THE INFLUENCE OF DIET ON CHOLESTEROL METABOLISM

by

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A THESIS

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(Chairman, Graduate Council)

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INTRODUCTION

Contained in this thesis, is a description of studies made on the relationship of dietary fat to tissue lipid levels and to sterol biosynthesis. The quantitative as well as the qualitative aspects of this relationship will be presented. Particular emphasis will be given to the metabolism of the sterol, cholesterol, and to the influence on it by corn oil or lard diets in the male, albino rat.

In surveying past literature, a logical separation can be made between the studies performed prior to the "isotope era" and those done after the application of isotopes to biology. Earliest publications reported on the amounts of lipid materials, fatty acids and cholesterol, in various experimental and clinical conditions. These studies were followed by balance studies, demonstrating conclusively that cholesterol and the other body lipids were closely related in a dynamic system. These balance studies gave the first indications that cholesterol was, indeed, synthesized in the animal body and that it had a finite biological life as well. Upon the introduction and development of isotopic techniques, better clarification of earlier problems was possible, and the intricate mechanisms of metabolism were made accessible for rigorous study.

Cholesterol was not the primary concern in many of the early studies, but was mentioned only incidentally. Fatty acids were the items of major interest, since clinical reports on lipemias and a host of animal studies on fatty livers cited these compounds as the materials influenced by altered metabolism (29, 8, 3, 9, 6, 83). Fatty livers, for example, could be produced by cholesterol feeding (the cholesterol

fatty liver) and the tissues were studied for their lipid content, the cholesterol considered being only a means to that end.

It was in the mid-1930s that the experimental focus shifted. Instead of cholesterol being used to mediate an effect on tissue fatty acids, fatty acids were administered to produce a change in the cholesterol concentrations of tissues. It was during this shift in interest that a publication by Okey et al. (48) appeared. This paper was entitled, "Factors Affecting Cholesterol Deposition in the Tissues of Rats", and told of experiments in which diets of 5, 10 and 15 per cent Crisco were fed to animals. It was in this paper, also, that the first difference was noted with respect to sex and the concentrations of cholesterol in tissues.

Indications that the fatty acids of the tissues were related in some way to exogenous sources of fat, as well as to the sterol content of the animal, were often present and speculations were common. Adequate evidence was, however, not available until balance studies were inaugurated for support of the proposition that a definite relationship existed.

Schoenheimer and Breusch (56) were led to the assumption that in the tissues, cholesterol is continually being formed and destroyed. Either a positive or a negative balance could be found, i.e. synthesis in excess of destruction or visa versa, depending upon the experimental conditions. When bread alone was fed to mice, the animals were able to synthesize, in a month, the amount of cholesterol initially contained in their bodies. When moderate amounts of cholesterol were administered, smaller amounts were found to be synthesized. When large amounts of cholesterol were fed, a considerable part was found to be destroyed.

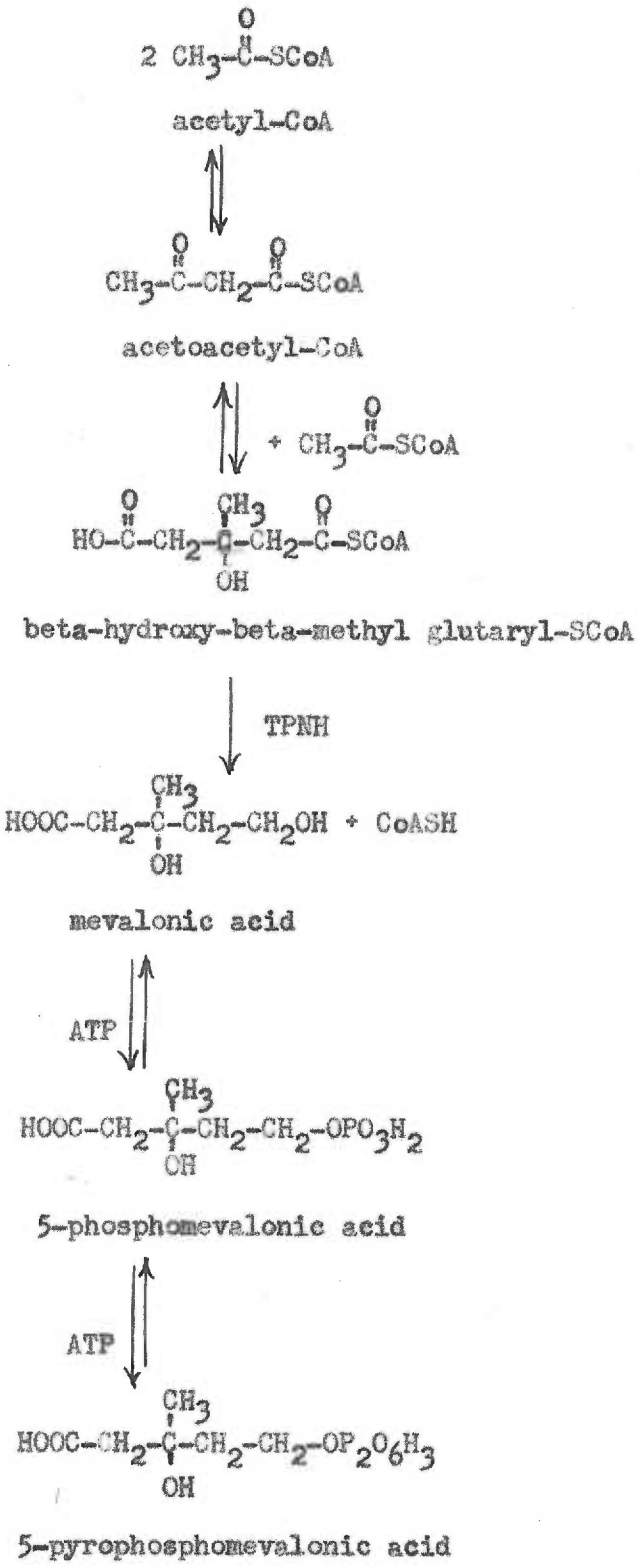
Large amounts of fat, on the other hand, were reported to have no effect on the cholesterol balance.

Simultaneously, other studies on cholesterol balance were being carried out (56). If large amounts of lard were fed to mice, no significant increase in cholesterol synthesis was found as compared with controls on a low fat diet. And again, cholesterol was found to be in a state of flux, continually being involved in the processes of formation and destruction.

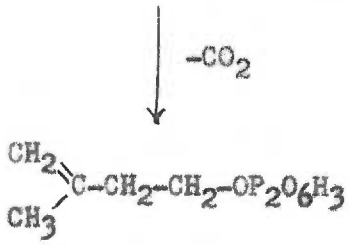
To growing rats, Eckstein and Treadwell (19) fed corn oil or soybean oil and found that the amounts of sterol in the livers were greater when the per cent of either oil was increased in the diet. A negative balance, i.e. fecal sterols exceeding fed sterols, was also observed when the dietary corn oil or soybean oil was increased.

Studies (18,19) also demonstrated sterol synthesis to be increased when corn oil (iodine number 118) was fed as compared to coconut oil (iodine number 8). Both of these dietary fats were fed at the level of 28 per cent of the diet. Eckstein remarked at the close of his report (18), "It is thus evident that more information concerning the relationship between the degree of saturation of dietary lipids and sterol formation is desirable".

As isotopes became available for biological research, a plethora of information emanated from scientific laboratories. Of particular value was the application of this tool to intermediary metabolism, and the pathways of cholesterol metabolism were no exception in this regard. At the present time, many of the intermediate compounds of cholesterologenesis are known and for general information, a brief schema of the known steps in cholesterol biosynthesis is listed as follows:

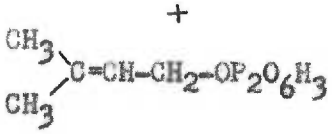

2 CH3-C-SCoA
acetyl-CoA
CH3-C-CH2-C-SCoA
acetoacetyl-CoA
+ CH3-C-SCoA
HO-C-CH2-C-CH2-C-SCoA
CH3
OH
beta-hydroxy-beta-methyl glutaryl-SCoA
TPNH
HOOC-CH2-C-CH2-CH2OH + CoASH
CH3
OH
mevalonic acid
ATP
HOOC-CH2-C-CH2-CH2-OPO3H2
CH3
OH
5-phosphomevalonic acid
ATP
HOOC-CH2-C-CH2-CH2-OP2O6H3
CH3
OH
5-pyrophosphomevalonic acid

5-pyrophosphomevalonic acid

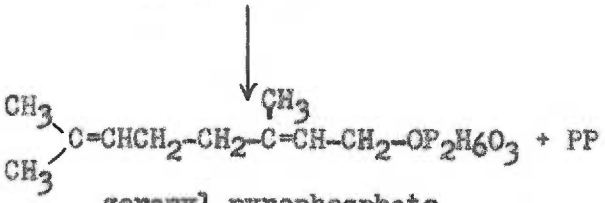


isopentenyl pyrophosphate

+

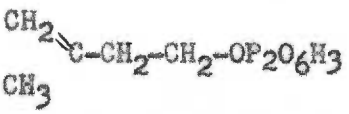


3,3-dimethyl allyl
pyrophosphate

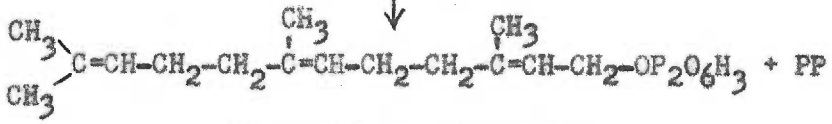


geranyl pyrophosphate

+



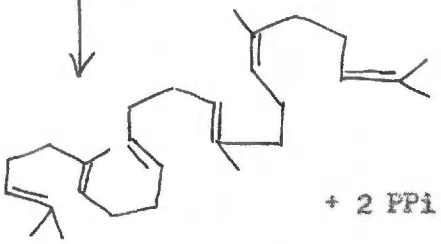
isopentenyl pyrophosphate



farnesyl pyrophosphate

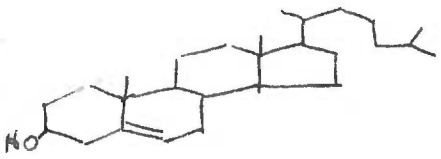
2 farnesyl pyrophosphate

(head to tail condensation)

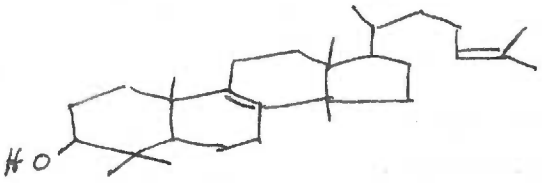


+ 2 PPi

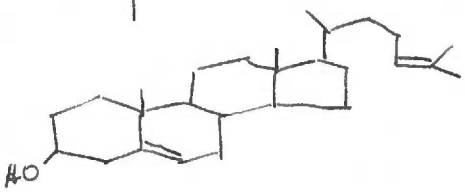
Squalene



Cholesterol

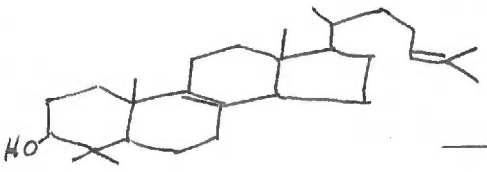


Lanosterol

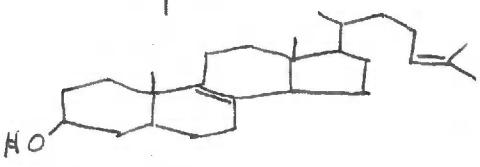


Desmosterol

$- CO_2$



$- 2\ CO_2$



Zymosterol

4,4'-dimethyl-cholestadiene-3-beta-ol

A number of factors have been found to influence the biosynthesis of cholesterol. These factors are as follows: (a) age of the animal, (b) nutritional state, (c) intake of cholesterol and cholesterol-like compounds, (d) intake of soy sterols, (e) the cations present and the hydrogen ion concentration, (f) hormones, (g) vitamin deficiencies, (h) tissue injury, and (i) fat feeding. Some mention should be made of the more outstanding points concerning the above factors, with particular stress being applied to the category of "fat feeding". The experimental animal is a complex system for study, and full knowledge of all the factors pertinent to cholesterol synthesis is of utmost importance.

Age

Synthesis of cholesterol is apparently most rapid at an early age. The formation of cholesterol in nervous tissue is accomplished in early life for the rat (63,20), and hepatic synthesis is increased at an early age (54).

Nutritional State

The nutritional state of the animal has a profound effect upon cholesterologenesis in the intact animal. Hutchens et al. (35) demonstrated decreases in this function in liver, gut, carcass, and skin tissues of rats fasted up to 240 hours. All of the fractions showed decreases in lipid labeling from an acetate precursor, but the liver seemed most susceptible to this prolonged fast. Restoration of the animal's ability for biosynthesis is possible if glucose, a protein hydrolysate, or fat is fed (66).

Cholesterol Intake

As was mentioned earlier, cholesterol included in the diet of experimental animals decreased cholesterol biosynthesis (56). This

report has been confirmed a number of time in studies in which labeled acetate was utilized as the cholesterol precursor. This suppression was apparently not a fleeting effect since the inhibition was still present 8 days later when the animals were maintained on a fat-free, cholesterol-free diet (2). This effect seems located mainly in the liver, since skin and intestinal mucosa studies *in vitro* did not demonstrate inhibition of cholesterol synthesis with the administration of exogenous cholesterol (27).

Cholesterol analogues, and precursors such as squalene, have a similar effect on cholesterol synthesis. Primarily from these observations, it has been postulated that the rate of cholesterol synthesis in the liver is subject to homeostatic regulation, and that the inhibition of cholesterol synthesis is a function of the level of liver cholesterol (68,24).

Effect of Soy Sterols

The soy sterols prevent accumulation of cholesterol in the liver by blocking cholesterol absorption from the gut. No affirmative answer has been given to the question of soy sterols depressing cholesterol synthesis.

Effect of Cations and Hydrogen Ion Concentration

In vitro studies have placed the optimum pH for cholesterol synthesis to be between 6.2 and 6.8. Variable results have been obtained in media in which potassium and magnesium concentrations were varied. Sufficient potassium and magnesium ions must, however, be present in the incubating medium if marked variations in the rate of cholesterol synthesis are to be avoided.

Hormones

Thyroid hormone has some effect upon cholesterol synthesis. Synthesis is stimulated in hyperthyroid conditions and depressed in hypothyroid conditions. This was found to be true for liver, intestine, kidneys and spleen, but not for lung tissue (42).

The influence of the pancreas upon cholesterol metabolism has been the subject of numerous studies with the diabetic rat (79,80,13,22,21). An early, postulated origin of the hypercholesterolemia of diabetes was that of the increased biosynthesis of cholesterol (34). With alloxan treated animals and with pancreatectomized animals, a decreased turnover of cholesterol in the liver was shown to cause the aberrant cholesterol metabolism, rather than any increase in synthesis. In fact, a decrease in cholesterologenesis has been demonstrated with acetate used as precursor. Mevalonic acid used as a cholesterol precursor did not demonstrate any decreased incorporation in the diabetic preparation. All of the available evidence indicates that cholesterol metabolism is affected by damage to the pancreas, as is reflected by the studies with the diabetic preparations.

According to Tomkins et al. (67) cholesterologenesis from acetate is depressed in hypophysectomized rats. This observation implicated the pituitary gland to be in control of steroid production in endocrine and non-endocrine target organs. However, this observation has subsequently been shown to be due to the effect of the poor eating (dietary) habits of the operated rats.

Vitamin Deficiencies

(a) Pantothenic acid deficiency

Deficiencies in the B-vitamins have been found to alter cholesterol

synthesis. Pantothenic acid, a structural moiety of coenzyme A, is presumed to alter cholesterol metabolism by limiting cholesterologenesis because of a lack of activated precursors. An interesting report in this regard is given by Rabinowitz and Gurin (51) who state that acetyl-CoA was incorporated more readily into cholesterol than was acetate alone.

(b) Biotin deficiency

There is a close relationship of biotin to fatty acid metabolism, but only a very limited or questionable relationship of biotin to cholesterol metabolism.

(c) Deficiencies of miscellaneous vitamins

Many of the early studies on lipids were done with fatty liver preparations. Cholesterol is only one among a list of many substances that cause fatty livers. Best et al. (2) found choline to be effective in relieving many of these fatty livers. But the role of the B vitamins in cholesterologenesis is not clear.

Tissue Injury

As might be supposed, cholesterol biosynthesis varies inversely with the degree of liver damage. Many of the studies on liver damage have been carried out in surviving livers perfused with C^{14}-acetate.

Effect of Fat Feeding

The experimental proof that cholesterol was not formed by cyclization of ingested fatty acids, but instead was formed by the coupling of smaller molecules, was a major contribution to lipid chemistry. This fact was ascertained in 1937 by Rittenberg and Schoenheimer (53) using deuterium as a label. The finding that the fatty acids did not directly participate in the formation of cholesterol did not eliminate

dietary fat from exerting indirect influences on cholesterol biosynthesis. Numerous investigators, however, have reported conflicting findings relating the effect of ingested fat to cholesterol synthesis and deposition (2,56,19,45,32,11).

Alfin-Slater et al. (2) found normal cholesterol synthesis at the end of 8 days of feeding a high fat diet. Also, the newly formed cholesterol present in the liver and plasma of rats prefed low fat diets was unchanged when the animals were placed on a high fat diet.

In recent years, a number of reports have appeared in which an alteration in cholesterol synthesis was observed in rats fed diets in which the fatty acid component was varied in kind and amount. In one of these studies, fatty acids were found to inhibit cholesterol synthesis by rat liver homogenates (81). Although apparently both saturated and unsaturated fatty acids were inhibitors, the inhibition increased with the degree of unsaturation of the acid. Oleic and vaccenic acids, which differ only in having a double bond in the 9-cis and 11-trans positions respectively, had approximately the same effect on cholesterol synthesis.

However, in a later report using acetate-1-C^{14} as precursor, Wood and Migicovsky (82) found that unsaturated oils and fatty acids stimulated the incorporation of the acetate into cholesterol both in vivo and in liver homogenates. When the unsaturated oil was corn oil, the increased synthesis could be demonstrated, as it could be when oleic acid, a major component of the oil, was fed. The corn oil was fed as 20 per cent of the diet and the oleic acid as 9 per cent of the diet. The ability of liver homogenates to incorporate the more specific cholesterol precursor, mevalonic acid, into cholesterol was enhanced by previously feeding the animals the corn oil diet. Concurring

evidence was presented by Avigan and Steinberg (4), who likewise found the rate of incorporation of acetate-1-C^{14} or of tritium oxide to be higher in corn oil fed rats than in others.

Contrary evidence was presented by Hill et al. (33) in which animals were injected with acetate-1-C^{14} and fed fat diets for three days. No change was observed in the $C^{14}O_2$ recoveries regardless of the amounts of corn oil fed in the diets. Increasing the percentage of corn oil in the diet from 0 to 15 per cent failed to change significantly the levels of the compounds resulting from acetate utilization. However, the short duration of the feeding regimen certainly could account for these results since 7-10 days of feeding seems to be necessary in order to cause any change in cholesterol metabolism.

Saturated lipids such as coconut oil or lauric acid had the opposite effect with respect to the in vivo incorporation, i.e. they depressed synthesis. The saturated compounds had no significant effect on synthesis in homogenates.

Other workers (46) compared the acetate conversion to cholesterol in animals fed cottonseed oil and hydrogenated coconut oil. Feeding 30 per cent hydrogenated coconut oil markedly reduced cholesterol synthesis. The maximum cholesterol synthesis was observed in the slices of livers of rats fed a diet containing 15 per cent cottonseed oil, which is rich in linoleic and oleic acids.

The essential fatty acid, linoleic acid, was fed to determine what its influence might be. This acid is the major fatty acid component of corn oil, and together with oleic acid, comprises more than 80 per cent of the unsaturated fatty acids of corn oil. Rats fed linoleic acid as the only fatty component, in an otherwise fat-free diet,

maintained normal cholesterol synthesis. It appears that the major influence of unsaturated oils on biosynthesis of cholesterol is due to discrete components of the oil and not to total unsaturation. The alteration is not dependent upon the "essential" nature of fatty acids, or at least, not in the case of linoleic acid.

Other animals fed 10 or 30 per cent lard, 10 or 30 per cent corn oil were found to incorporate acetate or mevalonate no differently than normal animals (76). These in vitro studies were interpreted as suggesting that the effects of saturated and unsaturated fats on cholesterol levels in rats were mediated at some phase of cholesterol metabolism other than upon the hepatic synthesis of the sterol.

In general then, the majority of reports indicate fat to affect the capability of the cholesterol synthesizing system in the livers of animals. Large amounts of corn oil, containing considerable amounts of linoleic and oleic acids, augment cholesterol synthesis as measured by incorporation studies with acetate as the tracer.

Correlative studies using hydrogenated oils or naturally saturated fats, such as lard, tended to depress cholesterol synthesis when ingested by rats. The more extensive the hydrogenation of cottonseed oil, for instance, the greater became the inhibition on cholesterol biosynthesis, indicating an inversely proportional relationship between degree of fat saturation and cholesterologenesis.

Influence of Fat on Tissue Cholesterol Concentrations

Dietary fat is in some way related to the amounts of cholesterol deposited in tissues. Blood levels of cholesterol have been extensively reported on in reviews on this subject; the review of Portman and Stone (50) covers this literature well. A few summary statements on this

subject would, however, appear to be desirable.

Vegetable fats generally, tend to lower blood cholesterol levels. The responsible ingredients seem to be linoleic acid and arachidonic acid (41,37,31). There has been shown to be a direct conversion of linoleic acid to arachidonic acid (43), so the active factor may in fact be arachidonic acid. At any rate, when adequate amounts of one or the other of these fatty acids is present in a dietary fat, drops in the blood cholesterol levels have been observed. Though this fact has not been shown universally (64,55), it is now generally accepted to be the case. The converse is also accepted, i.e. animal fats, or saturated fatty acids, tend to increase cholesterol levels in the blood.

Extensive work has been done on these and other factors relating to hypocholesterolemic influences of fats and sterols by Beveridge et al. (10). The summary of their results is as follows: (1) there is no simple relationship between the hypocholesterolemic effect of an oil and the degree of unsaturation, (2) sitosterol, or something closely associated with this sterol in the unsaponifiable matter, accounts for a large part of the hypocholesterolemic activity of corn oil, and (3) certain fatty acids of short chain length, or some other substances in butterfat and coconut oil, have a hypercholesterolemic effect.

Liver analyses have shown variations in cholesterol concentrations depending upon the past history of fat feeding. Reports have appeared in which unsaturated fats have produced increases, decreases or no changes in the livers of the animals (69,55,5,17). However, the majority of reports find cholesterol levels in the liver increasing with corn oil feeding, the major portion of this increase being in the cholesterol ester fraction.

Diets were fed to rats which contained coconut oil (iodine value 9) and corn oil (iodine value 127) for periods up to 80 days (4). There was very little, if any, increase in the cholesterol concentration in the livers of the coconut oil fed animals, whereas, striking increases were noticed for the corn fed animals. The increased cholesterol concentration of the corn oil fed animals was attributed to the presence of the linoleic acid in the corn oil.

Wood and Migicovesky (82) likewise confirmed these findings but were less specific and attributed the increase in liver cholesterol to the unsaturated oils and fatty acids. They found saturated material, viz. coconut oil or lauric acid, to lower the cholesterol concentration in the livers of rats. In their studies the amount of oil fed occupied 20 per cent of the total diet while the administered fatty acid was present as 9 per cent of the diet, determined on a weight basis. The actual amount of fat fed is an important consideration, since liver cholesterol levels are higher when dietary fat and linoleic acid are either in very low or very high amounts rather than when the levels of dietary fat are at an intermediary level (38).

In rats receiving 12.5 per cent cottonseed oil, cholesterol increases in the liver and adrenal glands, and plasma cholesterol levels decrease (1). In essential fatty acid deficiencies, an increase in esterified cholesterol accounts for most of the increased cholesterol. The conclusion seems to be that in the absence of essential fatty acids, the increase in cholesterol results from failure of cholesterol esters, containing other than polyunsaturated acids, to be available for proper metabolism.

In order to ascertain more specifically the location of cholesterol

in liver cells, a quantitative distribution of free and ester cholesterol was determined in five ultracentrifugal fractions (57). The five fractions were as follows: (i) nuclear, (ii) mitochondrial, (iii) submicroscopic particulate, (iv) supernatant, and (v) floating. The livers were from normal and cholesterol-fed rats. In the normal liver, more than 60 per cent was free cholesterol in the submicroscopic particles, while the major part of the esterified cholesterol was associated with the centripetally migrating fraction. With cholesterol feeding, increases in the esterified cholesterol in the centripetally migrating fraction was observed after 7 days.

To summarize the factors influencing the tissue cholesterol levels: The "active" fatty acids appear to be arachidonic acid and linoleic acid. The oils in which these acids are present are similar in action to the acids when fed separately. These oils or fatty acids have a depressing effect on serum cholesterol levels and mediate an increase in liver cholesterol concentrations, the majority of increase being in the ester form. The saturated fats and fatty acids have a slight increasing influence on blood cholesterol levels, and either do not affect the level of cholesterol in the liver or else decrease it very slightly.

Thesis objectives

Very few reports have presented a comprehensive study of the cholesterol levels in the whole animal as influenced by dietary corn oil or lard. This study was designed in an attempt to cover as completely as possible the changes mediated by this fat feeding regimen.

Tracer studies were done to study cholesterologenesis using

mevalonic acid, since mevalonate is a more specific precursor in cholesterol biosynthesis than is acetate. A paucity of information about mevalonate metabolism under these experimental conditions made mevalonate the more attractive tracer molecule for this study.

After studying the effects the diets had on normal animal tissues, a similar program was undertaken to compare with the normal, the alloxan diabetic animal. Thus far, no report has been presented to describe the influences that these diets might have on the alloxan diabetic rat.

MATERIAL AND METHODS

Animals

The animals used were male, albino rats of the Sprague-Dawley strain, obtained from a commercial colony maintained at Pullman, Washington. Upon arrival, the animals were fed ad libitum on Purina Rat Chow until the start of the experiment. The animals to be used as normals weighed approximately 160 gms. at the time of arrival, and the animals to be used as diabetics weighed approximately 200 gms. Drinking water was available to the animals at all times. All animals were housed in a cage-hood assembly developed in this laboratory (71).

Diets

The food consisted of synthetic diets containing 10 or 30 per cent corn oil (Mazola Corn Oil, Best Foods Division, Corn Products Company, New York), 10 or 30 per cent lard (Portland Rose Pure Lard, Portland Provision Company, Portland, Oregon), and a control diet containing 5 per cent lard that was designed to resemble Purina Rat Chow in fat composition. Table A lists the compositions of the diets used.

TABLE A

Rat Diets

Ingredient	Control %	10%	30%
Casein	18	18	18
Dextrin	44	39	19
Glucose	25	25	25
Yeast	4	4	4
Salt Mix	4	4	4
Lard or Corn Oil	5 (Lard)	10	30

Water soluble vitamins were mixed with the food and fat soluble vitamins were given orally, in daily doses to the rats by eye-dropper. Table B contains a list of the vitamins and the amounts given, as calculated for each kilogram of diet.

Table B

Vitamin Composition of Rat Diets

Fat soluble vitamins:

dissolved in triproprionate

Vitamin	mg/kilo of diet
B-carotene	5.4
Calciferol	0.03
2-methyl-1,4-naphthaquinone	2.0
2-tocopherol	60.0
Pyridoxine	4.0

Water soluble vitamins:

mixed with fat-free dry diet

Vitamin	mg/kilo of diet
Thiamine	4.0
Riboflavin	10.0
Calcium pantothenate	16.0
Inositol	1000.0
p-aminobenzoic acid	30.0
Choline chloride	1000.0
Folic acid	2.0
Biotin	0.1

In addition to the vitamin supplementation, 15 mg/rat/day of methyl linoleate was administered orally to the animals.

The above diets were fed for 28 days. The normal animals demonstrated a rapid gain in weight throughout the course of the experiment. In order to restrict this weight gain to approximately 100 grams for the 28 day period, the animals were fed ad libitum for the initial 14 days and trained to feeding for the final 14 days. The trained feeding regimen consisted of restricting each rat to 10 g of food per day and allowing them to eat for one hour at a time, twice a day.

Sacrifice

On the day of sacrifice, the animals were fasted for 12 hours, fed for an hour, fasted for another hour and injected with a tracer dose of mevalonic acid. This procedure was followed due to the influence of fasting and feeding upon lipogenesis (14,40,44,72). Immediately following the injection, the animals were placed in metabolism chambers and the urines collected for 2 hours. At the end of the 2 hours, the animals were anesthetized with chloroform, blood was taken by heart puncture and the animals were decapitated. The animals were separated into four fractions, liver, gut, carcass, and skin. These fractions were weighed, dissolved in alcoholic-KOH and refluxed for 2 hours. At the end of the reflux period, quantitative recoveries of cholesterol were made with petroleum ether extractions. The digest was acidified, and the fatty acids recovered quantitatively with petroleum ether extractions.

Condition of animals

Normal and alloxan diabetic animals were studied. The rats were made diabetic by an intramuscular injection of 0.06 ml of a 10 per cent alloxan solution per 100 grams of body weight (28). Just prior to the alloxan injection, an intraperitoneal injection of 5 ml of isotonic saline solution was administered. The animals were fasted for two hours after the injections and isotonic saline was given as drinking water for 48 hours. The criterion for diabetic animals was a blood sugar greater than 200 mg per cent. Three weeks were allowed for the diabetic state to develop before the blood was drawn for the sugar determinations. Blood sugars were determined again 26 days after the diet studies commenced. Animals not demonstrating sufficiently high blood sugar levels at this time were eliminated from the experiment.

Isotope

Mevalonic acid-2-C^{14} was the isotopic compound employed. It was obtained from Isotope Specialities Company, Inc. as the N,N-dibenzylethylenediamine salt of mevalonic acid. The 0.1 mc of material received was hydrolyzed and the mevalonic acid extracted. The free acid was dissolved in water so as to contain 0.67 uc/ml. This amount represented 6.88×10^4 cpm/ml counted as infinitely thick $BaCO_3$ under a D47 counter. The animals received 1 ml of this solution.

Chemical determinations and procedures:

Aliquots were taken from the alcoholic KOH digestion solutions,

extracted with petroleum ether as previously described (23), taken to dryness on a steam bath, and made to volume with 95 per cent ethanol. The extractions were carried out in 50 ml screw cap culture tubes using syringes and needles for the separation of phases. The non-saponifiable fraction will be referred to as cholesterol, although compounds closely related to cholesterol are known to be included in this fraction (see Appendix I). The saponifiable fraction is called the fatty acid fraction.

Cholesterol

Amounts of cholesterol were determined by the method of Zlatkis (84). This colorimetric procedure was used on digitonide samples and on non-digitonide samples in order to compare these two methods. The data and results for this comparison are found in Appendix I.

The non-digitonin procedure consisted of taking an aliquot of the non-saponifiable fraction to dryness; to this is added glacial acetic acid and a ferric chloride color reagent. The color is stabilized by the sulfuric acid used as solvent for the ferric chloride.

Digitonin was added to an aliquot of the non-saponifiable fraction and the digitonide precipiated (30). The digitonide was processed by a modification of the method of Sperry and Webb (62) which resulted in the washed sterol digitonide being dissolved in anhydrous methanol. An aliquot of this methanolic solution was removed and taken to dryness, and the Zlatkis method used for the sterol determination.

Color densities were measured with a modified Bausch and Lomb Spectronic 20 colorimeter (16) at a wavelength setting of 560 mu. Standard curves were prepared and standards were run with each set

of determinations. Dilutions of the cholesterol samples were made so that the concentration did not exceed 0.10 mg or fall below 0.025 mg per tube which gives optical density readings between 0.20 and 0.76 on our instrument. Samples were run in duplicate.

Fatty Acids

The extracts containing the fatty acids were transferred to shell vials that had been previously weighed. The ethanol solvent was evaporated, the shell vials being placed in a 60° C water bath, while a stream of nitogen was played on the surface of the evaporating solvent. The dried vials were reweighed and the amount of fatty acid residue obtained by difference. The amount of fatty acids in the total fraction was then calculated.

Blood sugars

The blood sugar determinations were done following the procedure of Somogyi (61). After a 24 hour fast, the end of the rat's tail was clipped and blood obtained for analysis. A protein free filtrate was made on 0.1 ml of blood with barium hydroxide and zinc sulfate. Final results were obtained from sodium thiosulfate titration of the excess iodine after reaction with the reducing copper.

Radioactivity analysis

The radioactive analyses were done using three instrument assemblies, all of which had Geiger-Mueller tubes. The samples were counted for at least 1600 total counts, and if very low count rates were encountered, the samples were counted for an additional 1600 counts. With the low

background counter, described below, it was sometimes necessary, with samples of low activity, to count less than 1600 counts, the time factor being a practical consideration. In these cases the samples were counted for 800 counts.

Automatic D47 Micromil gas flow system (Nuclear Chicago)

The D47 Micromil counter used is very suitable for low energy beta ray counting, i.e. carbon-14. The micromil window is an optional feature, but for all of the counting done and reported here, the window was always in place. The efficiency of this counter with infinitely thin samples was near 28 per cent with a background about 16 counts/minute. Auxiliary equipment used with the counter was a Nuclear Scaler (Model 183) with circuits for preset count or preset time assay, and a Nuclear automatic sample changer capable of handling up to 32 samples.

End window system

The Geiger-Mueller end window system was a Nuclear Scaler (Model 163) attached to a Tracer Lab 1.8 mg/cm^2 end window tube which is housed in an aluminum-lead shield. This equipment was used mainly for liquid sample counting, due to the easy accessibility to the counting area.

Automatic C115 Low Background gas flow system (Nuclear Chicago)

This low background counting system is designed to provide automatic detection with a low background of less than 2.0 cpm and a good efficiency of 21 per cent. This efficiency is 7 per cent lower than the efficiency of the D47 and is primarily due to a different sample-window geometry. Corrections for the two counting efficiencies were made for activity incorporation comparisons. The low background is

obtained because of an abundance of graded shielding, and a "guard" detector tube that acts as a cosmic ray shield, acting through an anti-coincidence circuit. The automatic sample changer and the Nuclear scaler are used with this detector also.

Samples

The samples assayed on these instruments were of two types, (i) the liquid sample, and (ii) the infinitely thin sample. Factors were determined previously in this laboratory permitting the reference of all samples to what they would have counted had they been infinitely thick $BaCO_3$ samples counted on the D47 counter.

Infinitely thin samples

A sample described as infinitely thin implies a condition of negligible self-absorption. This amount can be determined experimentally by adding radioactive material in small increments until further addition no longer gives a linear increase in activity. This deviation from liniarity is the point at which the infinitely thin criterion has been exceeded with the beginning of a measurable amount of self-absorption. This amount has been determined previously in our laboratory (36), and found to be less than 0.3 mg per cm^2 for both cholesterol and fatty acids. The sample was placed in the planchet as a solution and the solvent was then evaporated under a heat lamp. The dried samples were radioassayed in one of the counting devices.

Infinitely thick samples

Samples in our laboratory are all referred to infinitely thick $BaCO_3$ samples. An infinitely thick sample is one in which the

self-absorption of the sample is at a maximum and any further addition of material will not change the counting rate of the sample. At infinite thickness, a maximum count rate for that particular sample and sample holder has been achieved.

Liquid samples

The radioassay technique of aqueous samples containing carbon-14 was developed in and reported from our laboratory (73). The liquid sample is pipetted into a stainless steel planchet. A small drop of 1 per cent aerosol solution is added which reduces surface tension and allows the sample to cover the bottom of the planchet in an even film. A thin Mylar film (DuPont) is then placed, as covering, over the planchet and held in place by a rubber band. Counting is then done under the end window Geiger tube and the results converted to their equivalent as infinitely thick $BaCO_3$ samples.

RESULTS AND DISCUSSION

To present the results of these studies as clearly as possible, and without undue repetition, the Results section will be presented concurrently with the Discussion section of this thesis.

Statistical Methods

The statistical analyses used were of two types, the "analysis of variance test" and the "t-test". A confidence level of 0.95 was interpreted as indicating statistical significance, so that "p-values" less than 0.05 denoted that statistical difference existed between means.

t-test

The t-test was the statistical test used most frequently. This test is a small sample test, and is used to test for statistical differences between pairs of means. Two levels of significance (.05 and .01) are noted in the text, any value equal to or less than .05 being considered statistically significant. The assumptions for the t-test are that the samples come from normal populations, and that the variances are equal. The advantage in using the t-test is the test's insensitivity to violations of its two assumptions. The sampling behavior of "t" depends only upon the number of samples, and is independent of the population mean and standard deviation.

Analysis of variance

This statistical test is useful in comparing the means of more than two samples. The premise upon which this test is based takes advantage of the observation that, if means of subgroups differ greatly, the variance

of the group combined is much larger than the variances of the separate groups. A ratio, expressed as the F-value, is determined. This ratio is the variance of the "between" sub-groups divided by the "within" sub-groups. After determining the appropriate degrees of freedom, standard tables can be consulted giving the values for this variance ratio at the 0.95 or 0.99 level of significance. The assumptions upon which the analysis of variance is based are the same as for the t-test, viz. (1) both variables are drawn from normal distributions, and (2) the variances are equal.

In the material to follow, information relevant to the amount of sterol and to sterol labeling in individual tissues will be presented.

The concentration of cholesterol in four tissues

1. Liver tissue

A. Normal animals

Table I presents the mean values for the analysis of the livers of normal animals fed the various fatty diets.

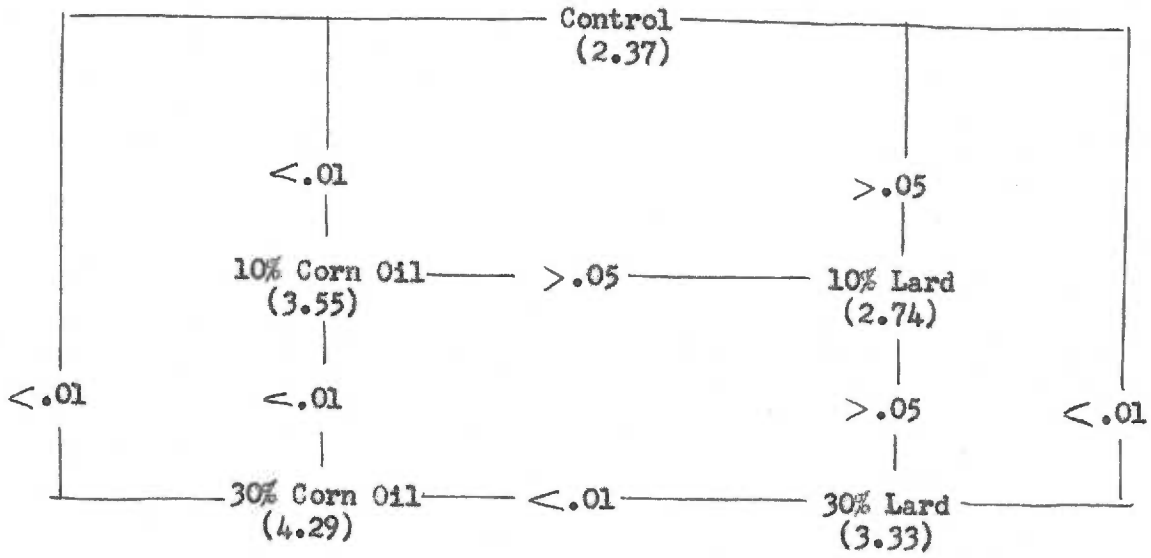
Table I

Diet	No. Animals	Mean (mg/gm tissue)
Control	10	2.37*±.07
10% Corn Oil	8	3.55 ±.36
30% Corn Oil	7	4.29 ±.20
10% Lard	8	2.74 ±.30
30% Lard	10	3.33 ±.20

* mean ± standard error

Diagram I summarizes the effects of the diets on the concentration of cholesterol in the livers of normal animals. The values in the parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram I



From these data two trends are noted. There is an increase in the liver cholesterol concentration as the fat in the diet is increased, and the amount of cholesterol found after the feeding of the 30 per cent corn oil diet is significantly higher than the amount found after feeding lard in similar amounts. The feeding of 10 per cent levels of lard and corn oil causes increases over control values, but these are not significantly different from each other.

B. Diabetic animals

Table II presents the values for the concentration of cholesterol in the livers of the diabetic animals.

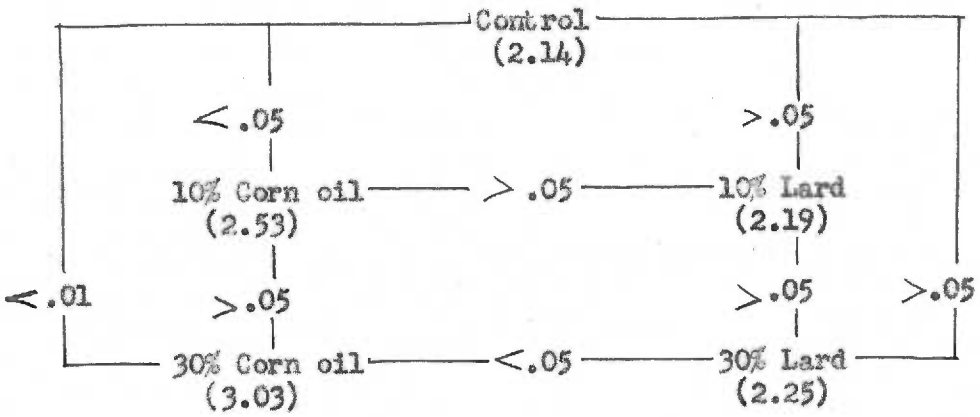
Table II

Diet	No. Animals	Mean (mg/gm tissue)
Control	10	2.14*±.07
10% Corn Oil	7	2.53 ±.13
30% Corn Oil	6	3.03 ±.23
10% Lard	5	2.19 ±.22
30% Lard	8	2.25 ±.12

* mean ± standard error

Diagram II summarizes the effects of the diets on the amounts of cholesterol found in the livers of diabetic animals. The values in the parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram II



The diabetic animals fed corn oil show similar trends with respect to increases in cholesterol as do the normal animals, but they are less extreme. Lard-fed, diabetic animals demonstrate no significant differences between any of the groups of animals.

Table III compares the findings from the normal and diabetic livers of animals fed the various diets. The values in the parentheses represent the number of animals in each group.

Table III

Liver:	Normal	Diabetic	p value
Control	2.37 (10)	2.14 (10)	$> .05$
10% Corn oil	3.55 (8)	2.53 (7)	$< .05$
30% Corn oil	4.29 (7)	3.03 (6)	$< .01$
10% Lard	2.74 (8)	2.19 (5)	$> .05$
30% Lard	3.33 (10)	2.25 (8)	$< .01$

The high fat diets show greater differences between normals and diabetics than do the diets with lower amounts of fat.

These results confirm earlier reports (4,36,49,60,82) that ingestion of corn oil increases the amount of cholesterol in the liver (Diagram I and Table I). There was found also a significant increase in cholesterol in the livers of the lard fed animals. In comparing animals fed the 30 per cent fat diets, it is seen that the corn oil diet initiated a greater cholesterol deposition than did the corresponding lard diet. This finding is not in agreement with that of Okey and Stone (49) and others (53,66) who found that lard-fed, male rats tended to store more liver cholesterol than did those fed equal amounts of vegetable fat.

The difference in the level of liver cholesterol cannot be attributed solely to the amounts of oleic acid in the two diets, since the lard and corn oil diets contain equal amounts of this acid (See Appendix II for analysis of lard and corn oil). The increase in hepatic cholesterol,

shown by others (17), was primarily in the ester form of the tissue sterol fraction, making a direct comparison with the present studies difficult.

It is of interest to note (Table II and Diagram II) that the diabetic and normal animals demonstrate similar liver tissue changes with corn oil feeding, but the changes are not as great in the diabetic. In nearly every case, increases in fat feeding produced higher values in normal than in diabetic animals (Table III).

2. Gut tissue

A. Normal animals

Table IV presents the mean values for the analysis of gut tissue of normal animals fed the various fatty diets.

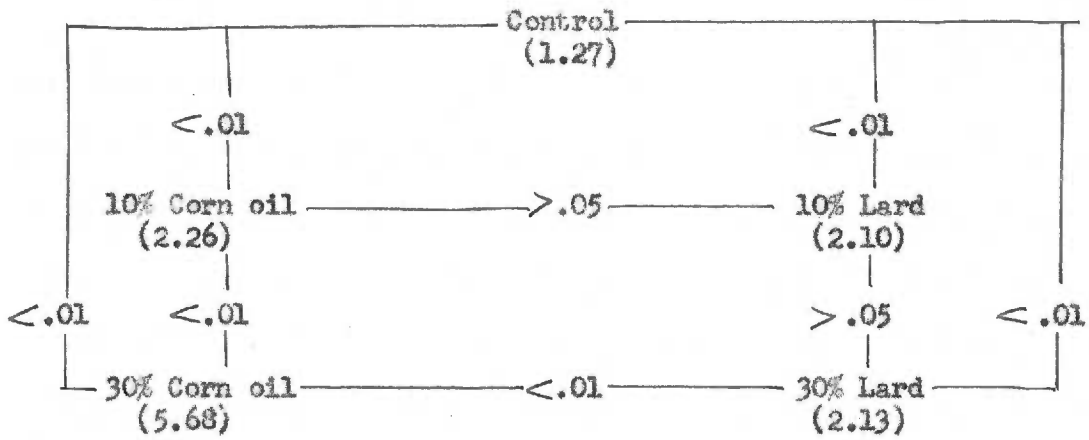
Table IV

Diet	No. Animals	Mean (mg/gm tissue)
Control	6	1.27*±.11
10% Corn oil	8	2.26 ±.18
30% Corn oil	7	5.68 ±.68
10% Lard	8	2.10 ±.18
30% Lard	10	2.13 ±.12

* mean ± standard error

Diagram III summarizes the effects of the diets on the cholesterol levels of the gut of normal animals. The values in the parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram III



In the gut tissue, there is an increased sterol concentration with 10 per cent fat in the diets. A further increase in fat, leads to a corresponding increase in sterol content in the corn oil animals only. The amount of sterol present following 30 per cent corn oil feeding was significantly greater than the amount of sterol found following the 30 per cent lard feeding.

B. Diabetic animals

Table V presents the data for the diabetic animals on the respective diets.

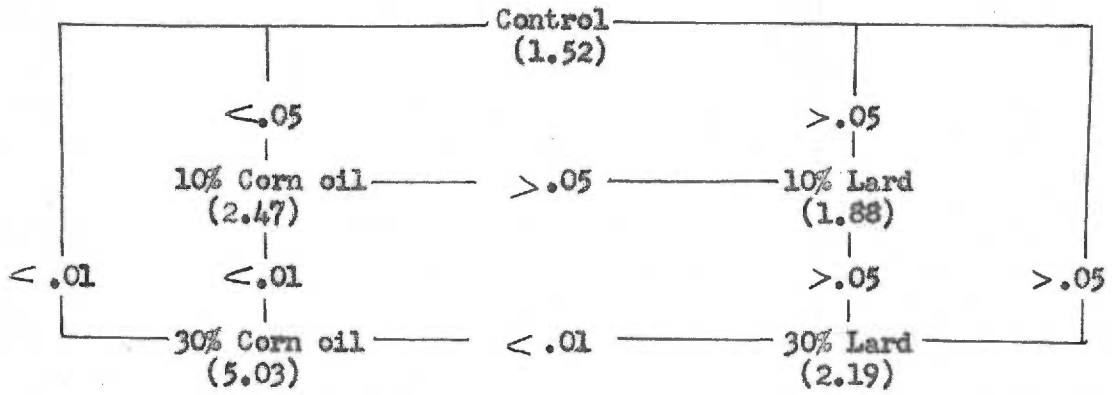
Table V

Diet	No. Animals	Mean (mg/gm tissue)
Control	3	1.52*±.03
10% Corn oil	7	2.47 ±.24
30% Corn oil	6	5.03 ±.30
10% Lard	5	1.88 ±.23
30% Lard	8	2.19 ±.13

* mean ± standard error

Diagram IV summarizes the effects of the diets on the cholesterol concentration as found in the gut tissues of diabetic animals. The values in the parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram IV



The diabetic gut tissue shows an increased sterol level only with the animals fed the corn oil diets, and it also shows an increase in this level as the amount of fat is increased in the diet.

Table VI compares the normal and diabetic gut tissue from animals fed the various diets. The values in the parentheses represent the number of animals in each group.

Table VI

Gut:	Normal	Diabetic	p value
Control	1.27 (6)	1.52 (3)	>.05
10% Corn oil	2.26 (8)	2.47 (7)	>.05
30% Corn oil	5.68 (7)	5.03 (6)	>.05
10% Lard	2.10 (8)	1.88 (5)	>.05
30% Lard	2.12 (8)	2.19 (8)	>.05

There is no statistical difference between the means of the normal and diabetic animals.

The gut presents an interesting picture. There is an unusually large amount of cholesterol in gut tissue of both the diabetic and normal animals fed the high corn oil diets, but there is no significant difference between the normal and diabetic animals at this or other dietary fat levels.

These results might be explained by the amounts of food remaining in the animal's gastrointestinal tract at the time of sacrifice. The gut was not washed out to remove residual food before alkali digestion, thus the values represent the tissue as well as the gut contents. There seems to be at least three possible ways for this to happen, (a) the cholesterol measured was of endogenous origin, (b) the cholesterol measured was contained in the food residue, thereby originating from exogenous sources, and (c) the color producing sterol was not cholesterol at all but rather some sterol contained in the two fatty components, but most predominantly in the corn oil.

The first consideration does not seem likely for two reasons. First of all, the concentrations of sterol differed widely between the animals fed the lard and corn oil diets. Secondly, the concentrations of sterol are high enough to cast doubt on the source of this sterol coming from intestinal bacteria or other endogenous sources. Though this possibility cannot be completely discounted, it does not seem to present the most feasible explanation for these observations.

The second and third possibilities seem more likely to afford an explanation of these results.

An analysis for cholesterol in the lard used for the diets, revealed

the presence of 0.77 mg of color producing sterol per gram of lard. The same procedure, when carried out on the corn oil indicated 9.25 mg of color producing sterol to be present per gram of corn oil. A published, commercial, analysis of Mazola Corn Oil (Appendix III) claimed the absence of cholesterol. There was reported, however, 1.5 per cent of the oil as sitosterols. Due to structural similarities between cholesterol and the sitosterols, it is possible that the color producing sterol of the gut fraction was B-sitosterol or some closely related compound. On the other hand, lard, being of animal origin, presents the possibility that cholesterol, per se, is the color reacting sterol present in the food residue in the gastrointestinal tracts of the lard fed animals.

Since each animal ate some 10 g of diet per day, this would be responsible for the presence of 1.7 mg of cholesterol in the 30 per cent lard animals and 15 mg of sitosterols in the 30 per cent corn oil fed animals. It is not possible to further quantitate these results with the tissue findings because of a lack of information on the homogenicity of the sterols of the gut tissue.

Bollman and Flock (12) could account for the absorption of 10-50 per cent of fed cholesterol in the intestinal lymph of rats within 24 hours after feeding. Vahouny et al. (70), found cholesterol absorption to be facilitated by the presence of oleic acid. Oleic acid is present as 26 per cent of lard. It is conceivable, then, that some influence may be exerted by the lard on the absorption of exogenous cholesterol.

Gould (25) found that rats fed B-sitosterol containing tritium had tritium labeled sterols in blood, liver and other tissues. B-sitosterol was found to be absorbed about 10 per cent as well as cholesterol. The possibility of B-sitosterol absorption is real, but the large changes

found in liver tissue sterol do not seem to be accounted for by the B-sitosterol alone. It is still entirely possible that some of the color producing sterol in the gut fraction is B-sitosterol, however.

3. Carcass

A. Normal animals

Table VII presents the mean values for the carcass tissue of normal animals fed the various fatty diets.

Table VII

Diet	No. Animals	Mean (mg/gm tissue)
Control	6	1.56*±.06
10% Corn oil	8	1.47 ±.04
30% Corn oil	7	1.41 ±.08
10% Lard	8	1.38 ±.05
30% Lard	10	1.42 ±.04

* mean ± standard error

There is no significant difference between any of the means in the carcass fractions of the normal diet animals.

B. Diabetic animals

Table VIII presents the values for the cholesterol concentration in the carcasses of the diabetic animals.

Table VIII

Diet	No. Animals	Mean (mg/gm tissue)
Control	3	1.64*±.09
10% Corn oil	7	1.93 ±.08
30% Corn oil	6	1.69 ±.04
10% Lard	5	1.85 ±.11
30% Lard	8	1.58 ±.10

* mean ± standard error

Concurrent with the results in the normal animals, the means of the carcasses do not differ significantly in the diabetic animals.

Table IX compares the normal and diabetic carcasses from animals fed the various diets. The values in the parentheses represent the number of animals in each group.

Table IX

Carcass:	Normal	Diabetic	p value
Control	1.56 (6)	1.64 (3)	$>.05$
10% Corn oil	1.47 (8)	1.93 (7)	$<.01$
30% Corn oil	1.41 (7)	1.69 (6)	$<.05$
10% Lard	1.38 (8)	1.85 (5)	$<.01$
30% Lard	1.42 (10)	1.58 (8)	$>.05$

The carcasses of the diabetic animals contained a higher concentration of cholesterol than did the normals at all diet levels except the control and 30 per cent lard diets.

4. Skin

A. Normal animals

Table X presents the mean values for the skins of normal animals fed the various fatty diets.

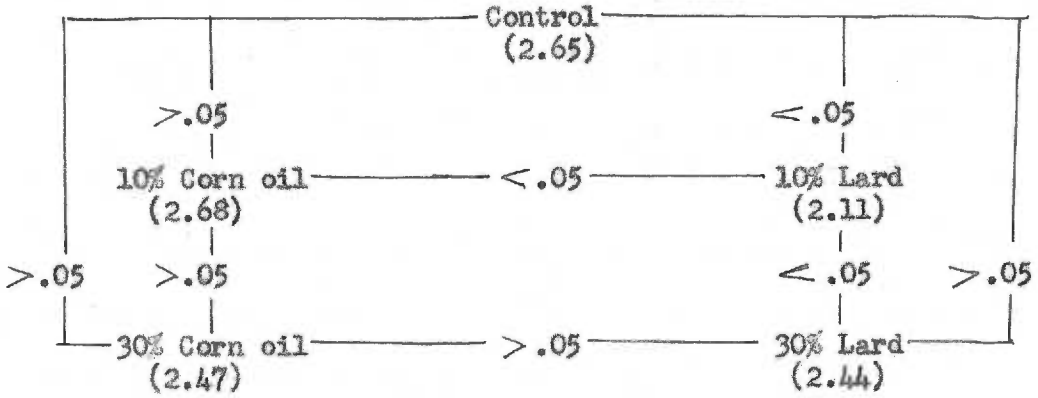
Table X

Diet	No. Animals	Mean (mg/gm tissue)
Control	6	2.65*±.21
10% Corn oil	8	2.68 ±.20
30% Corn oil	7	2.47 ±.15
10% Lard	8	2.11 ±.06
30% Lard	10	2.44 ±.07

* mean ± standard error

Diagram V summarizes the effects of the diets on the cholesterol concentration as found in the skin of normal animals. The values in parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram V



There appears to be no change in the cholesterol concentration in the skins of normal animals fed corn oil. Any change due to the lard feeding was reflected only in the animals fed the 10 per cent lard diet.

B. Diabetic animals

Table XI represents the data for the concentration of cholesterol in the skins of the diabetic animals.

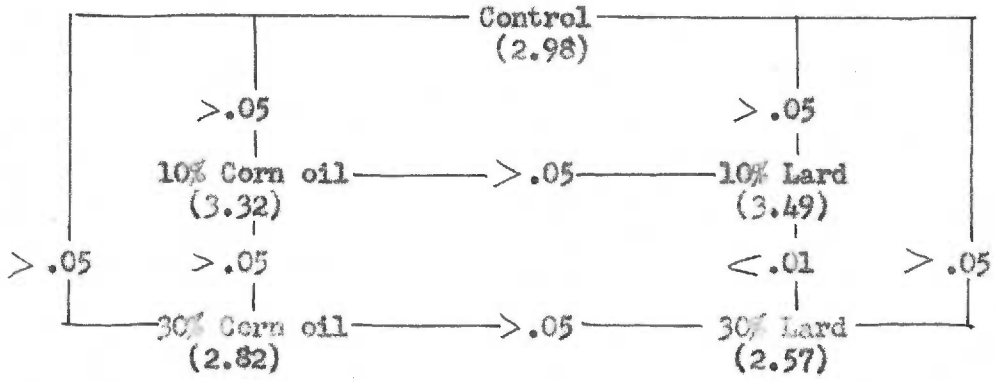
Table XI

Diet	No. Animals	Mean (mg/gm tissue)
Control	3	2.98*±.42
10% Corn oil	7	3.32 ±.28
30% Corn oil	6	2.82 ±.18
10% Lard	5	3.49 ±.14
30% Lard	8	2.57 ±.20

* mean ± standard error

Diagram VI summarizes the effects of the diets on the cholesterol concentration as found in the skins of diabetic animals. The values in the parentheses are the mean values in mg of cholesterol per g of tissue. The statistical difference of the means is indicated between all pairs of diets.

Diagram VI



No change in cholesterol concentration in the skin is noted among diabetic animals fed corn oil. Lard fed to animals seems to have changes dependent upon the amount of fat fed. The 10% lard diet and the 30% lard diet influence sterol levels to different extents, the concentration of sterol being the greatest in the animals fed the 10 per cent diet.

Table XII compares the skins from normal and diabetic animals fed various diets. The values in the parentheses represent the number of animals in each group.

Table XII

Skin:	Normal	Diabetic	p value
Control	2.65 (6)	2.98 (3)	$>.05$
10% Corn oil	2.68 (3)	3.32 (7)	$>.05$
30% Corn oil	2.47 (7)	2.82 (6)	$>.05$
10% Lard	2.11 (8)	3.49 (5)	$<.01$
30% Lard	2.44 (10)	2.57 (8)	$>.05$

The 10 per cent lard diet animals present an unusual picture with respect to their influence upon cholesterol in the skin. The normal animals demonstrated a depressed value compared to the control value and 30 per cent lard fed animals. The diabetic animal, on the contrary, displayed an increase over the 30 per cent feeding level of lard. The physiological meaning of this observation is not clear.

Amounts of fatty acids

Table XIII presents the means of the fatty acids as determined in the tissues of the animals on the various diets. The values represent the means for the normal and diabetic animals in each diet. The values are expressed as mg. of fatty acid per g. of tissue.

Table XIII

Liver	Normal (mg/gm)	Diabetic (mg/gm)
Control	32.3* ± 0.4	32.3 ± 3.7
10% Corn oil	38.5 ± 2.6	32.6 ± 2.1
30% Corn oil	46.3 ± 3.9	45.3 ± 4.9
10% Lard	35.1 ± 2.2	31.2 ± 2.4
30% Lard	52.7 ± 6.9	39.9 ± 1.8

Gut	Normal (mg/gm)	Diabetic (mg/gm)
Control	58.4 ± 4.1	30.9 ± 4.1
10% Corn oil	85.2 ± 8.9	36.8 ± 1.9
30% Corn oil	142.5 ± 13.3	87.3 ± 7.3
10% Lard	81.1 ± 8.9	34.9 ± 3.6
30% Lard	111.5 ± 10.0	86.9 ± 11.7

* mean ± standard error

Table XIII (continued)

Carcass	Normal (mg/gm)	Diabetic (mg/gm)
Control	69.1* ± 11.2	44.8 ± 7.6
10% Corn oil	78.4 ± 4.4	51.7 ± 5.1
30% Corn oil	75.2 ± 6.1	91.5 ± 9.1
10% Lard	68.5 ± 3.2	44.6 ± 2.5
30% Lard	82.6 ± 4.7	74.5 ± 5.8

Skin	Normal (mg/gm)	Diabetic (mg/gm)
Control	129.6 ± 5.7	58.0 ± 3.9
10% Corn oil	150.7 ± 7.5	79.6 ± 8.9
30% Corn oil	159.7 ± 9.0	136.3 ± 10.1
10% Lard	130.8 ± 6.2	39.7 ± 9.4
30% Lard	147.7 ± 8.6	110.4 ± 13.6

* mean ± standard error

In considering the data on the amounts of fatty acids, a few observations can be made. The fatty acids in the livers of normal and diabetic animals on the same diets do not differ from each other. This result is compatible with other observations on normal and alloxan diabetic animals (78). It is also apparent that as the amount of fat is increased in the diet, the concentration of fatty acids tends to increase. It would be expected that increased amounts of fat in the diet would increase the amount of fat in the liver, the liver being the organ so actively concerned with the metabolism and handling of fat.

These data are likewise consistent with the observations discussed above concerning the amounts of sterol found in the liver tissues after feeding corn oil or lard diets. If it is true that the increase in sterol found in the livers of animals fed corn oil is predominantly in the ester form, then there would be a sufficiently large increase in the fatty acid concentration of the liver with corn oil feeding to support that contention. If it is assumed that one molecule of cholesterol reacts with one molecule of fatty acid to form a cholesterol ester, then a 2 mg. per gram of tissue increase in the liver of corn oil fed, normal animals (Table I) would require a 1.5 mg. per g. of tissue increase in the fatty acids, assuming all of the cholesterol is esterified and that the fatty acid moiety is a C_{18} fatty acid. Since the average fatty acid increase of 14 mg. far exceeds the value of 1.5 mg. per g. of tissue for the 30 per cent diet animals, the increase in cholesterol as ester is not ruled out.

A problem arises when attempting to correlate the fatty acid changes in gut tissue with dietary fat intake as was the case in interpreting the data concerning amounts of cholesterol. How much of the fatty acid content found is due to the food residue in the intestine of the animals at the time of sacrifice, and how much is contained in the cellular structure of the gut itself, is impossible to determine with the techniques employed.

The carcass tissue of the diabetic animals showed an increase to 52 and 92 mg. fatty acid per gram, over the control level of 45 mg. when the animals were fed the 10 and 30 per cent corn oil diet. The 10 per cent lard had little effect on the fatty acid level but the 30 per cent lard diet caused an increase to 75 mg. per g. tissue.

The diabetic animals consistently showed a lower fatty acid concentration in carcass tissue on the low fat diets. The 30 per cent corn oil diet value for the diabetic, which seems to be higher, is not statistically higher. These findings on the decreased amounts of fatty acids in the diabetic might be explained by the increase in utilization of fatty acids by the diabetic, resulting in decreased tissue levels. In the animals fed high fat levels, it is interesting to see that the increased need for fatty acids as "fuel" was met, allowing some storage to take place.

The skin tissue demonstrated no difference in the amount of fatty acids between any of the normal diet animals. The diabetic animals showed increased amounts of fatty acids in the 30 per cent fat-fed animals only. This fact becomes more striking when the normal animals are compared to the diabetics. The amounts of fatty acids present in the skin tissues of the normal and diabetic animals fed the high fat diets are not significantly different. The high fat diets apparently do provide sufficient quantities of fatty acids for storage to take place in the diabetic animals.

Blood cholesterol values

Table XIV presents the values for the amounts of plasma total cholesterol. The values are the mean values and their standard errors and are listed as mg per 100 ml of blood plasma.

Table XIV

Diet	Normal (mg %)	Diabetic (mg %)
Control	89 ± 5 (4)	162 ± 34 (3)
10% Corn oil	71 ± 11 (8)	106 ± 15 (6)
30% Corn oil	67 ± 11 (7)	121 ± 16 (7)
10% Lard	110 ± 12 (8)	196 ± 20 (5)
30% Lard	100 ± 7 (10)	225 ± 47 (5)

The numbers in the parentheses represent the number of animals in each group.

The blood cholesterol levels of the diabetics are significantly higher than the normals, extending earlier reports (79) of hypercholesterolemia in alloxan diabetic rats. This increase was true, regardless of the kind of diet, or of the amount of fat, fed. If cholesterol is associated with the lipoprotein complex vehicles for fatty acid transport, the hypercholesterolemia seen in the diabetic animals could result from the increase demand of the animal for fatty acids, these compounds being transported so that they may be utilized as sources of energy. The lower cholesterol levels of the corn oil fed animals seem to indicate that there is a slight trend towards a decrease in blood cholesterol levels with corn oil feeding, and a trend towards an increase of blood cholesterol with lard diets.

Incorporation of mevalonate-2-C^{14} into sterol

Isotope incorporation was studied in order to gain information about the biosynthetic activity of the individual tissues. Mevalonic acid was used because of the specificity with which it labels cholesterol (65,15,77). The incorporation of label was calculated by determining the amount of

labeled sterol in a tissue and dividing that by the activity of the isotope injected, the results usually being presented as a percentage of the dose. Since the time lapse between the injection and sacrifice was constant (2 hours), the per cent incorporation is a reflection of the precursor label ending up in the sterol fraction at the end of 2 hours. Time course studies have shown (20) that tracer levels of labeled mevalonic acid are maximally converted to sterol within an hour after injection.

In the experiments to be described, each animal was given identical tracer amounts of mevalonic acid. The assumption was made that the specific activity of the precursor of biosynthesis was equal in all animals. If this assumption is valid, the comparative amounts of label incorporated is assumed to reflect the magnitude of the biosynthetic pathways.

Per cent incorporation

Liver

Table XV presents the mean values for the incorporation of C^{14} into cholesterol in the livers of normal and diabetic animals.

Table XV

Diet	Normal	Diabetic	p-Value#
Control	3.95* ± .11 (2)	5.34 ± .30 (2)	
10% Corn oil	3.92 ± .16 (6)	4.80 ± .28 (5)	< .05
30% Corn oil	4.69 ± .37 (6)	4.73 ± .24 (4)	
10% Lard	4.59 ± .20 (6)	4.31 ± .49 (3)	
30% Lard	3.84 ± .16 (7)	4.47 ± .85 (5)	
p-Value**	> .05	> .05	

* mean ± standard error

** F-test

t-test

The F-test is not significant for either the normal or the diabetic animals. This is interpreted to mean that within the group of normal animals, dietary variations did not alter the incorporation of mevalonic acid into cholesterol. Within the group of diabetic animals, dietary variations, likewise, did not influence the per cent of label incorporated.

The t-test analysis demonstrated only one difference between the per cent incorporations of normal and diabetics on comparable diets. The 10 per cent corn oil fed diabetic animals had a significantly higher incorporation than did the normals on the 10 per cent corn oil diet.

Since these findings indicate no change in the amount of label converted to cholesterol within 2 hours (with the one exception), it appears that the synthesis of cholesterol in this system is independent of any change in the size of the liver cholesterol pool within the normal group and within the diabetic group of animals.

Four possibilities seem available to explain this finding. First of all, the free cholesterol pool may remain constant, since ester cholesterol has been reported to be the predominant component to increase in corn oil fed animals (5). This could mean that any influence tissue cholesterol would have on the incorporation of labeled precursor would be from the free cholesterol, rather than from esterified cholesterol. Gould has shown (26) that liver ester cholesterol may be the more important controller of liver sterol synthesis. Secondly, the color producing sterol in the liver could be B-sitosterol rather than cholesterol. B-Sitosterol does not appear to affect cholesterol synthesis (25). Thirdly, these gross observations could be occurring in discreetly separate cellular compartments, essentially isolating the two events of synthesis and storage. And lastly, the influence of diet upon cholesterol metabolism could be mediated through some mechanism other than synthesis, e.g. breakdown or removal.

Wilson and Siperstein (76, 75) did study the biliary excretion of cholesterol by the rat, as well as the fecal excretion of cholesterol end products with diets containing varying amounts of lard or corn oil. They found that neither 30 per cent lard nor 30 per cent corn oil diets had any effect on either the excretion of total cholesterol end products or of cholesterol itself into the bile. Furthermore, there was found to be a marked excretion of non-digitonin precipitable neutral sterols in rats

in which corn oil was the dietary fatty component. It is inferred from these studies that the elimination of sterol from the body is not decreased, thereby eliminating this as a possible explanation for an increased retention in the body.

Rat liver mitochondria are able to oxidize the terminal methyl groups of the cholesterol side chain to carbon dioxide in the presence of a number of cofactors (74). When the rats were fed corn oil diets or commercial shortening for a period of 40 days, it was found that the liver mitochondria of the rats fed the saturated fat oxidized cholesterol to carbon dioxide to a much greater extent than did liver mitochondria from rats fed unsaturated fat (39). These in vitro studies do not support the in vivo studies of Wilson and Siperstein and imply a decreased catabolism of cholesterol in animals fed corn oil diets.

Gut

Table XVI presents the mean values for the incorporation of label into cholesterol in the gut tissues of normal and diabetic rats.

Table XVI

Diet	Normal	Diabetic	p-Values#
Control	1.41* ± .05 (2)	1.92 ± .28 (2)	
10% Corn oil	1.30 ± .18 (6)	1.89 ± .07 (5)	< .05
30% Corn oil	1.79 ± .24 (6)	1.54 ± .06 (4)	
10% Lard	1.40 ± .06 (6)	1.31 ± .10 (2)	
30% Lard	1.31 ± .09 (7)	1.48 ± .20 (5)	
p-Value**	> .05	> .05	

* mean ± standard error
** F-test
\# t-test

The F-test shows no significance for either the normal or the diabetic animals. This is interpreted to mean, that within the normal group and within the diabetic group, dietary variations did not alter the incorporation of mevalonic acid-2-C^{14} into cholesterol.

The t-test analysis demonstrated only one difference between the per cent incorporation comparing normal and diabetics on the same diet. The 10 per cent corn oil fed diabetic animals had a significantly higher incorporation than did the normals, i.e. the p value was <0.05.

Carcass

Table XVII presents the mean values for the incorporation of C^{14} into cholesterol in the carcass tissues of normal and diabetic animals.

Table XVII

Diet	Normal	Diabetic	p-Value#
Control	17.48* ± .32 (2)	15.23 ± .66 (2)	
10% Corn oil	16.27 ± .78 (6)	17.56 ± .41 (5)	
30% Corn oil	18.37 ± 1.30 (6)	15.65 ± .56 (4)	
10% Lard	19.66 ± .99 (6)	13.29 ± 1.07 (3)	< .01
30% Lard	20.41 ± .90 (7)	16.05 ± .85 (5)	< .05
p-Value**	> .05	< .01	

* mean ± standard error

** F-test

\# t-test

The F-test is not significant for the normal animals. This is interpreted to mean, that within the group of normal animals, dietary

variations did not alter the incorporation of mevalonic acid-2-C^{14} into cholesterol.

The F-test is significant for the diabetic animals. Upon further analyses with the t-test, the means from the 10 per cent corn oil and 10 per cent lard diets in the diabetic group were found to differ significantly from each other at the 0.01 level.

The t-test analysis further demonstrated a significant difference between the 10 and 30 per cent lard diet animals.

Skin

Table XVIII presents the mean values for the incorporation of C^{14} into cholesterol in the skin tissues of normal and diabetic rats.

Table XVIII

Diet	Normal	Diabetic	p-Value#
Control	0.65* ± .05 (2)	0.86 ± .18 (2)	
10% Corn oil	0.62 ± .10 (6)	0.65 ± 0.7 (5)	
30% Corn oil	0.58 ± .03 (6)	0.73 ± .08 (4)	
10% Lard	0.66 ± .06 (6)	0.39 ± .03 (3)	< .05
30% Lard	0.74 ± .06 (7)	0.62 ± .07 (5)	
p-Value**	> .05	> .05	

* mean ± standard error

** F-test

\# t-test

The F-test shows no significance for either the normal or the diabetic animals. This is interpreted to mean, that within the normal group and

within the diabetic group, dietary variations did not alter the incorporation of mevalonic acid-2-C^{14} into cholesterol.

The t-test analysis demonstrated only one difference between the per cent incorporations comparing normal and diabetics on the same diet. The 10 per cent lard-fed diabetics had a significantly lower incorporation than did the normals.

Thus far, data has been presented which compares isotope incorporations into sterol fractions of normal and diabetic rats. In certain instances, individual differences between normal and diabetic tissues have been shown. In order to properly evaluate these differences, it is necessary to relate the incorporation data to the corresponding data on the cholesterol concentrations in the particular tissue.

Table XIX presents the data for certain of the comparisons of per cent incorporation and concentration of cholesterol in both normal and diabetic animals.

Table XIX

Tissue	Diet	Per Cent Incorporation		Amounts (mg/gm)	
		Normal	Diabetic	Normal	Diabetic
Liver	10% C. O.	3.92	4.80	3.55	2.53
Gut	10% C. O.	1.30	1.89	2.26*	2.47*
Carcass	10% Lard	19.66	13.29	1.38	1.85
	30% Lard	20.41	16.05	1.42*	1.58*
Skin	10% Lard	0.66	0.39	2.11	3.49

* All diabetic values are significantly different from the corresponding control value with the exception of the values designated (*).

The liver tissue from animals fed 10 per cent corn oil, demonstrates a greater incorporation of mevalonate into cholesterol in the diabetic than in the normal. At the same time, a decrease in the concentration of liver sterol in the diabetic is noted (Table XVII). Previously it was shown (Table XV) that no significant difference could be demonstrated between the incorporations in the control (5 per cent lard) and in the 10 per cent corn oil animals, either in the normals or in the diabetics. Compared to the controls, however, significant increases in amounts of sterol are observed with 10 per cent corn oil feeding, the increase in the normal animal being greater than the increase in the diabetic (Table XIX). These relationships could imply a homeostatic controlling mechanism for the synthesis of cholesterol in the animals fed the 10 per cent corn oil diets.

Studies by Wong (78) demonstrated little change in the concentration of cholesterol in the livers of normal and diabetic rats maintained on Purina Rat Chow, but a decrease in the incorporation of acetate into cholesterol was noted. These findings, she attributed to the results of a homeostatic mechanism brought to bear by a defect in the mechanism of cholesterol degradation.

Although a direct comparison between Dr. Wong's work and the results presented in this thesis may not be possible, it is possible to postulate some homeostatic control, regardless of the actual mechanisms involved.

The gut tissue, as mentioned previously, cannot be objectively evaluated because of the difficulty in the present approach in differentiating between the intracellular sterol and the intraluminal sterol of the gastrointestinal tract.

The carcass tissue of the lard fed animals of both groups (Table XIX) displays an apparent decrease in synthesis of sterol in the diabetic animals. This observation is compatible with the observations of Wong and Van Bruggen (79) who found a decrease in sterol synthesis from acetate in the carcass fractions of animals fed Purina Rat Chow. Purina Rat Chow contains about 5 per cent fat, nearly all of which is saturated fat.

The skin tissue (Table XIX) of the animals fed 10 per cent lard shows an inverse relationship to that seen in the livers of the 10 per cent corn oil animals. There is observed in the skins a significantly higher value for the incorporation of label in the normal skin tissue over that of the diabetic. A reversed situation is noted when the amounts of sterol are considered, i.e. the normal skin tissue contains a lower cholesterol concentration than does the diabetic skin tissue. These observations can be accounted for by proposing some homeostatic mechanism to be active in these animals fed the 10 per cent lard diets.

The majority of the work done on cholesterologenesis, as influenced by diet, has been done using acetate as the cholesterol precursor. Avigan and Steinberg (4), using *in vivo* preparations, found the rate of incorporation of acetate higher in corn oil fed animals, as did Wood and Migicovsky (82), studying *in vitro* as well as *in vivo* systems. Wilson and Siperstein (76) studied this incorporation in corn oil fed animals with acetate and with mevalonate, and found no change in cholesterol biosynthesis with either precursor. This latter publication in which mevalonate was used, offers one of the few reports in which this specific sterol precursor has been used to evaluate changes in the synthetic abilities of the tissues of animals fed high fat diets. This thesis

presents *in vivo* data which further describes the influence of diet on cholesterol synthesis.

In an attempt to explain why corn oil diets are found to increase cholesterol biosynthesis from acetate and not from mevalonate in rat liver tissue, some recent reports seem pertinent. Siperstein (58,59) published evidence to support the hypothesis that cholesterologenesis was controlled by a specific homeostatic mechanism. From his *in vitro* studies he advanced the proposition that the site of this homeostatic regulation is at the first reaction of cholesterologenesis unique to cholesterol synthesis, i.e. the conversion of B-hydroxy-B-methyl glutaryl-CoA to mevalonic acid. This particular reaction was not positively shown to be the specific reaction involved, but Siperstein implied this to be the case after presenting evidence that the reactions from acetate to B-hydroxy-B-methyl glutaryl-CoA were not apparently affected by feeding cholesterol to rats. Cholesterol feeding is known to depress cholesterologenesis from acetate. If this proposal be true, the labeling results from acetate and mevalonate, as described above, could be accounted for by considering the mevalonic acid to be beyond the site at which the control of cholesterol is regulated.

More evidence is furnished for the localization of the chemical step controlling this homeostatic mechanism by Beher and Baker (7) who fed cholic acid to rats and compared the biosynthesis of cholesterol from acetate and mevalonate. The incorporation of acetate into liver cholesterol was inhibited by 65 per cent compared to an inhibition of 25 per cent for mevalonate.

Additional information on this problem is afforded from other studies. Working with alloxan diabetic rats, Elwood, Marcó, and Van Bruggen (21)

found a decreased incorporation of acetate, butyrate and acetoacetate but a normal incorporation of mevalonate into cholesterol. It appears that the control of cholesterol synthesis at the metabolic level suggested by Siperstein, could be similar to the step at which the metabolic block occurs in alloxan diabetes. It is clear that positive evidence for a definite location of a specific site of control must await further work.

The results presented in this thesis do confirm previous findings that the amounts of sterol do increase in the liver with corn oil feeding. Data are presented on the sterol levels in gut, carcass and skin tissues as well. Comparisons are made between changes induced by corn oil and lard feeding with various levels of fat considered. Further attention has been given to the synthesizing abilities of the tissues studied to gain some insight as to possible explanations for the observed changes. And finally, the study of the diabetic animals presents information about dietary influences on lipid metabolism in experimental diabetes.

It is uncertain how widely applicable the observations presented in this thesis are to other members of the animal kingdom, but it is always entertaining to speculate, especially in view of the extensive contemporary public interest centered about this particular area of study.

Summary and Conclusions

1. A comprehensive *in vivo* study of tissue cholesterol levels as influenced by dietary corn oil and lard is described. Cholesterologenesis was measured in the animals using the specific cholesterol precursor, mevalonic acid-2-C^{14}. Also, the alloxan diabetic rat was compared to the normal.

2. The diets fed were 10 or 30 per cent corn oil, 10 or 30 per cent lard, and the feeding was maintained for 28 days. The amounts of cholesterol and the fatty acids were determined in four tissue fractions, viz. liver, gut, carcass and skin. Total plasma cholesterol was also determined.

3. The liver tissues of both normal and diabetic rats showed an increase in concentration of cholesterol with increased fat feeding. The increase was most pronounced in the corn oil fed animals, and this finding was common to both normal and diabetic animals.

4. The increases in amounts of sterol that were observed in the gut tissue may be due to the food residue remaining in the gastrointestinal tract at the time of sacrifice.

5. There appears to be no influence of diet upon the cholesterol levels in the carcass tissue of either the normal or the diabetic animals.

6. There appears to be no change in the concentration of cholesterol in the skins of animals fed 10 or 30 per cent corn oil or 30 per cent lard diets. The only change observed was at the 10 per cent lard level.

7. The concentration of fatty acids in the livers of normal and diabetic animals does not differ significantly when each is fed the same diet. As dietary fat is increased, however, the amount of fatty acids tends to increase.

8. Carcass tissue demonstrated a greater concentration of fatty acids in the normal animals than in the diabetics when the animals were on the low fat diets. This difference was not apparent when normals and diabetics were fed the high fat diets. It is suggested that the requirement for fatty acids in the diabetic animals was satisfied by the high fat diets, and thus, fatty acids accumulated.

9. The diabetic animals displayed pronounced hypercholesterolemias, a finding that confirmed and extended earlier reports. The hypercholesterolemia was apparent regardless of the type of dietary fat and regardless of the level of fat fed. The plasma cholesterol lowering effects of corn oil and the plasma elevating effects of lard are clearly demonstrated in the diabetic preparations.

10. There could be demonstrated no influence of diet upon mevalonate incorporation into cholesterol in any of the tissues studied. It is suggested that perhaps the reason mevalonate labeling of cholesterol is independent of dietary fat, while acetate labeling of cholesterol is reported to be influenced by dietary fat, is because of some homeostatic regulation of cholesterologenesis that has its site of action above acetate and below mevalonate in the pathway of cholesterol biosynthesis.

APPENDIX I

Comparison of cholesterol concentrations in rat tissue with and without digitonin treatment

Tissue: Liver

Diet	Condition	No. Animals	Means (mg/gm tissue)		Ratio
			Digitonin	Non-digitonin	
10% Corn oil	Normal	4	2.70	2.91	0.93
10% Corn oil	Diabetic	7	2.45	2.61	0.94
10% Lard	Normal	4	2.84	3.00	0.95
10% Lard	Diabetic	2	2.24	2.48	0.90
30% Corn oil	Normal	4	3.84	4.14	0.93
30% Corn oil	Diabetic	4	2.48	2.62	0.95
30% Lard	Normal	4	3.31	3.52	0.94
30% Lard	Diabetic	2	2.36	2.54	0.93

Tissue: Gut

Diet	Condition	No. Animals	Means (mg/gm tissue)		Ratio
			Digitonin	Non-digitonin	
10% Corn oil	Normal	4	1.59	1.78	0.89
10% Corn oil	Diabetic	7	2.09	2.28	0.92
10% Lard	Normal	4	1.68	1.93	0.87
10% Lard	Diabetic	1	1.84	2.30	0.80
30% Corn oil	Normal	4	4.43	4.32	1.03
30% Corn oil	Diabetic	4	3.78	4.47	0.85
30% Lard	Normal	4	1.98	2.32	0.85
30% Lard	Diabetic	3	1.54	2.25	0.68

APPENDIX I
(continued)

Tissue: Carcass

Diet	Condition	No. Animals	Means (mg/gm tissue)		Ratio
			Digitonin	Non-digitonin	
10% Corn oil	Normal	4	1.50	1.45	1.03
10% Corn oil	Diabetic	7	1.97	2.13	0.93
10% Lard	Normal	4	1.40	1.39	1.01
10% Lard	Diabetic	2	1.72	1.72	1.00
30% Corn oil	Normal	4	1.30	1.29	1.01
30% Corn oil	Diabetic	4	1.60	1.62	0.99
30% Lard	Normal	4	1.33	1.30	1.02
30% Lard	Diabetic	3	1.42	1.40	1.01

Tissue: Skin

Diet	Condition	No. Animals	Means (mg/gm tissue)		Ratio
			Digitonin	Non-digitonin	
10% Corn oil	Normal	4	2.00	2.18	0.92
10% Corn oil	Diabetic	7	2.88	3.33	0.87
10% Lard	Normal	4	1.98	2.04	0.97
10% Lard	Diabetic	2	2.95	3.27	0.90
30% Corn oil	Normal	4	2.01	2.16	0.93
30% Corn oil	Diabetic	3	3.08	3.08	1.00
30% Lard	Normal	4	2.20	2.29	0.96
30% Lard	Diabetic	3	2.17	2.56	0.85

APPENDIX II

Contents of Corn Oil and Lard (47)

COMPOSITION OF CORN OIL AND LARD

Oil	Saturated fatty acids			Unsaturated fatty acids				Other	Unsap. Matter	Iodine Value
	Total	Palmitic	Stearic	Total	Oleic	Linoleic	Linolenic			
Corn Oil	10	8	2	84	28	53	1	2	2	127
Lard	38	31	7	57	46	10	1	trace	trace	64

APPENDIX III

Mazola Corn Oil (52)

One hundred grams of Mazola corn oil will yield:

53 grams of linoleic acid

12 grams of saturated fatty acids

28 grams of oleic acid

1.5 grams of sitosterols

No Cholesterol.

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