is broken down (107, 157).

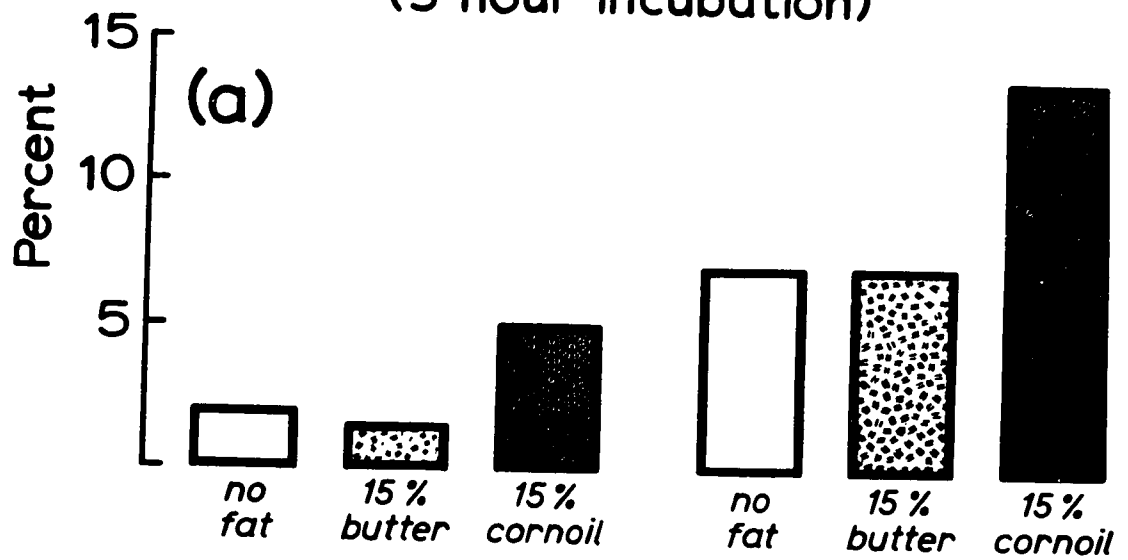
The finding that daily production of bile acid was markedly increased in rats fed the commercial diets compared to rats fed semi-synthetic diets means that there must be a corresponding increase in turnover rate of body cholesterol. Since bile acid is only formed from cholesterol and since cholesterol concentration in each commercial

diet compared with the respective semisynthetic diet is not much different, it is possible that cholesterol synthesis in the body may be increased in rats fed the commercial diets in comparison with those fed the corresponding semisynthetic diets.

Carroll (32, 33, 35) studied in detail the effect of commercial and semisynthetic diets containing various fats and oils on liver cholesterol synthesis by measuring both in vitro and in vivo the radioactive cholesterol incorporated from 1-¹⁴C-acetate. Figure 17(a) shows a part of his results and Figure 17 (b) shows the bile acid formed in the comparable groups. This gives some evidence that cholesterol synthesis in the intact rats fed the commercial diet is increased in comparison with those fed the semisynthetic diets. However, it is difficult to know whether changes in cholesterol synthesis are a consequence of those in bile acid excretion or vice versa. At any rate, the fact that cholesterol concentration of plasma and liver was not increased or decreased by feeding different basic diets (between rats in each group) means that cholesterol balance is well maintained; in other words, appearance and disappearance is well balanced.

Contrary to the effect of two basic diets on daily production of bile acid, that of fats is rather insignificant in the present study. Rats fed fat-free diet are not particularly different from those fed butter or corn oil diets. The most striking effect of corn oil can be seen in this study from the results on the recovery of the radioactivity from the

ACETATE-1-¹⁴C INCORPORATION INTO CHOLESTEROL BY LIVER SLICES. (3 hour incubation)



CONVERSION OF CHOLESTEROL TO BILE ACIDS.

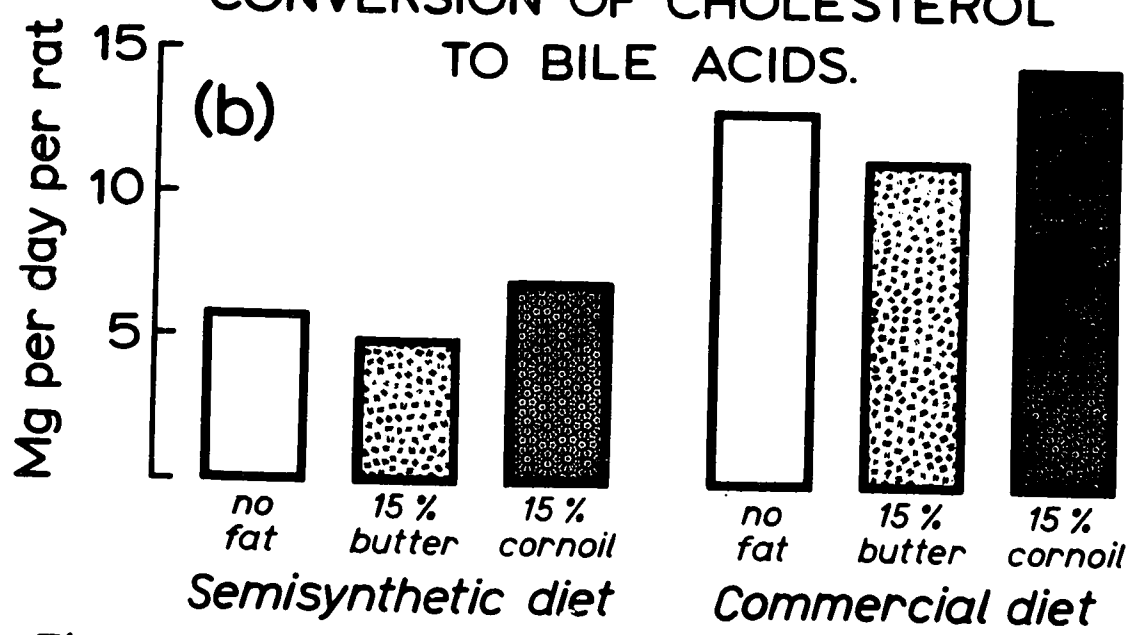


Figure 17.

whole intestine and feces (Table IV). Many investigators have shown that feeding corn oil or linoleic acid to rats increased excretion of fecal sterols (76, 207). Chevallier (38) and Mitteenen et al (142) demonstrated that fecal sterols originate from two sources: two-thirds of the sterols come from cholesterol synthesized in situ by intestine and excreted directly into the lumen without exchanging with the plasma pool; and the other one-third comes from the transfer space of cholesterol (plasma origin). It is not known whether increased excretion of fecal cholesterol by feeding corn oil or linoleic acid to rats originates mainly from the former or latter sources. Based on the fact that excretion of cholesterol from the former source is not markedly influenced by cholesterol feeding (38, 205), the increased influx of plasma cholesterol into intestine of rats fed corn oil diet may be an indication of increased excretion from the body-plasma cholesterol.

Since cholesterol concentration is rather negligible in diets containing no fat and corn oil* as compared with those containing butter, the rate of biosynthesis of cholesterol in the rats fed the butter diet may be lower than that suggested by data of bile acid formation in the present study, providing that cholesterol absorption is the same in both groups. On the other hand, the amount of daily production of bile acid in rats fed fat-free and corn oil diets represents most of the amount of cholesterol synthesized in the body.

The depressing effect of corn oil on serum cholesterol level in

* Corn Oil: 27.9 mg./100 g. Butter: 360 mg./100 g.

man led many investigators to study the mechanism and clinicians to suggest that patients should not take butter but corn oil. The present study showed that feeding corn oil to rats raised cholesterol concentration in liver as compared with butter or no fat. Increased liver cholesterol concentration as a result of feeding corn oil diet was also observed by several other investigators (6, 76). Gerson et al (76) found a raised cholesterol pool in the body as a whole and in many organs studied such as liver, testicles, muscle, intestine and aorta, etc.

The increase of liver cholesterol concentration in rats fed corn oil diets was mainly due to esterified cholesterol. The proportion of liver esterified cholesterol was 12% to 15% in rats fed fat-free and butter diets but increased up to 28% to 32% in rats fed corn oil diets (see Table XV). Table XIV indicates that the proportion of esterified- ^{14}C -cholesterol in plasma reaches that of endogenous esterified cholesterol around eight hours after the injection, which indicates equilibrium of free and esterified cholesterol in plasma after this time interval. This suggests that esterifying enzyme activity in plasma is the same in all dietary groups. Similarly, in livers of rats fed butter or fat-free diets, as shown in Table XV, the proportion of esterified- ^{14}C -cholesterol reaches that of endogenous liver esterified cholesterol and they have come into an equilibrium with free cholesterol of liver as well as that of plasma. On the other

TABLE XV
 PERCENTAGE OF ¹⁴C IN ESTERIFIED CHOLESTEROL OF LIVER AFTER INJECTION OF 26-¹⁴C-
 CHOLESTEROL

Rats' Diets	(a) Relative Percent of Injected 26- ¹⁴ C-Cholesterol as Esterified Form in Liver at Various Time Intervals							(b) Esterified Cholesterol in Liver (%)	
	5 min.	15 min.	30 min.	1.0 hr.	2.0 hr.	4.0 hr.	6.0 hr.		8.0 hr.
No Commercial Fat	0	1.3	3.3	3.6	7.4	10.2	10.5	11.8	12.0
Butter Semisynthetic	0	2.5	3.5	4.6	7.1	10.5	12.6	16.0	14.0
15% Commercial Butter Semisynthetic	0	----	3.5	4.4	6.4	10.2	12.5	14.8	15.0
15% Commercial Corn Semisynthetic Oil	0	----	4.4	5.9	7.0	12.6	14.4	15.9	15.0
Control	0	1.2	2.8	3.4	4.4	7.0	14.0	17.2	32.0
Triton-treatment	0	0.2	0.7	4.7	6.4	12.2	15.1	16.0	28.0
	0	0.2	4.9	8.0	8.1	9.6	10.6	13.2	12.0
	0	0	0	1.8	4.7	6.2	6.8	6.6	5.0

(a) Percentage of ¹⁴C in esterified cholesterol based on ¹⁴C of total cholesterol.
 (b) Percentage of endogenous esterified cholesterol in liver.

hand, the proportion of esterified- ^{14}C -cholesterol in livers of rats fed corn oil diets reaches only half of that in original liver esterified cholesterol and they have not achieved an equilibrium with free cholesterol of liver and of plasma. These results are shown in Figures 18, 19, and 20. Although the meaning of this finding remains to be elucidated, it may suggest that this remaining portion of esterified cholesterol in liver originates from some place other than plasma or the liver itself, possibly from the intestine. The same effect as observed by Chevallier and Lutton (42) in rats fed cholesterol, which gave rise to an increase in the absolute amount of esterified cholesterol in chylomicron of intestinal chyle, also probably occurs in rats fed corn oil. It may be interesting to see where this esterified form originates in rats fed this oil in a future study. At any rate, an important fact here is that corn oil feeding deposits mainly cholesterol esters in the body, which are metabolically less dynamic than free cholesterol (79). The finding in the present study that liver cholesterol concentration is increased by feeding corn oil but not butter suggests that cholesterol balance is not as well maintained in this group as in groups fed butter diets or fat-free diets. In other words, appearance of cholesterol is greater than disappearance, resulting in an accumulation of cholesterol in the body. In addition, feeding cholesterol with polyunsaturated oil to rats was found to enhance absorption and accumulation of cholesterol more than feeding it with saturated fat (24)

**FREE-AND ESTERIFIED -¹⁴C-CHOLESTEROL
IN PLASMA AND LIVER
FOLLOWING INJECTION OF 26-¹⁴C-CHOLESTEROL.**

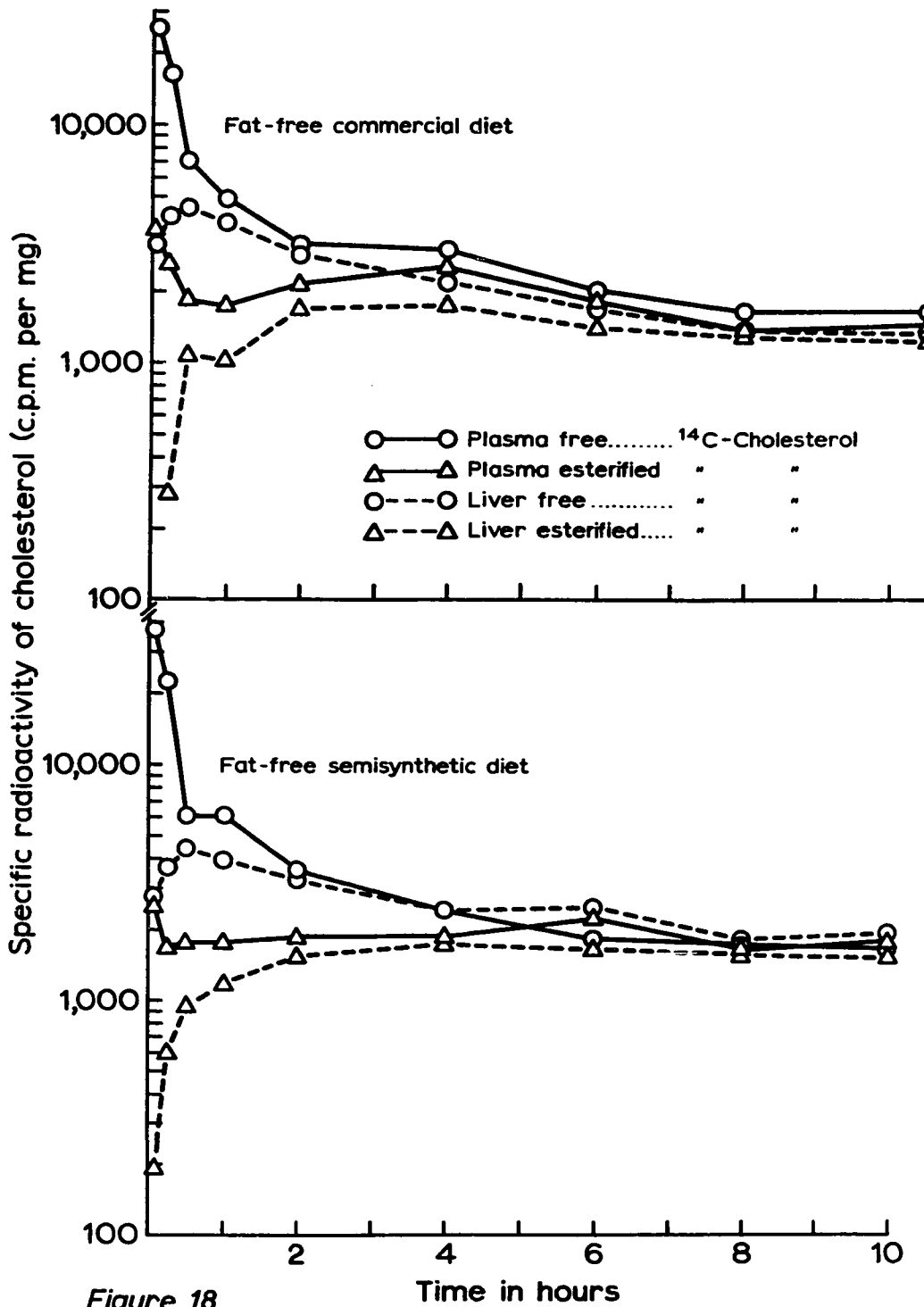


Figure 18.

FREE-AND ESTERIFIED -¹⁴C-CHOLESTEROL
 IN PLASMA AND LIVER
 FOLLOWING INJECTION OF 26-¹⁴C-CHOLESTEROL.

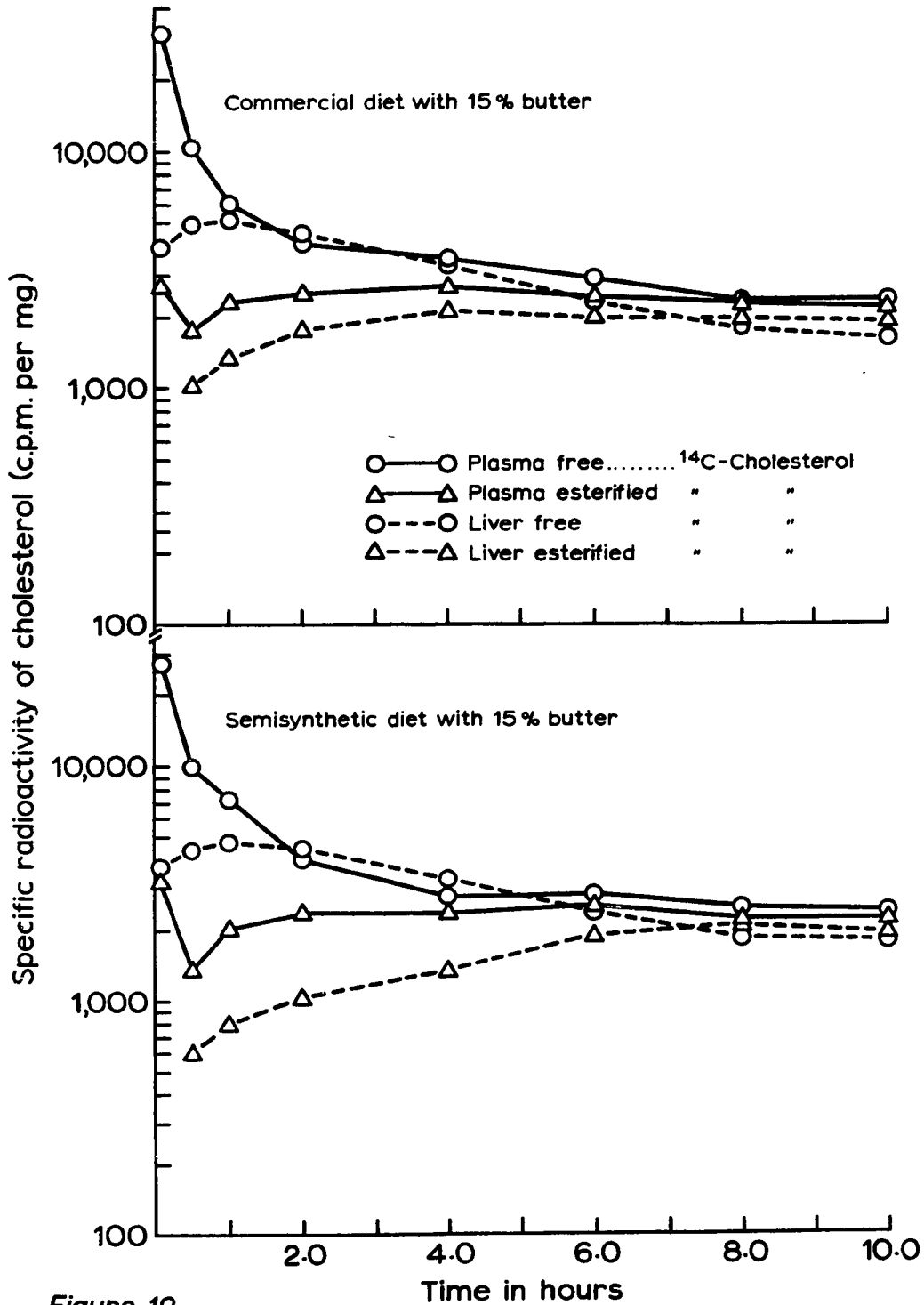


Figure 19.

FREE-AND ESTERIFIED ^{14}C -CHOLESTEROL
 IN PLASMA AND LIVER
 FOLLOWING INJECTION OF $26\text{-}^{14}\text{C}$ -CHOLESTEROL.

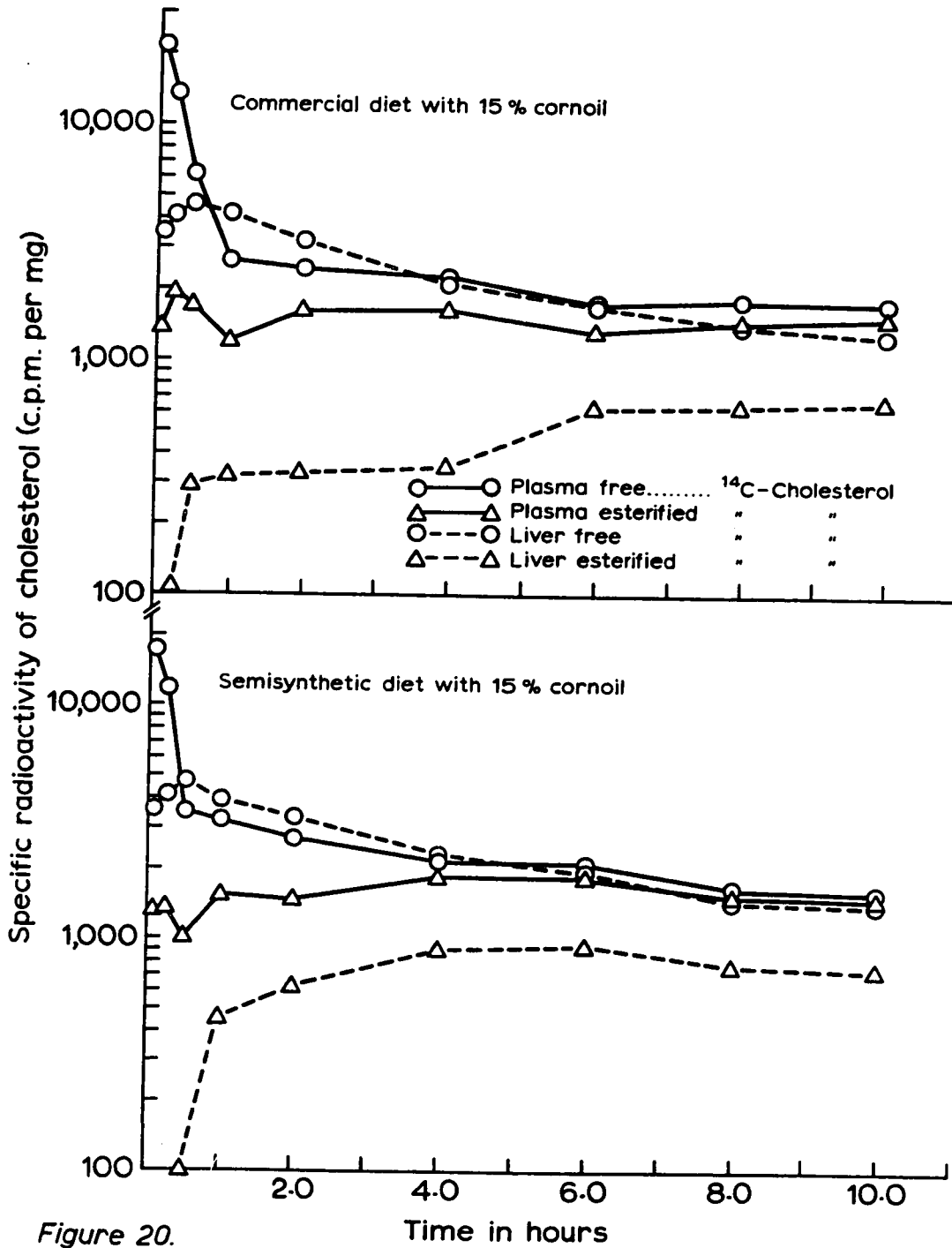


Figure 20.

and feeding polyunsaturated oil does not prevent atherogenesis in the rabbit (75).

There is no agreement or firm evidence to suggest that corn oil increases or decreases the biosynthetic rate or concentration (except serum cholesterol concentration) of cholesterol in the human body (79). Considering the fact that the greater proportion of cholesterol accumulated in atherosclerotic plaques is in esterified form (79) and that feeding corn oil to rats mainly increases esterified cholesterol in liver and other organs as well as in the whole body, the value or meaning of corn oil for the replacement of butter in human diet for the treatment of atherosclerosis appears very unattractive, although the present study cannot completely argue against the original concept on unsaturated fats. However, caution must be exerted in extrapolating experimental results of animals to humans.

3. Studies on Effect of Other Conditions

The present study showed that bile acid production is lower in suckling rats than in weaned and young adult rats. The latter two groups were fed on commercial Master Meal in which the total sterol concentration calculated as cholesterol is 0.23 mg. per g.* The actual amount of cholesterol in the diet is lower than that shown above because other plant sterols are present. In rat milk, the water content comprises 80% and cholesterol concentration is 0.6 mg. per g. of dry weight (34, 39). If weaned and young adult rats ingest 10 and 20 g.

* Data furnished by R. M. Hamilton at this Laboratory.

of food every day, respectively, the intake of cholesterol will be around 2.3 and 4.6 mg. per day, correspondingly. In suckling rats at the age of 15 to 17 days, around 2 mg. of cholesterol is absorbed every day from milk (39). If daily absorption or intake of cholesterol is calculated based on 100 g. of body weight, 8 mg. is absorbed per day in suckling rats and 3 mg. per day taken by weaned rats and young adult rats, respectively. Since the cholesterol concentration of the entire plasma in the body and of whole liver are 1.0 and 2.1 mg., respectively; the plasma-liver pool is 3.1 mg. in suckling rats. On the other hand, in weaned and young adult rats, the plasma-liver pool is 8.7 mg. and 20.1 mg. respectively. Therefore, the amount of cholesterol provided by milk per day is about 65% of the cholesterol in the plasma-liver pool whereas dietary exogenous cholesterol contributes, at most, 25% of the plasma-liver pool in weaned and young adult rats, providing that the ingested dietary cholesterol is completely absorbed. Considering the fact that dietary cholesterol is absorbed only to the extent of 60% to 70%, then percentage in the latter two groups will be even further decreased.

Carroll (34) showed that liver slices of suckling rats incorporated very little acetate into liver cholesterol as compared with recently-weaned and young adult rats. In addition, Chevallier (39) demonstrated that in suckling rats two-thirds of the exchangeable cholesterol (transfer space) is exogenous (milk cholesterol) and one-third is endogenous (synthesized cholesterol = internal secretion)

whereas in weaned and young adult rats, which were fed on the diet containing 0.03% of cholesterol, 80% originated from the latter source. Therefore, the low rate of bile acid formation in suckling rats is related to the low rate of internal secretion in comparison with weaned and young adult rats. Harris et al (97) reported that the low rate of acetate incorporation into liver cholesterol in suckling rats was increased 2 to 3-fold within 24 hours after severing the bile duct, suggesting that bile acid is an inhibitor of cholesterol synthesis by the liver. However, caution must be given to the interpretation of results obtained from this kind of experiment, based on the fact that bile acid is required for absorption of cholesterol as well as fat in the milk and is also required for the transport into lymphatic channels of cholesterol synthesized by intestinal wall (209), which is known to be one of the major organs for internal secretion.

Considering the fact that in suckling rats a large proportion of cholesterol in the transfer space deposits in the body and that two-thirds of the transfer space of cholesterol originates from exogenous sources, the quantity of bile acid formed from biosynthesized cholesterol per day would be a very small fraction of the total as shown in the present study. In other words, the magnitude of synthesized cholesterol excreted as bile acid would be far smaller than that suggested by the value in this study.

The finding that weaned rats at the age of 37 days produced slightly less bile acid than young adult rats (60 days old) is easily

understandable on the basis that the magnitude of deposition in the former group would be slightly greater than in the latter group (39). Thus, even though the synthetic rate is of the same order of magnitude in both groups, the fraction of synthesized cholesterol for excretion would be less in weaned rats than in young adult rats.

Several investigators observed hypercholesterolemia in suckling rats (19, 34). Since the previous diet experiments showed that the effect of fat versus no fat or saturated fat versus unsaturated fat on bile acid formation was not markedly different and since the rate of internal secretion in suckling rats is found to be low, the hypercholesterolemia cannot be ascribed to high content of fat or of highly saturated fat of milk. Rather milk cholesterol may be a possible cause of hypercholesterolemia in suckling rats.

Thus, the supply of exogenous cholesterol by milk in suckling rats may result in a decreased rate of internal secretion, in turn influencing the disappearance rate of cholesterol as bile acid and there may be simultaneous development of transformation of cholesterol into bile acid with the secretion of synthesized cholesterol as suggested by Chevallier (39).

A reduced rate of bile acid formation in the present study was also found in rats fasted for 48 hours. It is well known that cholesterol biosynthesis by liver of fasted rats is only 10% to 15% of that in normal control rats (195). However, liver and plasma cholesterol

concentration are slightly increased in fasted rats compared with controls (see Table XI). On the other hand, cholesterol biosynthesis in the whole body does not decrease as much as suggested by liver synthesis. Thus, Van Bruggen et al (198) demonstrated that fasting for 48 hours reduced acetate incorporation into the whole body cholesterol to 63% of the controls, whereas cholesterol concentration was not decreased. Therefore, the total body cholesterol concentration is maintained in the presence of a decreased incorporation and this is associated with a reduced rate of cholesterol catabolism in fasted rats as found in this study.

Fasting is nevertheless a complex situation. During a complete fast, carbohydrate stores are first depleted in one or two days and this may account for the low rate of biosynthesis. In the present experiments, using $^{14}\text{CO}_2$ measurement, the possibility that the results are affected by other factors such as pool size of propionate or TCA cycle intermediates through which the three end atoms are oxidized to CO_2 , can not be ruled out. It is hard to know whether the maintenance or slight increase of cholesterol concentration per gram of tissue or body weight in fasted rats is due to a greater decreased rate of catabolism of cholesterol relative to biosynthetic rate or due to a higher proportion in loss of other cell constituents relative to cholesterol. At any rate, the present study clearly suggests that fasting, in which the biosynthetic rate of cholesterol is decreased, also depresses cholesterol catabolism to bile acid which in turn results

in the maintenance of cholesterol concentration in the whole body.

In X-irradiated-fasted rats, the conversion of cholesterol to bile acid was not significantly different from that in fasted rats and was lower than in fed controls. Several investigators (27, 28, 83, 84) showed that X-irradiation increased the rate of acetate incorporation into liver cholesterol (15 times greater than fasted control in vivo) as compared with fasted or fed controls, whereas the carcass did not show any changes. Cholesterol concentration is slightly lower in liver but higher in plasma. Although plasma cholesterol concentration is higher in X-irradiated rats than in fasted controls, the difference between two groups in total plasma pool is only about 1.7 mg. and the liver-plasma pool is almost similar because of a lower liver cholesterol concentration in the X-irradiated rats. The slight decrease in liver cholesterol concentration may be due to the net increase in liver weight by hypertrophy of liver tissue or by the accumulation of other substances (84). The paradoxical situation in X-irradiated rats is that liver cholesterol biosynthesis is accelerated to such an enormous extent as suggested by acetate incorporation in comparison with fasted controls but conversion of cholesterol to bile acid as shown in the study is not much different from fasted control and the liver-plasma pool is almost the same in both groups. Then where does all the cholesterol synthesized by liver go? It is very doubtful whether the magnitude of cholesterol biosynthesis in the body of X-irradiated rats as suggested by acetate incorporation study bears

any significance to the true picture of cholesterol metabolism taking place in the body in the particular situation.

The fact that X-irradiation caused an increase in liver weight and a decrease in liver cholesterol concentration as compared with fasted or fed controls suggests that the rate of biosynthesis may be in a normal range. On the other hand, the slight increase in plasma cholesterol concentration in X-irradiated rats as compared with fasted or fed controls may be due to the decreased cholesterol excretion as bile acid, as shown in this thesis, in the presence of a normal rate of cholesterol biosynthesis.

Injection of Triton WR-1339 caused a tremendous increase in all the lipid fractions of plasma, including cholesterol. Table X shows that there was a nine-fold increase in plasma total cholesterol, corresponding to a net increase of 25.7 mg. over control in the whole plasma cholesterol in the body. The fact that there was a marked increase in plasma cholesterol with constant liver cholesterol concentration suggests an increased rate of cholesterol biosynthesis taking place in the body under this circumstance.

As already mentioned, the level of radioactivity in plasma or in liver was the function of the plasma cholesterol concentration. The findings reported in Table XII and Table XIII indicate that the influx of plasma cholesterol into intestine and liver does not increase proportionately to the increased plasma cholesterol concentration but remains relatively constant. This may be due to the fact that Triton-injection does not increase the rate of incorporation of plasma cholesterol

into a proper lipoprotein fraction (170) or may be related to the lower proportion of esterified cholesterol in these rats. At any rate, this suggests that outflow of cholesterol from tissues into the circulatory system must be greater than influx, resulting in an increased plasma cholesterol concentration. In addition, the finding that there is a constant amount of influx of plasma cholesterol into tissue in the presence of nine-fold increase could be related to the finding (113) that Triton-injection was able to prevent atherogenesis in cholesterol-fed rabbits.

In this connection, the finding in corn oil-fed groups that the magnitude of the influx of plasma cholesterol into intestine and liver is greater than in other dietary groups is interesting. The accumulation of cholesterol in tissues may not necessarily be related to increased plasma cholesterol concentration but more to the physical properties of cholesterol present in plasma, which in turn may govern the dynamic transport of cholesterol from plasma to tissue or vice versa. It may also be related to the interaction of plasma cholesterol with tissue itself.

Although the mechanism by which this detergent causes hyperlipemia is not known, Friedman and Byers (73) showed that hypercholesterolemia is related to an increased rate of cholesterol biosynthesis. The increased rate of biosynthesis which results in an increased plasma cholesterol concentration is not accompanied by an increased rate of conversion of cholesterol to bile acid which is in

the normal range as shown in this study. This imbalance between appearance and disappearance of cholesterol may secondarily contribute to cause an increase of cholesterol concentration in the body.

Injection of Triton into rats caused a change in the proportion of esterified to free cholesterol in plasma and in liver. As shown in Table XIII, plasma esterified cholesterol comprises two-thirds of the total cholesterol in control or other dietary groups whereas it comprises only one-half in Triton-treated groups. An interesting finding in our study is that the proportion of ^{14}C -esterified cholesterol in plasma in Triton-treated rats reaches only half that of endogenous cholesterol which corresponds only to one-third of control. This may be due to the fact that the amount or the activity of esterifying enzyme in plasma is lower in proportion to the amount of cholesterol or that there is insufficient amounts of substrate for the enzyme activity. Scanu and Page (171) demonstrated that most of the injected 4- ^{14}C -cholesterol in controls was found in high-density lipoprotein but only 10% was found in this fraction in Triton-treated animals and most of it was in low-density lipoprotein. Since high-density lipoprotein is a possible immediate substrate for acyltransferase for esterification of cholesterol in plasma (77), the lower proportion of esterified form in endogenous and injected radioactive cholesterol in Triton-treated rats may be due to a lower rate of incorporation of cholesterol into high-density lipoprotein.

CONCLUSIONS

CONCLUSIONS

1. The quantity of bile acid formed in rats, measured by collecting radioactive CO_2 after a single injection of 26- ^{14}C -cholesterol intravenously, is in good agreement with that obtained by other authors who used the same basic principle. A modified and simpler method of the quantitative estimation of conversion of cholesterol to bile acid after the single injection is also proposed in this thesis.
2. The rate of bile acid formation in rats is mainly influenced by the basic diet, commercial or semisynthetic, and is only slightly influenced by the presence or type of fat and by cellulose content. The effect of basic diet on bile acid formation is directly related to the rate of biosynthesis of cholesterol as measured by acetate incorporation into liver cholesterol. Therefore, it was concluded that the turnover rate of cholesterol is markedly increased in rats fed the commercial diet as compared with those fed semisynthetic diet.
3. The findings on the synthesis, breakdown, concentration of cholesterol and influx rate of plasma cholesterol into tissues in rats fed corn oil diet raises the question of the significance of the depressing effect on serum cholesterol level of corn oil for the treatment of atherosclerosis.

4. The rate of bile acid formation was decreased in suckling rats and this may be related to the lower rate of cholesterol synthesis and to increased cholesterol deposition in the body as compared to weaned or young adult rats. It is suggested that the hypercholesterolemia in suckling rats cannot be ascribed to the high content of highly-saturated fat of milk but rather to the large supply of exogenous cholesterol by milk.

5. In fasted rats the rate of bile acid formation was lower than in controls. Although fasting is a complex situation, the findings observed here may account for the maintenance of cholesterol concentration in the body in the presence of a decreased rate of biosynthesis of cholesterol in this condition.

6. A reduced rate of bile acid formation was also observed in X-irradiated-fasted rats, as in the fasted rats. This observation and other findings discussed already indirectly suggest that the rate of cholesterol synthesis may be in a normal range in rats so treated and that a slight increase in plasma cholesterol concentration in X-irradiated rats as compared with fasted or fed controls may be accounted for by the decreased rate of cholesterol excretion as bile acids in the presence of the same rate of cholesterol synthesis as in fed controls.

7. Injection of Triton WR-1339 in saline into rats caused a nine-fold increase in plasma total cholesterol and the rate of bile acid production was not increased proportionately but was similar to that in controls. This imbalance may secondarily contribute to an increased cholesterol concentration in plasma. The magnitude of influx of plasma cholesterol into tissues was not increased proportionately to the increased plasma cholesterol concentration in Triton-treated rats. In addition, the esterified cholesterol concentration both in plasma and liver was lower than in controls. These findings may be due to the effect of Triton on lipoprotein structure, and could be related to the finding that Triton injection was able to prevent atherogenesis in cholesterol-fed rabbits.

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APPENDIX I (a)

COMPOSITION OF SEMISYNTHETIC DIETS

	Fat-Free	High Cellulflour	Butter	Corn Oil
Casein	18 g.	18 g.	22 g.	22 g.
Dextrose	72 g.	52 g.	52 g.	52 g.
Fat	1.5 g.*			
	(corn oil)	1.5 g.*	15 g.	15 g.
Salt Mixture	4 g.	4 g.	5 g.	5 g.
Cellulflour	5 g.	25 g.	5 g.	5 g.

Added to each kilogram of diet:

Water-Soluble Vitamins: 15 ml. of freshly prepared mixture**

Fat-Soluble Vitamins:

Vit. A acetate, 20 mg.
(1,000,000 units/mg.)

Vit. E, 110 mg.
(DL- α -tocopherol acetate)

Vit. K, 30 mg.
(2 methyl-naphthoquinone)

* For dissolving fat-soluble vitamins.

** Each 100 ml. of mixture contained 50 mg. of thiamine hydrochloride, 100 mg. of riboflavin, 50 mg. of pyridoxine hydrochloride, 2 mg. of biotin, 500 mg. of nicotinic acid, 590 mg. of calcium pantothenate, 5 mg. of folic acid, 10 mg. of choline, and 1 g. of inositol.

APPENDIX I (b)

COMPOSITION OF COMMERCIAL DIET

"Master Fox Cubes"

Brewer's dried yeast, dried beet pulp, dehydrated alfalfa, cooked wheat flakes, wheat bran, wheat germ, dicalcium phosphate, potassium iodide, ground lime stone, 5% iodized salt, manganese sulphate, iron sulphate, Vitamin A and B supplement, riboflavin, Vitamin E.

Crude Protein	20%
Crude Fat	3%
Crude Fibre	5%
Metabolizable energy	2.8 Cal./g.

For the preparation of the commercial diets containing no fat or 15% by weight of butter or corn oil, the Master Meal was extracted with ether twice to remove most of its endogenous fat.

APPENDIX II (a)

TESTS FOR THE IDENTIFICATION OF INTESTINAL BACTERIA
GROWN ON MINERAL SALT-CHOLESTEROL MEDIA (Test No. 2)

Colonies on	A	B	C	D
Tryptose Agar Plate				
Gram Stain	Neg. Rods	Neg. Rods	Neg. Rods	Neg. Rods
Motility	Non-Motile	Non-Motile	Motile	Non-Motile
Gelatin Stab	Surface Growth	Surface Growth	Growth in Melted Gelatin	Surface Growth
Liquefaction	-	(+)	+ 2/5	-5
Litmus Milk	-	-	-	-
Indole	+1*	-5	-2	-1
H ₂ S	+2	-5	-5	+
Catalase	+	(+)	(+)	+
Dextrin	A ¹ G ¹			A ¹ G ¹
Glucose	A ¹	0	0	A ¹ (G)
Inositol	(G) ¹			A ¹ (G) ¹
Inulin	(A) ¹	07	07	(A) ¹ (G) ¹
Lactose	A ¹ G ¹	07	07	(A) ¹ G ¹
Maltose	A ¹ G ¹	07	07	A ¹ G ¹
Mannitol	A ¹ G ¹	07	07	A ¹ G ¹
Rhamnose	A ¹	0	0	A ¹ G ¹
Salicin	A ¹	01	01	A ¹ G ¹
Sucrose	A ¹ G ¹	0	0	A ¹ G ¹
M. R.	(+)	-5	-5	-5
V. P.	-	No growth	No growth	+
Citric Acid	-			+
Citric Salt	-			+
Nitrate	+1	-5	+2	+2
Urease	-	-	+1	-

Remarks	<u>Escheri-</u> <u>chia coli</u> (or <u>Aero-</u> <u>bacter</u>)	<u>Alcaligen</u> <u>fecalis</u>	<u>Alcaligen</u> <u>fecalis</u>	<u>Aerobacter</u> (or <u>E. coli</u>)

* Days taken for positive or negative reactions.

G = Gas Formation

A = Acid Formation

() = Slight reaction

M. R. = Methyl Red Test

V. P. = Voges-Prostauer Reaction

APPENDIX II (b)

TESTS FOR IDENTIFICATION OF INTESTINAL BACTERIA GROWN ON
MINERAL SALT-CHOLESTEROL MEDIA (Test No. 3)

Colonies on	Colony A	Colony B	Colony C	Colony D	Colony E
Tryptose Agar Plate	Neg. Rods	Irregularly stained long rods	Neg. Rods	Neg. Rods	Neg. Rods
Gram Stain	Non-Motile	Motile	Non-Motile	Motile	Very actively motile
Gelatin Stab	Gas Formation	Surface Growth	Surface Growth	Surface Growth	Growth in the melted Gelatin
Liquifaction	-	-	+7	-	rapid +2
Litmus Milk	-7	-7	Digested 4	-7	Digested 2
Indole	+1	+1	+1	+1	+1
H ₂ S	-	-	-	-	+
Catalase	-	+	(+)	-	+
Arabinose	A1 GI	07	0	0	0
Dextrin	A1 GI	07	(A)7	07	07
Glucose	A1	07	07	A1	A1
Inositol	A1 (GI)	07	07	07	07
Inulin	(A)1	07	07	07	07
Lactose	A1 GI	07	07	07	07
Maltose	A1 GI	07	07	07	07
Mannitol	A1 GI	07	07	07	07
Raffinose	A1 GI	07	07	07	07

Rhamnose	A	07	07	07	07
Sucrose	AI GI	07	07	07	07
Sorbitol	AI GI	07	07	07	07
M. R.	-7	-7	-7	-7	-7
V. P.	+2	-7	-7	-7	-7

Remarks and Possible Organisms

Aerobacter
(or E. coli)

Methylene blue staining - long rods containing metachromatic granules and having barred appearance.

Corynebacterium

Good bipolar staining.
Yellow pigment on the surface of sugar media. Not soluble in water.

Pseudomonas

Yellowish pigment on the surface of sugar media. Not soluble in water.

Pseudomonas

Yellowish pigment - soluble. Stained the medium. Swarming on agar slant.

Proteus

G = Gas Formation.
A = Acid Formation.
() = Slight reaction.
M. R. = Methyl Red Test.
V. P. = Voges-Prostauer Reaction.