STUDIES ON PATHWAYS OF LIPID METABOLISM

by

John C. Elwood, B.S., M.S.

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

June 1960

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

The author considers it a great pleasure to be able to express his sincere gratitude and indebtedness to Dr. John T. Van Bruggen who has given so freely of his time and talent in being both teacher and friend. I would also like to express appreciation to Dr. E. S. West and Dr. W. R. Todd for numerous stimulating discussions. I am grateful to Dr. C. H. Beatty, Dr. R. D. Peterson, Dr. B. Ross, and Alicia E. Marco', for their valuable technical advice and criticism. The very capable technical assistance of Jean C. Scott has aided in the completion of this work, and appreciation to Mary Lou Zurcher for her participation in the preparation of this manuscript is gratefully acknowledged.

CHAPTER I

INTRODUCTION

"The rapid progress true science now makes occasions my regretting sometimes that I was born so soon. It is impossible to imagine the height to which may be carried, in a thousand years, the power of man over matter. O that moral Science were in as fair a way of improvement, that man would cease to be wolves to one another, and that human beings would at length learn what they now improperly call humanity." - Benjamin Franklin.

From the foregoing, it is evident that Franklin had keen insight into the accelerated growth that was to take place in science. However, I wonder if he truly realized how rapid this progress was to become. Did he conceive of the multitudinous disciplines that were going to evolve in all areas of science, so numerous and specialized that scientists of different specialties find it difficult to correspond with each other because of the limitations of each ones technical jargon.

This thesis is concerned with one of the quite specialized areas of biochemistry, and yet, lest we forget the contributions and influence of those in other fields, I would like to enumerate briefly a few of the highlights in the history of biological chemistry or, as it is also called, physiological chemistry. In broad general terms, biochemistry is involved in trying to understand living phenomenon from the single cell form to complex organism, in terms of biology, chemistry, physics and mathematics.

To this end the biochemist utilizes the tools and techniques of each of these so called "basic scientific fields".

A great hinderance to progress in early scientific thinking was the philosophy of vitalism, i.e., that the chemical compounds of living organisms contained a "vital spirit" which made them different from non-living chemical entities. Probably the first well marked revolution in scientific thinking was accomplished by Newton in 1687 with his publication of mechanics, Philosopiae Naturalis Principia Mathematica. Antoine Lavoisier changed the structure and outlook of chemistry when in 1789 he published Traite Elementaire de Chimie. It was Lavoisier more than anyone else who showed that when substances burn they do so by combining with the element oxygen, not by releasing "fire-stuff". He also carried his thinking into the areas of agriculture and from this attention began our modern concept of respiration; the utilization of oxygen by living organisms for the combustion of foodstuffs and the production of heat. The synthesis of urea by Frederick Wöhler in 1828 had a tremendous impact on the scientific world and did much to show that "organic" chemicals were not products of living organisms alone, but could be produced in the laboratory, thus starting the decline of the "vitalism theory". The work of the Buchner brothers in 1897 showed that fermentation of sugar could be carried out by cell-free extracts in a manner similar to intact living cells. The early studies of the Buchners led ultimately to the defining of the cell in terms of cell structures such as membrane, nucleus, mitochondria, microsomes, etc. The name of Emil Fischer is another which requires mention as a giant in the evolution of modern scientific thinking. During the latter portion of the 19th and early part of the 20th century,

Fischer formulated the structure of and synthesized many organic compounds. From this point in history, prominent men and women of science have increased in number and have become too numerous and their contributions too varied to observe separately in any detail. The contributions of Curie, Einstein, Urey, Cori and Fermi are each classics in their respective fields. The full influence of these contributions are only beginning to be visualized.

The tools and techniques developed in such areas as physics, physical chemistry, organic synthesis, etc. have been adopted, modified, and put to work in unraveling the beautifully complex nature of living material. It has become clear that the discipline of biochemistry can advance only as the other sciences advance.

The investigations to be reported in this thesis are concerned with the intermediary metabolism of the rat and particularily with the anabolic and catabolic reactions of fatty acid and cholesterol metabolism. Also, disturbances in metabolism associated with the pathological condition, alloxan diabetes, have been studied. The techniques employed in these investigations were numerous but the use of tracer compounds and tracer methodology were of paramount importance in following the various pathways of metabolism.

Present concepts of fatty acid and cholesterol metabolism.

The numerous advances in the fields of fatty acid and cholesterol biosynthesis in recent months, and the elucidation of the new pathways involved, makes necessary a brief review of the present status of these areas of research endeavor.

Until 1958, fatty acid synthesis was considered by many to be accomplished by the reverse of the mechanisms of fatty acid oxidation. The Knoop theory of beta oxidation of fatty acids had been well verified and expanded and most of the reactions had been studied in detail in reconstructed soluble enzyme systems (46, 47, 78, 83, 128). The fatty acids were known to be broken down or to be built up with the liberation or addition of 2-carbon units. A few of the most pertinent of the details of the accepted scheme of metabolism will be presented below.

Oxidation:

The breakdown of long chain fatty acids is mediated by four enzymatic reactions that result in the liberation of 2-carbon fragments in the form of acetyl-S-CoA* with the possibility of a subsequent condensation of the active acetate with oxaloacetic acid.

It is essential that the fatty acid be in the form of its coenzyme A ester prior to the start of the degradative reactions. The formation of an acyl-S-CoA (a thiol ester) from the acid and CoASH** requires energy (8,000 cal/mol) (30). Therefore, the participation of ATP in the reaction of acyl activation was not an unexpected finding, and it has in fact been shown to be a requirement in the activation of fatty acids (2, 44, 68, 129). The general reaction can be written as follows:

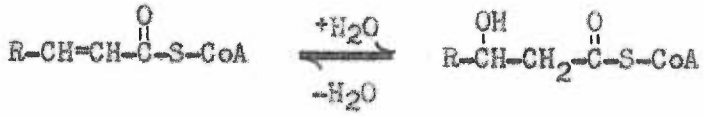
fatty acid + CoASH + ATP ⟶ acyl-S-CoA + AMP + PP

* CoA-S- = coenzyme A
** CoASH = reduced coenzyme A

The first step in shortening an acyl-S-CoA chain involves the dehydrogenation of the alpha-beta position with the formation of an unsaturated acyl-S-CoA (30,46,58,62).

This reaction is mediated by the enzyme acyl dehydrogenase and TPN is the hydrogen acceptor.

The second step involves the hydration of the unsaturated thiol acyl ester to form a beta-hydroxy acyl-S-CoA derivative (71,103,104) and is accomplished through the participation of the enzyme crotonase, more recently called enoyl hydrase (84). The unsaturated acyl incorporates a molecule of water as in the following reaction:



An oxidation reduction reaction is next encountered. However, DPN is the hydrogen carrier rather than FAD as is the previous redox reaction. The following sequence is representative of the beta-hydroxy acyl-S-CoA oxidation to the beta-keto form, utilizing DPN as the electron carrier and mediated by the enzyme beta-hydroxy acyl dehydrogenase (64).

The final reaction sequence in the breakdown of fatty acids involves the cleavage of beta-keto acyl-S-CoA to an acyl-S-CoA derivative shorter by two carbon atoms with formation of acetyl-S-CoA

by the enzyme beta-keto thiolase (42,105).

Sequence of reactions:

1) fatty acyl-S-CoA $\underset{FADH}{\overset{FAD}{\rightleftharpoons}}$ alpha-beta unsaturated fatty acyl-S-CoA

2) alpha-beta unsaturated fatty acyl-S-CoA $\underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}}$ beta-hydroxy fatty acyl-S-CoA

3) beta-hydroxy fatty acyl-S-CoA $\underset{DPNH}{\overset{DPN}{\rightleftharpoons}}$ beta-keto fatty acyl-S-CoA

4) beta-keto fatty acyl-S-CoA + CoASH $\rightleftharpoons$ fatty acyl-S-CoA + acetyl-S-CoA

Repetition of the foregoing sequence of reactions results in the generation of additional acetyl-S-CoA, i.e. palmitic acid giving rise to 8 molecules of active acetate. The further utilization or fate of this acetate depends on the nutritional and physiological state of the organism. Oxidation via the Krebs cycle accounts for the major portion of the energy generated, although energy also becomes available from the reoxidation of the coenzymes reduced in the reactions cited above. It is thus through beta oxidation that the energy stored in fatty acids is made available to the cell.

Fatty acid synthesis:

The reversal of the reactions of fatty acid oxidation were for a time used to describe the formation of fatty acids, but they are

no longer considered to describe the true (June 1960) mechanism of fatty acid synthesis.

Acetate activation:

A requirement necessary for the incorporation of C_2 units into long chain fatty acids is the activation of acetate to a higher energy level. Two enzyme systems have been elucidated for the activation of carboxylic groups.

The first of these, found thus far only in bacteria, consists of two enzymes 1) acetokinase (66,69,80), and phosphotransacetylase (67). The reactions catalyzed by these two enzymes are the following:

a) acetokinase

$$\text{acetate} + \text{ATP} \rightleftharpoons \text{acetyl-PO}_4 + \text{ADP}$$

b) phosphotransacetylase

$$\text{acetyl-PO}_4 + \text{CoASH} \rightleftharpoons \text{acetyl-S-CoA} + \text{PO}_4$$

The phosphorylation of acetate is carried out at the expense of ATP and results in the formation of ADP. The phosphate is then replaced by transfer of CoASH to the acetyl group by a phosphotransacetylase promoting the formation of acetyl-S-CoA.

The second system for acetate activation is found in plants, animals and micro organisms. The overall reaction is as follows:

$$\text{ATP} + \text{acetate} + \text{CoASH} \rightleftharpoons \text{acetyl-S-CoA} + \text{AMP} + \text{PP}$$

This reaction is catalyzed by the enzyme acetothiokinase (23,38,55,68).

Evidence for the mechanism of this reaction has been presented

by Berg (2,3,4). The suggested mechanism consists in the formation of adenyl-acetate from acetate and ATP. The adenyl acetate then participates in the thiol transfer of CoASH giving acetyl-S-CoA and liberating AMP.

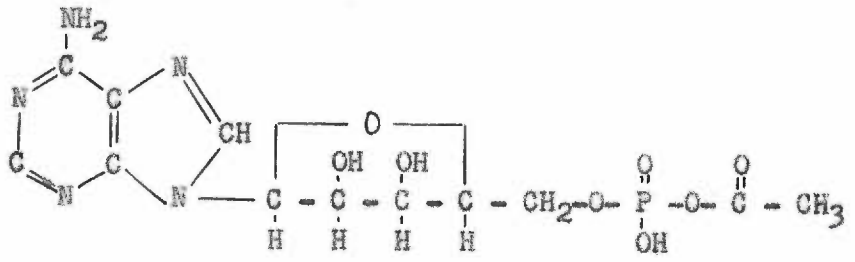
$$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{PP}$$

$$\text{adenyl acetate} + \text{CoASH} \rightleftharpoons \text{acetyl-S-CoA} + \text{AMP}$$

The above mechanism is supported by the following observations.

1) The exchange of labeled phosphorus between PP and ATP requires acetate.
2) Synthetic adenyl acetate is readily converted to ATP and acetate in the presence of PP, and to acetyl-S-CoA plus AMP with the addition of CoASH.
3) The CoA-independent accumulation of acetohydroxamic acid, PP, and AMP in the presence of hydroxylamine.
4) The requirement of acetate and CoASH for the exchange of AMP with ATP.
5) The requirement of AMP and PP for the exchange of acetate with acetyl-S-CoA.

The formula of acetyl adenylate is as follows:



Sequence of reactions:

According to the view held before 1958, the next reaction of synthesis would be the condensation of 2 molecules of acetyl-S-CoA to form acetoacetyl-S-CoA. Recent investigations offer considerable evidence to indicate that such may not be the case. Gibson et al. (130, 132), using a partially purified enzyme system (extra mitochondrial), presented evidence for bicarbonate participation in long chain fatty acids synthesis along with the need for Mg^{++} and acetyl-S-CoA. That the bicarbonate ion is not incorporated into fatty acid *in vivo* had previously been shown in this laboratory. Labeled bicarbonate, given either intravenously or intraperitoneally (121) was shown not to label tissue fatty acids or cholesterol to any significant degree. This did not seem too surprising since it had been shown by degradative studies that acetate or acetyl-S-CoA is the sole source of the carbon chain of the fatty acids synthesized. Thus, some function for bicarbonate, other than that of a carbon source for synthesis, had to be devised.

No intermediates between acetate and decanoic acid could be demonstrated at the level of purity to which the preceding enzyme fractions had been taken. Wakil carried out further purification of the fatty acid synthesizing enzyme fractions (E_1 and E_2) and it then became possible to conduct stepwise synthesis (131, 133, 134), and it also suggested a new metabolic pathway. Malonic acid was isolated from this system in the presence of acetate-C^{14}, CoASH, Mn^{++}, ATP, and bicarbonate ion along with the enzyme fraction E_1. When TPNH was added plus the second enzyme E_2, long chain fatty acids were

formed. Various enzymes concerned with the breakdown of fatty acids could not be found in this system, i.e. enoyl hydrase, beta-hydroxy acyl dehydrogenase or thiolase.

The above evidence suggests that the first step in fatty acid synthesis after acetate activation is the carboxylation of acetyl-S-CoA to a malonyl derivative with subsequent successive condensation and reductive steps in the presence of TPNH. Malonic acid per se is not the intermediate. Green has suggested (48) that the intermediate is malonyl-S-CoA. The reaction steps are illustrated below.

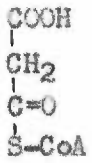
1) $CH_3-\overset{O}{\overset{\|}{C}}-S-CoA + HCO_3^- + E_1 \xrightarrow[ATP]{Mn^{++}}$ COOH / CH_2 / C=O / S-CoA

2) COOH / CH_2 / C=O / S-CoA $+ E_2 \xrightarrow{TPNH}$ palmitic acid

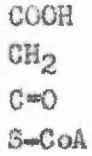
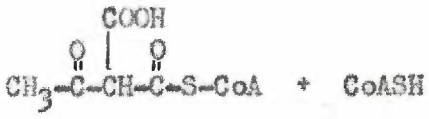
Wakil et al (130,132) have also demonstrated that biotin is a requirement in the above system of fatty acid synthesis. Utilization of the terminal phosphate bond of ATP supplies the energy of the reaction by combining with biotin to form phosphoryl biotin. The phosphoryl group is replaced by CO_2 forming carbonyl biotin, an activated CO_2. It is in this activated form that CO_2 combines with acetyl-S-CoA forming malonyl-S-CoA (48). Lynen has suggested that adenosine diphosphoryl biotin may be the intermediate (48).

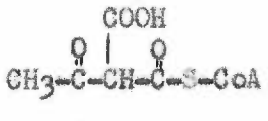
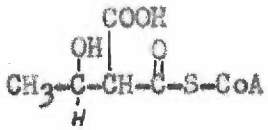
There exists at least two possible mechanisms for the further reaction of malonyl-S-CoA, i.e. condensation with itself or condensation with a molecule of acetyl-S-CoA. Wakil feels that the

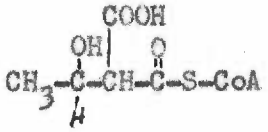
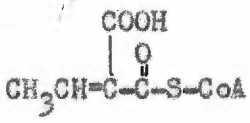
following mechanism is the most likely:

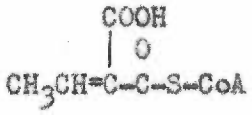
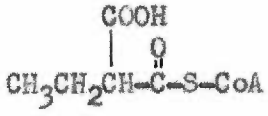
1) $CH_3COO^- + ATP + CO_2 \xrightarrow[CoASH]{Mn^{++}}$  $+ ADP + Pi$

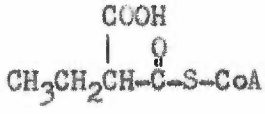
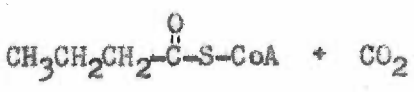
2) $CH_3COO^- + ATP \xrightarrow[CoASH]{Mn^{++}} CH_3C(=O)-S-CoA + ADP + Pi$

3) $CH_3C(=O)-S-CoA +$  $\longrightarrow$ 

4)  $\xrightarrow{TPNH}$ 

5)  $\xrightarrow{-H_2O}$ 

6)  $\xrightarrow{TPNH}$ 

7)  $\longrightarrow$ 

The butyryl-S-CoA that is formed is then capable of condensation with another molecule of malonyl-S-CoA. The addition of another 2-carbon unit thus takes place through the preceding sequence of reactions.

Another group of workers, Shulman and Westerfeld (143) have

suggested that the "unknown compound" in their studies concerning ethanol metabolism may well be the postulated alpha-carboxy-beta-hydroxy butyryl-S-CoA intermediate. The alpha-carboxy compounds of the above reaction mechanism have not been isolated.

Another mechanism of fatty acid synthesis, postulated by Brady (26,27), involves an aldol condensation similar to that shown for the formation of dihydrosphingosine (25). Using a soluble fraction from pigeon liver, Brady prepared malonyl-S-CoA by the enzymatic activation of malonate. This reaction is also dependent upon ATP, CoASH, and Mg^{++}. Brady also reported that malonyl-S-CoA could be enzymatically formed from acetyl-S-CoA utilizing ATP, Mn^{++}, and bicarbonate. The scheme suggested by Brady is the following:

1) $R-\overset{O}{\overset{\|}{C}}-S-CoA + TPNH \rightleftharpoons R-\overset{O}{\overset{\|}{C}}H + TPN + CoASH$

2) $R-\overset{O}{\overset{\|}{C}}H + \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ C=O \\ | \\ S-CoA \end{array} \longrightarrow R-CH_2CH_2-\overset{OH}{\overset{|}{\underset{O}{\underset{\|}{C}}}}-S-CoA + CO_2$

The above scheme involves reduction of the acyl-S-CoA ester to the corresponding aldehyde and subsequent condensation with malonyl-S-CoA followed by decarboxylation. The enzymatic reduction of acetyl-S-CoA to acetaldehyde was followed by observing TPNH oxidation spectrophotometrically. The longer chain fatty acids were separated as their hydroxamic acids and the data suggested the presence of acids having chain lengths of 8 to 12 carbon atoms.

Cornforth (36) has considered these two postulated mechanisms (Wakil and Brady) in terms of ionic organic reaction mechanisms. He concluded that the condensation of acetyl-S-CoA with malonyl-S-CoA

would have no energetic advantage over the condensations of two acetyl-S-CoA molecules. The primary effect of the extra carboxyl group would be to promote the enolization of the ester. "The condensation of this enolate with an S-acyl coenzyme A by an enzyme of the thiolase type does not seem likely to lead to a greater degree of synthesis than occurs in the condensation of two molecules of S-acetyl coenzyme A." Cornforth further states that the condensation of an aldehyde with a malonic acid derivative is easily carried out, but that after condensation, dehydration or decarboxylation or both usually takes place. The final conclusion of Cornforth is the following:

"Chemically, condensation is well supported by analogy. However, the actual utilization of a fatty aldehyde as such in the biosynthesis of a higher fatty acid has yet to be demonstrated."

It becomes clear from the foregoing statements and observations that the evidence is incomplete for any of the proposed mechanisms and that the final scheme of fatty acid synthesis may not as yet be known. Considerable information will undoubtedly be forthcoming in the near future as regards individual enzymes and pathways concerned with this very interesting problem of fatty acid synthesis. The fact that fatty acid synthesis is now being considered as something other than the reversal of oxidation is not too surprising and is actually to be welcomed in the field. Langdon's original suggestion that oxidation and synthesis might take place at different geographical locations in the cell (62) now also seems more tenable. Langdon

showed all of the enzymes of fatty acid biosynthesis to be present in extramitochondrial fractions (62), and Kennedy and Lehninger (65) established that the mitochondria represents the only subcellular fraction of liver capable of the oxidation of fatty acids at the expense of molecular oxygen.

Cholesterol biosynthesis

The observation that acetate can serve as a carbon source for the biosynthesis of cholesterol in mammalian systems is well documented (6, 7, 8, 9, 40, 41). That every carbon in cholesterol may be derived from acetate was established by studies which have been in progress for a number of years in several laboratories (82). Many compounds have been utilized over the years in attempts to characterize intermediates in cholesterol synthesis but little progress was made until 1956. A number of C^{14} labeled branched chain mono- and dicarboxylic acids were studied. Some of these were shown to be synthesized from acetate and also converted to cholesterol, but in each case the efficiency of conversion was low (6, 10, 22, 51, 52, 91, 92, 93, 94). Greenberg et al. (49) excluded many of these compounds as intermediates in cholesterol synthesis with the possible exception of beta-methyl glutaconic acid. The finding of a growth factor for Lactobacillus acedophilous by Skeggs (101) and the subsequent identification of it (144) as the optically active beta-hydroxy beta-methyl pentano-5-lactone (mevalonic acid) prompted Tavormina, Gibbs and Huff to test labeled mevalonic acid as an intermediate in cholesterol synthesis (108). They found that synthetic DL mevalonic acid-2-C^{14} was converted into cholesterol with an efficiency of 43 per cent by liver preparations.

The almost quantitative conversion of MVA into cholesterol has stimulated research in all areas of the field of cholesterogenesis. The pathway of cholesterol biosynthesis from acetate can now be described, perhaps not completely, but it would seem with only a few uncertainties. Chronologically, the four major contributions to the elucidation of this pathway were the discoveries of acetate (7), squalene (10), lanosterol (11) and mevalonate (108) as precursors of cholesterol.

The first reaction in the utilization of acetate in mammalian systems is its activation, that is, the formation of acetyl-S-CoA. The next reaction is one of condensation of 2 molecules of acetyl-S-CoA. Whether this involves malonate formation isn't known with certainty at this time. The following formulas illustrate the next reaction, the condensation of acetoacetyl-S-CoA with a molecule of acetyl-S-CoA giving rise to beta-hydroxy beta-methyl glutaryl-S-CoA with the liberation of free CoASH (93, 94, 95).

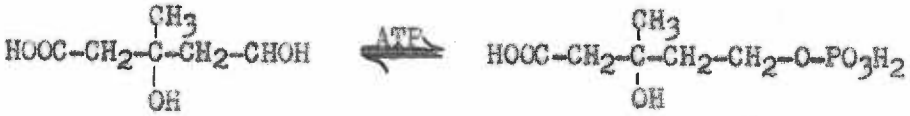
acetoacetyl-S-CoA acetyl-S-CoA beta-hydroxy beta-methyl glutaryl-S-CoA (HMG-S-CoA)

The enzyme mediating this condensation is known as the HMG-S-CoA condensing enzyme and has been partially purified (79).

The reduction of HMG-S-CoA to mevalonic acid has been described by Lynen et al. (72). TPNH is a requirement in the reported yeast system. One of the findings concerning this reaction is its apparent irreversibility.

HMG-S-CoA mevalonic acid

The further conversion of MVA to cholesterol requires the addition of energy in the form of ATP. The partial purification of the enzyme mevalonic kinase has been achieved. Its action results in the formation of 5-phosphomevalonic acid from MVA and ATP (73, 89, 110).



mevalonic acid 5-phosphomevalonic acid

Block et al. (20) showed that the next reaction was another phosphorylation resulting in 5-diphosphomevalonic acid.

5-phosphomevalonic acid 5-diphosphomevalonic acid

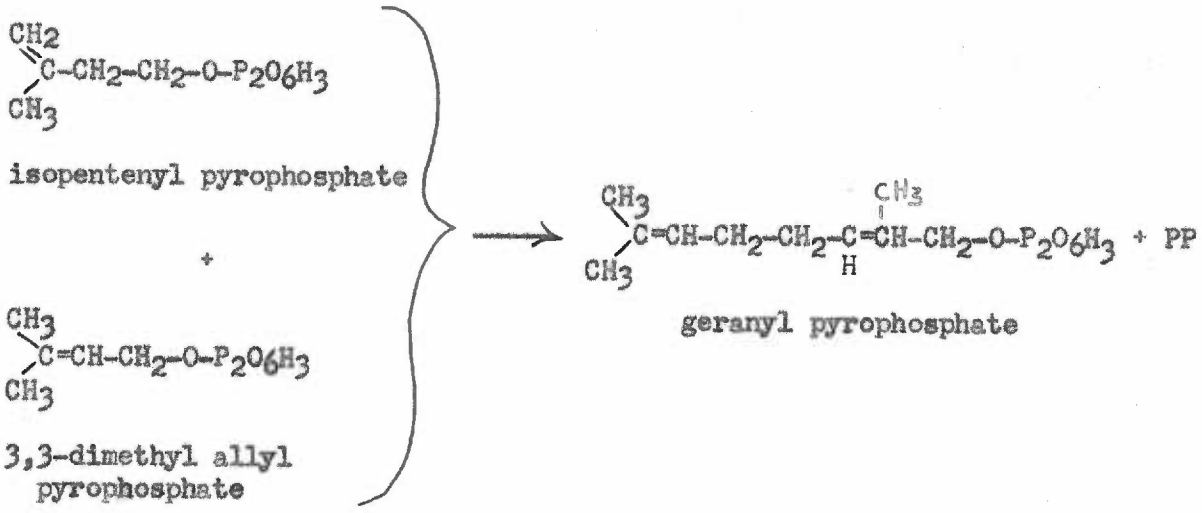
The requirement of a third molecule of ATP in the next reaction suggests a triphosphorylated compound, and indeed Block has suggested its structure, 3-phospho-5-diphosphomevalonic acid (21). Thus far there is no real proof of its existence. The next isolated metabolite to be discussed is isopentenyl pyrophosphate, the carboxyl group of 5-diphosphomevalonic acid having been lost as CO_2 (109).

5-diphosphomevalonic acid isopentenyl pyrophosphate

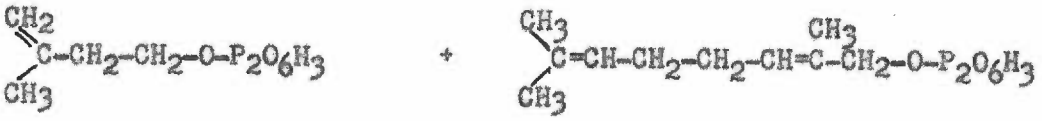
A double bond shift to form the beta beta-dimethyl allyl pyrophosphate is the next predicted intermediate from organic reaction mechanistic theory and Lynen has shown the formation of this compound in yeast extract (74).

isopentenyl pyrophosphate 3,3-dimethyl allyl pyrophosphate

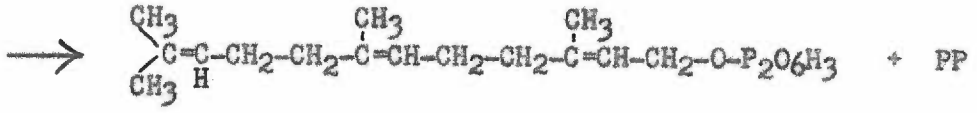
Dimethyl allyl pyrophosphate reacts at the double bond position of isopentenyl pyrophosphate while losing its pyrophosphate group thus causing a shift of the methylene double bond to form the compound, geranyl pyrophosphate.



This C-10 derivative has the same reactive site as isopentenyl pyrophosphate and thus adds another molecule of dimethyl allyl pyrophosphate to form farnesyl pyrophosphate.

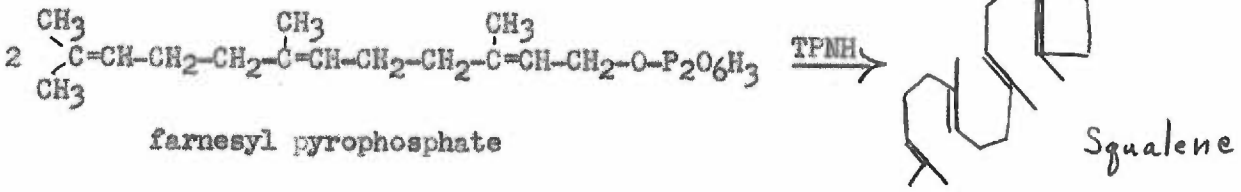


isopentenyl pyrophosphate geranyl pyrophosphate



farnesyl pyrophosphate

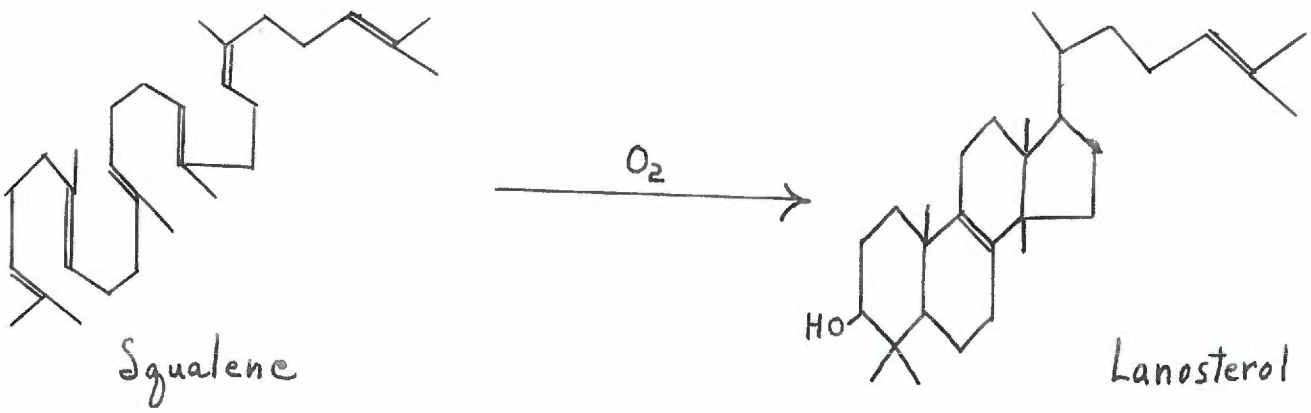
Lynen and also Block have proposed the above scheme from data obtained with both yeast and liver systems. The condensation of 2 molecules of farnesyl pyrophosphate (head to tail condensation) and the loss of two pyrophosphate groups gives rise to a molecule of squalene. This is a reductive step and TPNH has been shown to be required (75).



farnesyl pyrophosphate Squalene

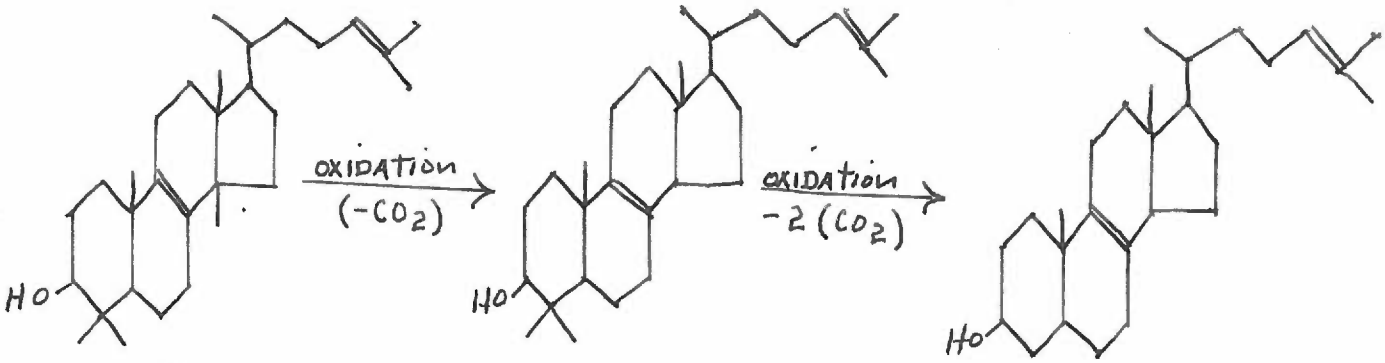
The cyclization of squalene to lanosterol is a very complex reaction and involves 16 active centers (13, 14).

The formation of lanosterol from squalene requires an oxidative step.



Squalene Lanosterol

Lanosterol (4,4',14-trimethyl cholestadiene) has presented some biochemical problems since its postulated participation in cholesterol synthesis. Lanosterol must of necessity be demethylated in its role as cholesterol precursor. The question of the sequence in which methyl groups are eliminated plus the method, i.e. demethylation or oxidation followed by decarboxylation, has been under investigation by a number of laboratories. Block et al. (15) showed that oxidative conditions were required for lanosterol to cholesterol conversion thus indicating that decarboxylation was the mechanism involved. Recent evidence by Block et al. (12, 16, 19) has shown that the 14 methyl group is lost first, yielding the compound 4,4'-dimethyl cholesta-8,24-diene-3-beta-ol. Oxidation of the alcoholic group to a ketone forming 4,4'-dimethyl-cholesta-8,24-diene-3-one, followed by further oxidation and decarboxylation of the 4,4'-methyl groups yields the compound zymosterol (96, 18).



Lanosterol

4,4' dimethyl cholesta-8,24-diene-3-beta-ol

Zymosterol

Stokes et al (106) have shown desmosterol to be the immediate precursor of cholesterol, thus completing the total scheme of cholesterol biosynthesis as it is felt to exist at the time of this writing.

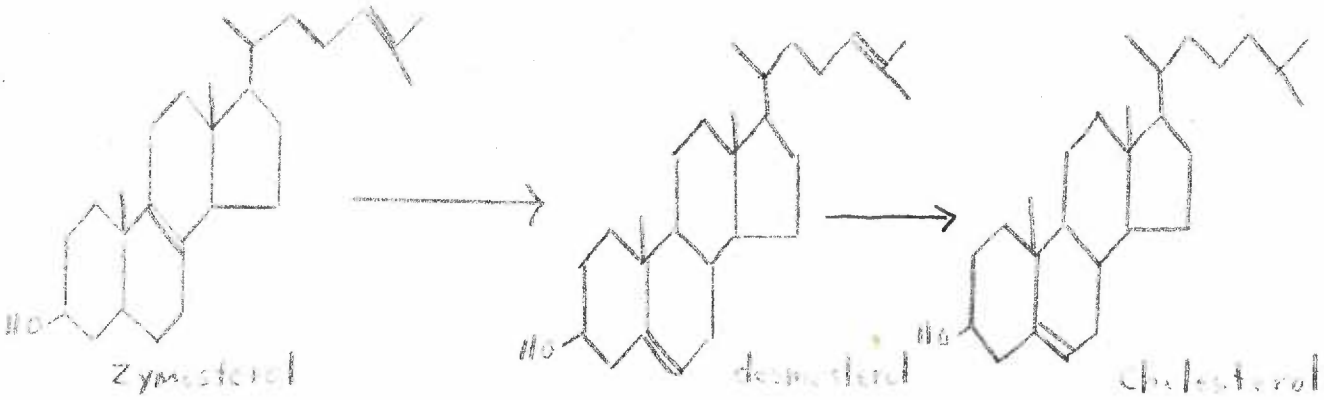


Figure 1 lists the word summary of cholesterol synthesis from acetate. The arrows in the summary all point to synthesis, however most of the reactions are reversible as listed previously.

The present studies: Research objectives and statements regarding results.

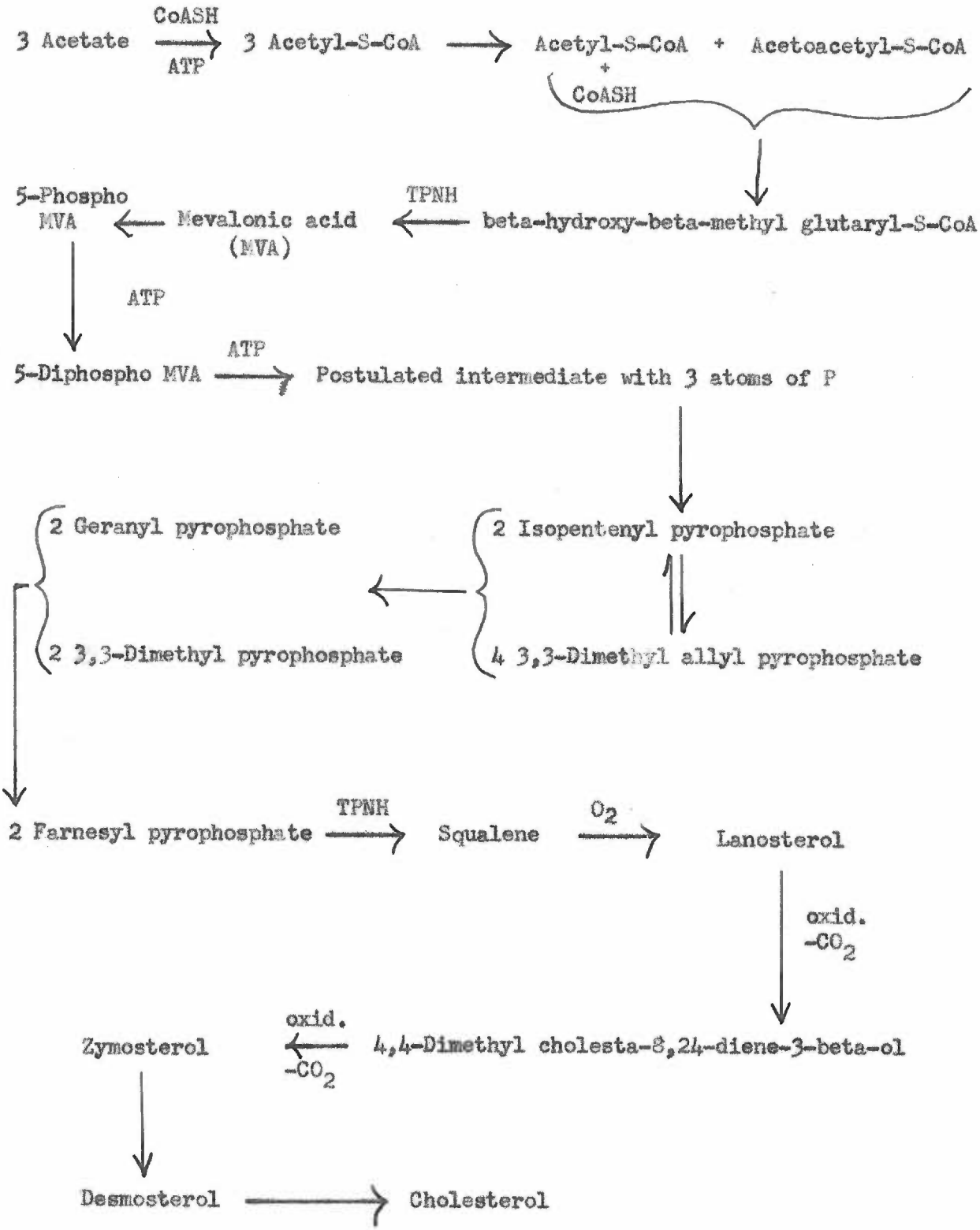
Studies concerned with the qualitative and quantitative utilization of acetate-C^{14} by rat liver tissue preparations has been, and continues to be, a profitable field of investigation for many laboratories.

Lipogenesis (the anabolic reactions of fatty acids and cholesterol metabolism) is dependent upon the kind of diet and upon the relation of feeding and fasting periods. Weinhouse showed that lipid formation from acetate in liver slices is greatly impaired by fasting or under nutrition (136). Chaikoff et al presented evidence that the liver loses most of its ability to convert acetate to fatty acids when the animal is fasted

Fig. 1: A summary of the general pathway of cholesterol metabolism:

The sequence listed shows the conversion of acetate to cholesterol via the intermediates known to exist at this time. The reactions are written in one direction only to indicate synthesis. The equilibrium reactions are listed in the text.

Fig. 1: A summary of the general pathway of cholesterol metabolism.



for 18 hours (33,31). Carbohydrate administration was shown to alleviate the depressed synthesis. Tepperman and Tepperman found a high positive correlation between initial glycogen content and lipogenic response in liver slices (112). Van Bruggen et al have shown the variability of hepatic lipogenesis as affected by non-chronic refeeding and/or fasting using in vivo preparations (116,118,123). Similar responses have been shown to occur when liver slice preparations were studied (122,124).

Findings somewhat like the above have been observed when alloxan diabetic preparations were used for study (32,53,140). The similarity, in many respects, between fasting and experimental diabetes has been known for some time. Gurin has suggested (53) that a common feature of fasting and diabetes could include a deficiency of glycolytic intermediates. Teleologically these observations are valid. If carbohydrate calories become less available either by fasting or by the presence of diabetes, there is necessitated a shift to other substances for caloric needs, and there thus follows a consequent disturbance in biosynthetic pathways.

Shaw, Dituri and Gurin presented evidence in 1957 that a decrease in the reduction of alpha-beta unsaturated crotonyl-S-CoA to butyryl-S-CoA was a block in the flow of acetate to higher fatty acids (53). Langdon (61) showed a requirement for TPNH in the hydrogenation of crotonyl-S-CoA to butyryl-S-CoA in long chain fatty acid synthesis. From these observations, it was inferred that TPNH is a requirement at all of the subsequent alpha-beta reductions in fatty acid synthesis. Siperstein has proposed that, because of decreased glucose oxidation via the hexose monophosphate shunt, there is a reduced amount of TPNH available

for this reductive step in the diabetic (99, 100). Because of the similarity of decreased lipogenesis in both starvation and in diabetes, it has been postulated that the block in the incorporation of acetate into fatty acids is at the TPNH requiring step. Siperstein also indicated that the cholesterol synthesizing system is in need of TPNH as well as are the lipogenic pathways. Little attention has been paid to the importance of DPNH in the obligated step of ketone body reduction. In the absence of proof of inadequate DPNH-TPNH transdehydrogenase action, it would appear that undue emphasis has been placed upon this TPNH dependency as the sole rate determining reaction.

Objectives:

The experimental work to be presented is concerned with the utilisation of selected substrates by normal and by diabetic tissues, and in this thesis the data and discussion have been separated into four series and are presented in the sequence in which the work was carried out. Series I constitutes an *in vitro* study of liver slices from normal and alloxan diabetic preparations. Acetate-1-C^{14} was the labeled tracer and the incorporation of this molecule into fatty acids, cholesterol, ketone bodies, and CO_2 was determined. Series II is also an *in vitro* study of normal and alloxan diabetic liver slices. The tracer molecules employed were acetate-1-C^{14}, acetoacetate-3-C^{14}, butyrate-1-C^{14} and mevalonic acid-2-C^{14}. Analysis of lipid components and oxidative products were carried out as in the first series. Series III is the result of an *in vivo* investigation concerned with following the intraperitoneal injection of mevalonic acid-2-C^{14}. Label from this isotope was determined in blood, urine, CO_2, and numerous tissues of the rat. It also includes an early time course study

concerning cholesterol and fatty acid synthesis in the entire animal and in kidney tissue as well. Series IV was initiated in an attempt to clarify the role of kidney tissue in cholesterogenesis. The experiments were done in vitro and again use was made of normal and alloxan diabetic preparations. The label from acetate-1-C^{14}, acetoacetate-3-C^{14}, and mevalonic acid-2-C^{14} was determined in cholesterol, fatty acids, and CO_2 following incubation of the kidney tissue in Warburg flasks.

Summary of results:

The findings reported in Series I of this work are in agreement with the observations of others that there is a block in the flow of acetate at some point or points in the pathway of fatty acid and cholesterol synthesis of alloxan diabetic liver slices. That TPNH deficiency could be a part of this block is compatible with these findings, however, indirect evidence is presented to show that a TPNH deficiency does not account for the entire diminution in these synthetic pathways.

The present studies also reveal that in addition to the suggested controlling steps presented above, there exists a limiting step prior to the participation of crotonyl-S-CoA. Decreased acetoacetate synthesis by liver slices of fed alloxan diabetic rats, and decreased production of CO_2 is suggested to be related to a decreased availability of acetyl-S-CoA in these diabetic preparations. This decrease is thought to be due to a decreased supply of acetate. In addition, the decrease in beta-hydroxy butyrate formation, which was observed in the diabetic, is also suggested to be related to decreased availability of DPNH.

In the Series II study, butyrate-1-C^{14} (which as butyryl-S-CoA is seemingly peculiar to fatty acid pathways), acetate-1-C^{14} , and

acetoacetate-3-C^{14} (as CoASH derivatives), are common to both fatty acid and cholesterol synthesis, and mevalonic acid-2-C^{14} (which as mevalonate pyrophosphate is peculiar to pathways of sterol synthesis) were chosen to better elucidate the sites of "metabolic lesions" in the diabetic.

These studies reveal that butyrate does not constitute a selective label for pathways of fatty acid synthesis, and that acetoacetate does preferentially appear in lipid products. It is also shown that both acetoacetate and butyrate fail to be incorporated into diabetic liver lipid in normal amounts, the defect being similar in magnitude to that with acetate. Mevalonate is shown to be a unique tracer of cholesterol synthesis in that it is selectively converted to sterols. There does not appear to be a further defect in diabetic cholesterogenesis beyond the stage of mevalonate participation.

Most of the work of others pertaining to mevalonic acid utilization was obtained using *in vitro* methods, especially homogenates. Observations presented in this thesis (Series II) describe the participation of mevalonic acid in cholesterol synthesis by rat liver slices.

The Series III investigations describe the *in vivo* time course of cholesterol and fatty acid labeling in male rats following intraperitoneal injection of mevalonic acid-2-C^{14}. The problem of cholesterol biosynthesis (studied with acetate-C^{14}), transport and turnover has been under investigation in this laboratory for some time. A previous publication has given values for the turnover of cholesterol in normal and alloxan diabetic rats (126). One of the problems encountered when acetate is used to label cholesterol relates to the numerous reactions in which acetate is involved. The fact that acetate, after conversion to

acetyl-S-CoA, is an intermediate of numerous pathways somewhat limits its usefulness as a tracer of specific pathways. Multiple pathways may contribute secondary amounts of label obscuring both the primary labeling event and subsequent time course studies. Because of the apparent preferential labeling of cholesterol by mevalonic acid without its participation in major side pathways and because of its preferential rapid conversion to sterol, it was felt that this compound would be an ideal one for elucidating some of the dynamics of cholesterogenesis.

Series III thus presents data concerning the early time course of cholesterol labeling from mevalonic acid-2-C^{14} in the liver, gut, "carcass", kidneys and skin of normal rats. Also presented are the amounts of label appearing in blood and urine during the first four hours following administration of mevalonic acid-2-C^{14}. A detailed study of the "carcass" fraction shows the relative distribution of label in the kidneys, adrenals, adipose, muscle, spleen, lungs, heart and testes. An interesting observation, is the relative amount of radioactive cholesterol found in the kidneys of these animals. With the finding that the kidney contained most of the activity of the carcass fraction, it became necessary to carry out an in vivo time course experiment using this organ. Approximately 70 per cent of the labeled cholesterol of the rat was found to be present in the kidneys 30 minutes following the injection of mevalonic acid-2-C^{14}, and this activity remained in the kidney during the period of observation. The changes in specific activity of the various tissues is considered to be due to transport. The half life ($t_{\frac{1}{2}}$) values of excretion by the liver and accumulation by the gut are given. The rapid liver component is shown to have a $t_{\frac{1}{2}}$ = 1.5 hours, and a slower component $t_{\frac{1}{2}}$ = 7.5 hours. The gut is thought to be the recipient of the liver

cholesterol excreted during the four hour period discussed.

With the finding in Series III, that the kidney played an important role in cholesterogenesis from mevalonic acid-2-C^{14}, it was felt desirable to investigate the synthetic capacity of this tissue under *in vitro* conditions. This approach should allow for a better comparison of liver and kidney tissue in respect to utilization of mevalonic acid-2-C^{14}. Since the kidney tissue appeared from the previous experiments to be very active metabolically, it was considered pertinent to allow the kidney tissue access to other labeled compounds.

Alloxan is a general tissue poison and causes marked changes in the lipogenic pattern of rat liver slices. Therefore, the possibility existed that kidney function might also be altered in alloxan diabetic rat kidney slices. Kidney slices of normal and alloxan diabetic rats were incubated in the presence of acetate-1-C^{14}, acetoacetate-3-C^{14}, and mevalonic acid-2-C^{14}. Utilizing these labels in normal and diabetic preparations gives rise to a number of parameters for comparison.

The incorporation of mevalonic acid-2-C^{14} into cholesterol by kidney tissues does not differ in the diabetic from that of normal. The incorporation of this tracer into the fatty acid fraction of the kidney is also found to be the same in the diabetic, and the amount of mevalonic acid-2-C^{14} converted to CO_2 is also seen not to differ in the two preparations.

The incorporation of acetate-1-C^{14} into CO_2 is slightly reduced in the diabetic. Fatty acids and cholesterol labeling by kidney tissue shows a response different that that of liver tissue.. The incorporation of acetate-1-C^{14} into kidney cholesterol is not different in the normal and alloxan diabetic, nor is the incorporation into fatty acids decreased as is in the diabetic liver.

The observations concerning the utilization of acetoacetate-3-C^{14} by kidney tissue are similar to those obtained from acetate. Acetoacetate-3-C^{14} is oxidized to CO_2 to the same extent in the two experimental preparations. The synthesis of cholesterol from acetoacetate-3-C^{14} is also seen to be similar in the normal and diabetic as well as is the incorporation of acetoacetate-3-C^{14} into fatty acids.

CHAPTER II

METHODS AND MATERIALS

Animals:

The animals used in the following experiments were male albino rats of the Sprague-Dawley strain. They were obtained from the commercial colony maintained at Pullman, Washington. On arrival, all animals were placed on an ad libitum feeding regime (Purina Rat Chow) for three days before initiating a trained feeding routine (123). Trained feeding consists of allowing each rat to eat 10 grams of chow in one hour, twice daily. Within a day or two, all animals eat their entire ration during the one hour feeding period. Fasting periods were considered initiated at the time uneaten food was removed from the cage. Drinking water was available at all times. This type of nutritional control (trained feeding), has been shown to be necessary in minimizing the variability of lipogenesis and cholesterogenesis which is dependent upon fasting and feeding periods (33,112,123,124,136). Such trained animals maintain a fairly constant weight gain of 5 grams per day on this feeding routine up to a weight of approximately 300 grams, at which time their rate of gain begins to diminish. The weights of the animals at time of experimentation fell into two groups: 1) 80 - 100 grams, 2) 200 - 225 grams, depending on the area of investigation; the weights of animals utilized being listed at appropriate places throughout the text. All animals were maintained in a cage-hood assembly developed in this laboratory (117), except when it was desired to collect urine and/or CO_2.

Conditions:

The conditions investigated in the following experiments were two, normal and diabetic. A normal animal is defined as one which has had a satisfactory weight gain during a five day period, is within the weight range for the group being studied, and has no apparent physical defects. The diabetic animals were prepared, after a 48 hour fast, by intramuscular injection of 0.60 mg per gram body weight of purified alloxan as a 10 per cent solution in normal saline. At the same time, 5 ml of saline were injected intraperitoneally and normal saline was given as drinking water during the next 48 hours. The diabetic animals were not trained to feeding, but were given Purina chow ad libitum until just prior to use. At that time, the animals were fasted for 12 hours and fed for one hour to insure a postprandial state. Controls and diabetics were used between three and seven weeks following alloxan injection if 24 hour fasting blood sugars were greater than 200 mg per 100 ml of blood.

Level of organization:

Two approaches were utilized in the present investigations, *in vivo* and *in vitro*. The *in vivo* technique involved the intraperitoneal injection of radioactive tracer following a specified fasting period. Subsequent collection of CO_2 and urine was carried out, after which time the animal was sacrificed and various fractions assayed. The *in vitro* technique was concerned with kidney and liver tissue slices. These slices were incubated in flasks containing labeled tracer and measurements of respiratory gas exchange, suspending medium components, and analysis of tissue were carried out.

In vivo:

Following the intraperitoneal injection of label, the animals were placed in a metabolism chamber for urine collection and for the collection of respiratory CO_2 using the apparatus previously described (120). After the specified time interval, the animals were killed by decapitation. Blood was collected and radioassayed for activity. Initially, the animals were separated into five fractions: liver, gut, carcass, skin, and blood. In subsequent experiments, the animals were further fractionated into kidney, adrenal, epididymal fat pads, brain, lung, spleen, muscle, diaphragm, heart and testes. These fractions were weighed and placed in flasks containing 25 per cent KOH in 95 per cent alcohol. Each fraction was digested under reflux for two hours.

In vitro:

After a specified fasting period, the animals were killed by decapitation, their tissues immediately removed and placed in cold buffer (59) prior to slicing. Slices were obtained using a Stadie-Riggs slicer. The capsular tissue of liver and kidney was discarded. The kidneys were placed on their long axes and sliced horizontally using only the upper third and lower third of the organ to minimize the amount of connective tissue per slice. The kidney tissue slice was dissected free of pelvic area tissue leaving the cortex and medullary tissue. One gram samples with 27 ml of buffer in 125 ml Warburg flasks and 0.110 gram samples with 3 ml of buffer in 15 ml Warburg flasks, were incubated at 37° for 1 - 3 hours in the standard Warburg apparatus. C^{14}-tracers were placed in the side arm of the flask and tipped in after 30 minutes equilibration. When the macro and micro procedures were compared,

using tissue from the same animal, essentially identical results were obtained.

Oxygen utilization was measured manometrically. At the end of the incubation period, total CO_2 was collected in a KOH center well by H_2SO_4 acidification of the suspending medium. When acetoacetic acid was to be determined or isolated from the suspending medium, duplicate flasks were incubated and the flask contents were not acidified, but deproteinized. At the end of the CO_2 collection period, the tissues were removed, washed with distilled water and digested in 11 per cent KOH for two hours.

C^{14}-tracers:

The following C^{14} compounds were utilized in the present investigations: acetate-1-C^{14}, acetoacetate-3-C^{14}, mevalonic acid-2-C^{14}, and butyrate-1-C^{14}.

Acetate-1-C^{14}:

The acetate-1-C^{14} was prepared in this laboratory (115) and 0.57 umoles containing 19.4 uc of activity were used per 27 ml substrate. One-ninth of this amount was used in the small flasks.

Ethylacetoacetate-3-C^{14}:

Ethylacetoacetate-3-C^{14}, New England Nuclear Corporation, was dissolved in water and aliquots hydrolyzed with NaOH (60). The acetoacetate solution was neutralized and diluted to contain 0.41 uc (0.44 umoles) per ml. One ml of this solution was added from the side arm of the small flasks. The final 3 ml buffer solution contained 1.5 mg acetoacetic acid per 100 ml.

Mevalonic acid-2-C^{14}:

DL-mevalonic acid-2-C^{14} was obtained as the dibenzethylenediamine salt (DBED) from Tracer Lab Inc., and had a specific activity of 2.26 mc/mmole. The salt was treated with dilute NaOH and the DBED removed by ether extraction. 0.46 umoles of the DL form of the free acid, containing 0.42 uc, were added per gram of tissue per flask. The purity of the mevalonic acid was checked by paper chromatography in a butanol: acetic acid: water system (4:1:5). A single peak with an Rf of 0.75 was obtained and there was no indication of a radioactive impurity. A single peak was also obtained in a methyl ethyl ketone: propionic acid: water system (75:25:30). At the start of this work labeled mevalonic acid was obtained from Radioisotopes Specialties Co. and was used for some metabolic experiments. It was subsequently determined that there was a radioactive contaminent in the preparation. The results from these early studies are not included in the presentation below.

Butyrate-1-C^{14}:

The sodium butyrate-1-C^{14}, Nuclear Chicago Corporation, had a specific activity of 4.7 uc/mmole. 0.37 umoles of the salt (1.8 uc) were added in 1 ml of solution per flask per gram of tissue.

Chemical determinations and procedures:

Aliquots were taken from the alcoholic KOH digestion solutions, extracted with petroleum ether as described previously (122), and after being taken to dryness on a water bath, were 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 nonsaponifiable fraction will be referred to as the cholesterol

fraction with the reservation that a small portion of this fraction may not be cholesterol per se. The saponifiable fraction is called the fatty acid fraction.

Cholesterol:

Cholesterol was determined by the method of Zlatkis (146) which involves the formation of a stable color using acetic acid, sulfuric acid, and ferric chloride. The color density was measured on a modified Bausch and Lomb Spectronic 20 colorimeter (37)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 whenever possible.

Fatty acids:

The fatty acids were determined gravimetrically by adding known volumes of the extracted sample to weighed shell vials. The alcohol was evaporated by heating the shell vials in a 60° water bath while applying a stream of nitrogen to the liquid surface. After evaporation, the shell vials were reweighed and the total weights of fatty acids per fraction were calculated.

Ketone bodies:

Ketone bodies were determined by the method of Bessman and Anderson (5). The technique required the precipitation of protein material with barium hydroxide and zinc sulfate prior to chemical analysis. An aliquot of the protein free filtrate is then heated in a sealed screw cap tube

in the presence of sulfuric acid which causes decarboxylation of the acetoacetic acid with the formation of acetone. Added salicylaldehyde in the presence of KOH then forms a yellow color with the acetone. The value obtained represents the combined acetoacetic acid and acetone present in the original sample. This sum is referred to as acetoacetic acid in the experiments to follow. The total ketone body concentration is determined by adding potassium dichromate to the acid digested sample which oxidizes the beta-hydroxy butyric acid to acetoacetic acid which is then decarboxylated to form acetone. The difference between the acetoacetic acid concentration and total ketone body concentration gives the value for beta-hydroxy butyric acid. The color developed with salicylaldehyde was measured spectrophotometrically using the Spectronic 20 at a wavelength setting of 500 mu. Standard curves were prepared using ethylacetoacetate. Concentration of 0.02 to 0.20 umoles of ketone bodies can be measured with this method resulting in optical density readings in the range of .20 to .70. The amount of ketone bodies contained in the liver tissue was small in relation to the amount put into the medium by the slice. For this reason, only analyses of the media are reported.

Blood sugar:

Blood sugar determinations were carried out according to the method of Somogyi (101). After a 24-hour fast, the end of the rat's tail was clipped and blood obtained for analysis, generally using 0.1 ml samples of blood. A protein free filtrate was made with barium hydroxide and zinc sulfate. Final results were obtained from sodium thiosulfate

titration of the excess iodine after reaction with the reduced copper. Standard curves were prepared and standard glucose samples run with each set of sugar determinations.

Radioactivity analysis:

A number of techniques were utilized in determining the amount of radioactivity present in the various fractions investigated. Four different instruments were made use of in these analyses. All samples were counted for at least 1600 total counts and if exceptionally low count rates were encountered, an additional 1600 counts were obtained. This routine was carried out in an attempt to maintain a less than $\pm$ 5 per cent (the 0.95 error) error in the radioassay determinations.

Automatic D47 Micromil Gas flow system (Nuclear Chicago):

The D47 Micromil counter has a high efficiency of counting with low background characteristics and is especially suitable for low energy beta ray counting, i.e. carbon-14. This assembly can be used with or without the micromil window. The assays to be reported in this thesis were all done with the window in place. Associated with this counter as auxillary equipment is a Nuclear Scaler (Model 183) with circuits for pretime or precount assay, and a Nuclear automatic sample changer capable of handling 32 samples.

Manual D47 Micromil Gas flow system (Nuclear Chicago):

The manual instrument is similar to the one mentioned in the paragraph above except that each sample is engaged manually using the Nuclear Manual Sample Changer (Model M-5) attached to a Nuclear Scaler (Model 183). The efficiency of counting and background activity for the manual instrument and the automatic instrument are identical.

End window system:

The Geiger Mueller end window system is 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. The sample holder has three shelves so that samples with a greater than normal radioactivity can be placed at a distance from the window giving greater latitude to the counting.

Metabolism chamber for gas analysis system:

The metabolism assembly has been described previously (120), and will be discussed only in general terms. The chamber which holds the rat has inlet connections for CO_2 free air and an exit connection for expired CO_2. The air from the chamber is drawn through an H_2SO_4 drying tube to take out moisture prior to assay in a lead shielded chamber containing a G-M end window tube. The $C^{14}O_2$ is monitored by a Berkeley Decimal Scaler (Model 100) which then activates a Berkeley Count Rate Computer (Model 1600) to which is attached an Esterline Angus chart recorder. The rate computer allows visual observation of the count rate and also sends rate information to the recording device which charts the time course of $C^{14}O_2$ collection. The gas is drawn from the chamber into CO_2 collection flasks containing NaOH. The flow rate of the gas is kept constant by the use of a Cartesian Manostat in series with a small oscillating diaphragm vacuum pump.

The samples assayed in these various instruments included ones that were infinitely thick, infinitely thin, liquid, and also paper chromatographic strips. Factors were determined experimentally in this laboratory which allowed the assaying on any of the above assemblies of various kinds of samples, with subsequent conversion to any other assembly, and inter-

conversion to any other kind of counter. Consequently, all final counting was calculated in terms of what that sample would have assayed as an infinitely thick $BaCO_3$ plate on the D47 counter.

Infinitely thin samples:

Samples are considered infinitely thin when the addition of small increments of sample shows a linear increase in count rate. This situation is obtained when the sample on the plate is so thin that no measurable self-absorption is taking place. Experiments in this laboratory (57) have shown that this amount of cholesterol or fatty acid is less than 0.3 mg per planchet. The planchets used were aluminum with a surface area of 5 cm^2. The sample is placed in the planchet with a pipette and then evaporated to dryness under a heat lamp. The dried samples were assayed in one of the counting devices.

Infinitely thick samples:

Samples that are infinitely thick are those which after the addition of more sample to the plate show no further increase in count rate because the activity underneath can no longer penetrate to the surface of the plate and thus cannot be counted. Therefore, at infinite thickness, you have the maximum count rate obtainable with that particular sample and sample holder.

Liquid:

The radioassay technique of aqueous samples containing carbon-14 was developed here and has been described previously (125). The liquid sample to be assayed is pipetted into a stainless steel planchet (Nuclear

Chicago SS-10). A small drop of 1 per cent aerosol solution is added which reduces surface tension and allows the sample to spread out in a film completely covering the bottom of the planchet. The sample holder is then covered with a thin Mylar film (Du Pont) which is held in place by a rubber band. The sample is counted under the end window Geiger Mueller tube and the results converted to their infinitely thick $BaCO_3$ equivalent.

Chromatographic strip assay:

Ketone bodies were assayed after being isolated by ascending paper chromatography, utilizing a butanol, acetic acid, water system (4:1:5), and Whatman #3 filter paper. After chromatography, the strips were cut into 1 cm. sections, attached to aluminum planchets with rubber cement, and the samples counted under the D47 counter. The values obtained were computed as infinitely thick samples and the counts per minute were plotted with respect to Rf value. Ethyl acetoacetate-1,3-C^{14} prepared in this laboratory and commercial ethyl acetoacetate-3-C^{14} were hydrolyzed and chromatographed. The Rf of acetoacetic acid in our system was 0.74. Ethyl acetoacetate-1,3-C^{14} prepared in this laboratory* was hydrolyzed and chromatographed, the Rf in our system being 0.74 (Fig. 2). The additional peak (Rf 0.87) in Fig. 2 was felt to be due to unhydrolyzed ethylacetoacetate-1,3-C^{14}. Fig. 3 represents the chromatography of ethyl acetoacetate-1,3-C^{14} with an Rf of 0.87, confirming the identity of the second peak in Fig. 2. The Rf of beta-hydroxy butyric acid was found to be 0.80 as determined by color development with ferric chloride. As determined on standard samples, loss of radioactivity due to decarboxylation

* The assistance of Mr. William Kemnitz in the preparation of the doubly labeled acetoacetate is gratefully acknowledged.

Fig. 2: Radiochromatograph of hydrolyzed ethyl acetoacetate.

A chromatographic strip was cut into 1 cm strips after chromatography of hydrolyzed ethyl acetoacetate. Each strip was counted for a total of 1600 couts on a D47 Nuclear gas flow counter. The counts per minute obtained were graphed with respect to the Rf value on the chromatogram.

Fig. 3: Radiochromatograph of unhydrolyzed ethyl acetoacetate.

Same technique as in Fig. 2 above except that the radioactivity originated from unhydrolyzed ethyl acetoacetate.

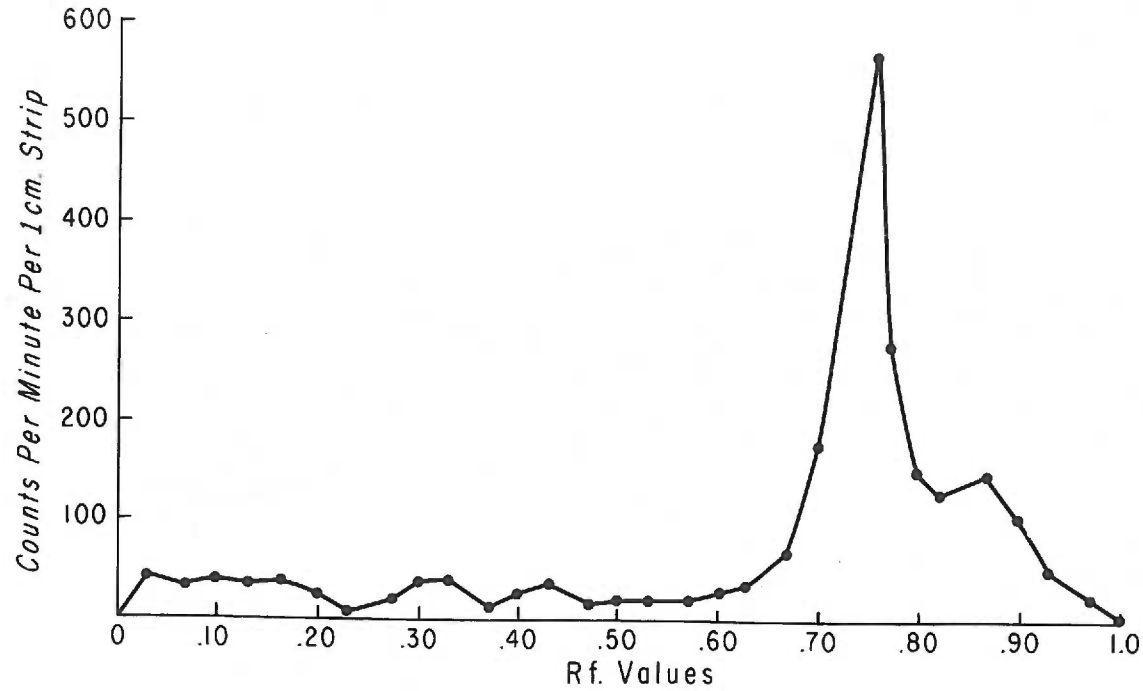


Figure 2

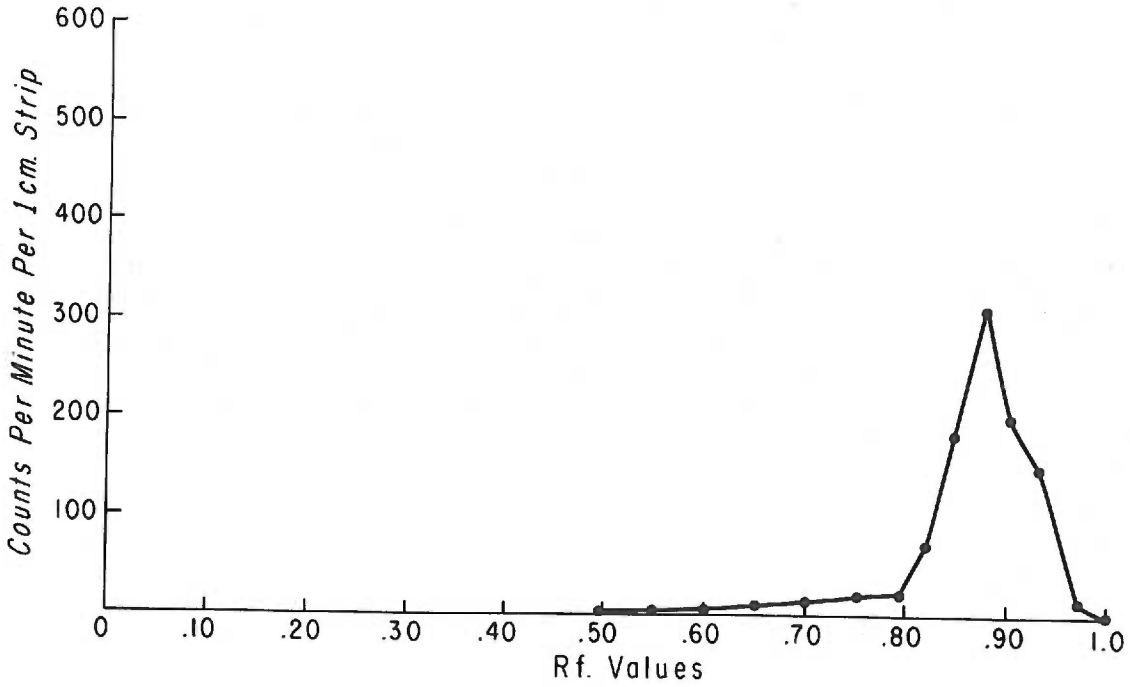


Figure 3

of acetoacetic acid during chromatography and radioassay did not exceed 8 per cent. No correction has been made for this loss. The total radioactivity recorded between Rf 0.57 and 0.82 is considered to be due to acetoacetic and beta-hydroxy butyric acid (39). The per cent incorporation of the labeled acetate into the C_4 fraction of the substrate was calculated as follows:

$$\% \text{ Inc.} = \frac{\text{radioactivity of specified Rf range x total vol.of medium}}{\text{total radioactivity of the dose x vol.of aliquot}} \times 100$$

$BaCO_3$ plates:

The preparation of $BaCO_3$ from alkaline solutions has been previously described (125) in detail. In the experiments to follow there were two types of samples that were prepared for $BaCO_3$ precipitation. The in vivo CO_2 collection was made in 100 ml containers, and it was necessary to use aliquots from each flask due to the large amount of CO_2 collected from the entire animal. On the other hand, the in vitro technique required the quantitative transfer of the absorbed CO_2 into conical centrifuge tubes (50 ml). This transfer and plating has also been described previously (122).

Acetate-1-C^{14} was assayed as $BaCO_3$ under a Geiger Mueller D47 Micromil Counter after persulfate combustion (113) and also as Mylar covered liquid samples using the techniques developed in this laboratory (125).

CHAPTER III

EXPERIMENTAL RESULTS

Statistical Evaluations:

The evaluation of the significance of the difference between mean values reported in the following sections was made using the t test at a confidence level of 0.95. The "p" values are reported in each of the tables, and those equal to or less than 0.05 are considered to indicate a significant difference. In most instances, the standard error is listed in the table with the mean value. The time course information for liver and gut tissues (Fig. 5 and 6) was plotted on semilog paper. The best fitting curve for each component was obtained using the method of least squares after transformation of the numbers to their logarithmic equivalent. The analysis of the difference between the slopes of lines was made utilizing linear regression analysis and the t test. A "p" value equal to or less than 0.05 was taken to indicate a high probability that the two values were from different populations.

Series I: In vitro metabolism of acetate in normal and diabetic rat liver.

The animals utilized in this series weighed between 200 and 225 grams at time of sacrifice and all animals had been maintained on a trained feeding regime at least 5 days prior to use. The diabetic animals were not trained to feeding (see Methods). Control and diabetics were used one hour after the hour feeding period. Acetate-1-C^{14} was the tracer molecule employed. The other experimental procedures followed were as outlined under Methods (In vitro, page 31).

In Table I there are reported the manometrically measured oxygen uptakes per hour per gram of liver tissue. Also recorded are the umoles of CO_2 produced per hour per gram, the calculated respiratory quotients, and the per cent incorporation of acetate-1-C^{14} into $C^{14}O_2$. Normal and diabetic groups consisted of at least 10 animals each, with a minimum of duplicate flasks being run on each animal, thus providing a total of 20 samples from which to determine the mean values for each group.

Table I. Oxygen uptake and CO_2 production by normal and diabetic rat liver slices[+]

Animals	O_2 uptake umoles/hr /g	CO_2 produced umoles/hr /g	Respiratory Quotient	% Acetate incorporated into CO_2
Normal	62.1 ± 0.8[*]	56.7 ± 1.7	0.91 ± 0.07	7.9 ± 0.5
Alloxan Diabetic	53.4 ± 1.8	49.1 ± 2.2	0.92 ± 0.08	13.5 ± 0.2
"p" values	< 0.01	< 0.01	> 0.10	< 0.01

[+] One gram of slices in 27 ml buffer containing 0.57 umoles of tracer acetate; time of incubation = 1 hour.

[*] Mean ± standard error

It is seen that both the amounts of O_2 utilized and the amounts of CO_2 produced by the diabetic animals are lower than those for the controls. The R.Q. values, however, do not differ. The percentages of the tracer dose oxidized to $C^{14}O_2$ by the diabetic and control slices were 13.5 and 7.9 respectively, the 70 per cent increase of the diabetic over the normal being significant.

Table II presents a comparison of the amounts of fatty acid and cholesterol per gram of tissue and the acetate-1-C^{14} activity incorporated into these two classes of lipides. It is clear that the amounts of fatty acid and cholesterol are the same for both preparations. The lipids of the diabetic rats, however, contain significantly less label. The fatty acid labeling is seen to be reduced 92 per cent and the cholesterol labeling 83 per cent for the diabetic as compared to normal slices.

Table II. Amounts of lipid isolated and per cent incorporation of acetate-1-C^{14} into lipid of liver slices

Animal	Fatty Acids		Cholesterol+	
	mg/g tissue	Acetate incorp. %**	mg/g tissue	Acetate Incorp. %
Normal	23.9 ± 1.0*	0.21 ± 0.05	2.4 ± 0.1	0.27 ± .630
Alloxan Diabetic	25.3 ± 0.9	0.02 ± 0.01	2.2 ± 0.1	0.05 ± .009
"p" values	> 0.10	< 0.001	> 0.05	< 0.001

+ Nonsaponifiable

* Mean ± standard error

** Per cent inc. per hr per g tissue

Table III gives data obtained from the analysis of the medium for ketone bodies. The radioactivity values represent the total activity present in the combined acetoacetate and beta-hydroxy butyrate fraction.

The amounts of ketone bodies released to the medium by the diabetic liver slice are seen to be considerably lower than those for the normal

livers. The decrease in total ketone bodies is caused by a decrease in the beta-hydroxy butyrate fraction; the acetoacetate fraction of both preparations being similar. The amount of acetate-1-C^{14} incorporated into the C_4 fraction (acetoacetate and beta-hydroxy butyrate) is significantly depressed in the diabetic.

Table III. Amounts of acetoacetic and beta-hydroxy butyric acid isolated from the substrate and the incorporation of acetate-1-C^{14} into these fractions

Animal	Ketone bodies of the medium+			
	Total ketone bodies produced	Beta-hydroxy butyric produced	Acetoacetic produced	Acetate inc.*
Normal	13.7 ± 0.9**	7.0 ± 0.6	5.7 ± 0.3	5.2 ± 0.27
Alloxan Diabetic	8.5 ± 1.1	3.6 ± 0.9	5.0 ± 0.3	3.3 ± 0.14
"p" values	< 0.01	< 0.02	> 0.05	< 0.001

+ umoles/hr/g

* Per cent inc./hr/g

** Mean ± standard error

Series II: Metabolism of acetate, acetoacetate, butyrate and mevalonate*.

The animals used in this group of studies were similar in weight (220 - 225 grams) to those of the first series. The nutritional regime was, however, altered slightly as compared to the others. The

* The acetoacetate studies listed in Series II were carried out by Miss Alicia Marco', and are included in this thesis only because they complement the acetate, butyrate, and mevalonic acid data. Sincere appreciation for this courtesy is acknowledged.

The mevalonate studies were done on animals fasted 1 hour and experiments on acetate, acetoacetate and butyrate with animals fasted 3 hours. In investigations not being reported here, there was found less variable lipid labeling of 3-hour fasted animals as compared to 1-hour fasted animals. The liver slices of animals of the present series were incubated in the presence of either acetate-1-C^{14}, acetoacetate-3-C^{14}, butyrate-1-C^{14}, or mevalonic acid-2-C^{14}.

Since in liver slice studies, an excess of labeled metabolite is always present, it was necessary to determine the stability and distribution of the label of the tracer mevalonate in the lipid fractionation procedure. For this experiment, 0.85 umoles of MVA-2-C^{14} were added to a gram of liver tissue suspended in alcoholic KOH, and the mixture was digested and fractionated as described under methods. It was found that 0.05 per cent of the label was subsequently recovered in the saponifiable fraction, 0.07 per cent in the nonsaponifiable fraction and that more than 98 per cent of the added C^{14} was present in the acid aqueous phase that is usually discarded. Since radiochromatograms of suspending medium, after 1-hour incubation with 1 gram of liver slices, showed the presence of 87 per cent of the added C^{14} mevalonate, it is likely that the "contamination" of fatty acid and cholesterol fractions would not exceed 0.007 and 0.01 per cent respectively. In view of this negligible activity, no correction was made for this slight contamination from mevalonic acid in lipid activities reported below.

To determine that the present series of animals was comparable to the first series described above, analysis was made of ketone bodies present in the medium after incubation of the slices for 1 hour. As

indicated by the data in Table IV, the diabetic preparations produced less ketone bodies than the normals and as before, this was shown to be due to a reduction in beta-hydroxy butyrate since the amounts of acetoacetate were normal.

The amounts of lipid in the tissues were determined and found to be in agreement with those reported in Table II. With such similarities in findings, it is concluded that the responses studied are common to normal and diabetic preparations of this laboratory, justifying comparisons between groups.

Table IV: Ketone body production by normal and by diabetic liver slices*

Animal No.	Kind	Total ketone bodies umoles/hr/g	Beta-OH butyrate umoles/hr/g	Acetoacetate umoles/hr/g
8	Normal	14.7 ± 0.73**	9.2 ± 0.60	5.5 ± 0.08
6	Alloxan Diabetic	10.4 ± 0.63	5.6 ± 0.59	5.5 ± 0.30
"p" values		$< .05$	$< .05$	$> .10$

* One gram of slices in 27 ml buffer containing 0.57 umoles of tracer.

** Mean ± standard error.

The respiratory gas exchanges of 12 normal and 14 diabetic animals from the butyrate and mevalonate experiments were followed. The normals used 59.8 ± 3.1 and the diabetics 56.0 ± 2.2 umoles O_2 per gram per hour; the difference is not significant ($p > .05$). The normals produced

59.3 ± 2.4 and the diabetics 51.6 ± 23 umoles CO_2 per gram per hour; the difference of 7.7 umoles is significant ($p < 0.05$). The decrease in CO_2 production is the same but the decrease in O_2 utilization is not quite as large as reported in Series I.

The incorporation of C^{14} of the four tracer substances into CO_2, fatty acids, and cholesterol is reported in Table V. Comparison of differences in the response of normal and diabetic animals is offered by the appropriate p values. It is evident that with tracer acetate, acetoacetate, and butyrate, there was a real decrease in the labeling of fatty acids and cholesterol of the diabetics. There was no significant change in the formation of $C^{14}O_2$ by the diabetics, but the decreased production of CO_2 in the diabetic, in the presence of normal C^{14} labeling of the CO_2, gives rise to a $C^{14}O_2$ of higher specific activity as was reported before.

Essentially the same amounts of each of the three test compounds, acetate, acetoacetate, and butyrate, were converted to lipids by the normal and by the diabetic animals. The diabetics, however, showed a lower level of incorporation of all three compounds. A grossly different pattern of CO_2 labeling was present for these same three substrates. $C^{14}O_2$ appearing from acetoacetate was only about one-sixth that coming from acetate and butyrate.

Mevalonate was incorporated into the three fractions in a pattern quite different from the other substances. Although about the same amount of C^{14} activity was incorporated into fatty acids as in the case of the other three metabolites, there was an increase in cholesterol and a decrease in CO_2 labeling. The responses of the diabetic

animals did not differ from those of the normals in any of the fractions studied. Studies on systems other than liver slices (36,145) have shown greater labeling of cholesterol from mevalonate than observed here. A time course study was carried out to gain more insight into the dynamics of mevalonate metabolism in the liver slice preparation.

Table V. Incorporation of tracer substances into CO_2, fatty acids and cholesterol

Animal		Tracer Substrate	% Incorporation per gram of tissue per hr.		
No.	Kind		Fatty Acids	Cholesterol	CO_2
6	Normal	Acetate	0.23 ± 0.05*	1.22 ± 0.15	11.9 ± 1.22
6	Diabetic	Acetate	0.01 ± 0.002	0.02 ± 0.002	15.6 ± 1.14
"p" values			< 0.05	< 0.005	> 0.10
6	Normal	Butyrate	0.18 ± 0.09	0.83 ± 0.032	12.8 ± 1.2
7	Diabetic	Butyrate	0.01 ± 0.003	0.03 ± 0.010	14.2 ± 1.0
"p" values			< 0.05	< 0.01	> 0.10
8	Normal	Acetoacetate	0.11 ± 0.024	0.49 ± 0.95	1.6 ± 0.2
4	Diabetic	Acetoacetate	0.02 ± 0.001	0.01 ± 0.001	2.0 ± 0.1
"p" values			< 0.05	< 0.02	> 0.10
6	Normal	Mevalonate	0.26 ± 0.04**	5.8 ± 0.6	1.34 ± 1.0
6	Diabetic	Mevalonate	0.38 ± 0.04	5.6 ± 0.2	0.88 ± 0.2
"p" values			> 0.10	> 0.05	> 0.05

* Mean ± standard error

** As per cent of the active isomer.

In Table VI there are recorded the mean values obtained from this time course study. The data is not presented with a statistical evaluation, but it is clear that the rates of labeling decline after the first two hours. The high rate of label incorporation in the first hour indicates that tracer permeability (i.e. availability to the cell) was not seriously limiting. It is likely that the equilibrium period permitted attainment of cellular mevalonate levels commensurate with the high rate of utilization recorded for this first period. Since adequate levels of tracer mevalonate remained in the medium, it is likely that the decline in incorporation is due to a lack of accessory factors.

Table VI. Rates of labeling from mevalonate-2-C^{14}

Hour	No. Animals	% Incorporation per gram per hour*			
		Fatty Acids	Cholesterol	CO_2	Total
1st	6	0.13	2.90	0.67	3.70
2nd	2	0.07	2.50	1.50	4.07
3rd	2	0.04	2.10	0.80	2.94
4th	2	0.08	1.30	0.20	1.58

* Mean values

Series III: In vivo metabolism of mevalonate.

These studies constitute the observations obtained after intraperitoneal injection of mevalonic acid-2-C^{14} into intact rats. The animals weighed 80-100 grams at time of sacrifice. Young animals were utilized because of the observation of Wright (145) that tissue

from young rats favored incorporation of MVA into cholesterol in rat liver homogenates. In vivo work in our laboratory has indicated, however, that young adult rats (200 grams) show no less incorporation of label nor a grossly different labeling pattern. A total of 27 animals was used in the present studies.

In Table VII are recorded the amounts of cholesterol found in the major fractions studied, as well as a detailed separation of the components of the carcass fraction. The carcass tissues contain approximately 55 per cent of the animals total cholesterol, while the gut, skin and liver contain 23, 16 and 6 per cent respectively. It is interesting that the amount of cholesterol per gram of tissue is relatively constant in the four large fractions. Brain and adrenal tissue have higher cholesterol concentrations than other tissues. The amounts of cholesterol in the various fractions are in agreement with Chevallier's findings (35).

Table VIII presents the per cent of the administered dose found in the tissues in the time periods between 15 minutes and 4 hours. The molecular species containing the radioactivity of the blood and urine were not characterized. The first observation of interest from Table VIII is that the cholesterol labeling is 95 per cent maximal in 30 minutes and is maximal at the 1 hour post injection time. The same is true for the acidic fraction. It appears that almost 1/4 of the tracer dose is the maximum which can be incorporated into the sterol fraction. Of the total dose, 3.8 per cent is directed to the acidic fraction. Urinary excretion of C^{14} activity was 90 per cent complete within the first hour, paralleled by a decrease in activity

Table VII. Cholesterol content of tissue

Fraction	No. of Animals	mg/gm tissue	Weight tissue grams	Wgt. Total cholesterol mg	% of total body cholesterol
Liver	16	2.6± 0.6*	4.5	11.7	6.2
Gut	16	2.0± 0.6	21.4	42.8	22.8
Skin	17	2.9± 0.9	10.3	29.9	15.9
Total carcass	17	2.0± 0.3	51.7	103.4	55.1
Kidney	7	4.3± 0.4	.88	3.8	2.0
Brain	4	13.1± 2.9	1.70	22.3	11.9
Adrenals	8	34.5± 11.0	.02	.7	.4
Leg muscle	3	.85	1.00	.85	.5
Diaphragm	3	.73	.32	.23	.1
Gonads	2	1.9	.84	1.6	.85
E.F.P.	2	.51	.80	.41	.2
Lung	2	4.5	.64	2.88	1.53
Spleen	2	3.8	.31	1.18	.63
Heart	3	1.33	.34	.45	.23

* Mean ± standard error.

Table VIII. Per cent* recovery of injected radioactivity

Time	No. of Animals	CO_2**	Cholesterol	Acidic	Urine	Blood	Total Recovery
15 min.	3	0.46	15.9	2.5	16.0	7.0	41.9
30 min.	2	1.44	22.7	3.0	23.3	5.0	55.4
1 hour	3	3.50	23.8	3.8	52.5	2.5	85.8
2 hour	8	6.40	21.0	2.3	59.6	2.4	91.3
3 hour	3	8.00	21.9	3.3	58.6	2.5	94.3
4 hour	5	9.00	18.0	3.6	57.0	2.7	90.4

* Per cent of the total dose of D,L MVA-2-C^{14}

** The CO_2 data was obtained from a total of 5 animals, the serial samples being so spaced in time as to yield a minimum of 3 values for each time interval.

of the blood to a low level by the end of the 1-hour time period. At the 15 and 30 minute time intervals, total recovery of activity is seen to be only 41.9 and 55.4 per cent, respectively. It has been shown previously that MVA is not extracted in the procedures for isolating fatty acids or cholesterol so that it can be assumed that the total activity unaccounted for in the early time periods was as MVA per se. A larger amount of activity is accounted for in the urine in the 30-minute to 1-hour periods. By the end of the first hour, 86 per cent of all the administered radioactivity was accounted for in the total recovery .

The respiratory $C^{14}O_2$ activity is seen to rise regularly (Fig. 4) during the first 2 hours and then to decline somewhat during the subsequent 2 hours. Of the total of the 9 per cent incorporation in 4 hours, 3.5, 2.9, 1.6 and 1 per cent were excreted per 1st, 2nd, 3rd and 4th hour respectively.

Table IX presents data on the activity found in the cholesterol fraction of liver, gut, carcass, skin, and brain over a 24-hour period following injection. The radioactivity of both carcass and skin fractions appear to reach high values within a short time. The brain tissue is, as reported by others, relatively inert as regards cholesterol synthesis even in these young animals. There was a loss of cholesterol fraction activity from the liver during the 24-hour time period and there was a concomitant increase in the labeling of the cholesterol of the gut fraction. The labeled cholesterol movement from the liver, after synthesis from MVA-2-C^{14}, into gut, occurs presumably via the bile duct (ductus choleductus). If the information concerning liver and gut (Table IX) is plotted as in Fig. 5, a biphasic curve becomes apparent for both fractions. The liver fraction has a rapid component with a half life ($t_{\frac{1}{2}}$) of 1.5 hours and a slower component of 7.5 hours. The activity in the gut fraction is seen to increase (Fig. 6) and the curve of response is resolvable into 2 components that have half times of 1.3 and 7 hours. The initial fast turnover component of liver is of course made up of two processes, the slow and the faster fraction. However, the effective rate of cholesterol movement was of more interest than the individual contributions of either component.

Fig. 4: Respiratory $C^{14}O_2$ activity from MVA-C^{14}.

The per cent of the dose of mevalonic acid-2-C^{14} converted to $C^{14}O_2$ by intact rats after intraperitoneal injection of the label is plotted with respect to time. The CO_2 data was obtained from a total of 5 animals, the serial samples being so spaced in time as to yield a minimum of 3 values for each time interval.

Figure 4

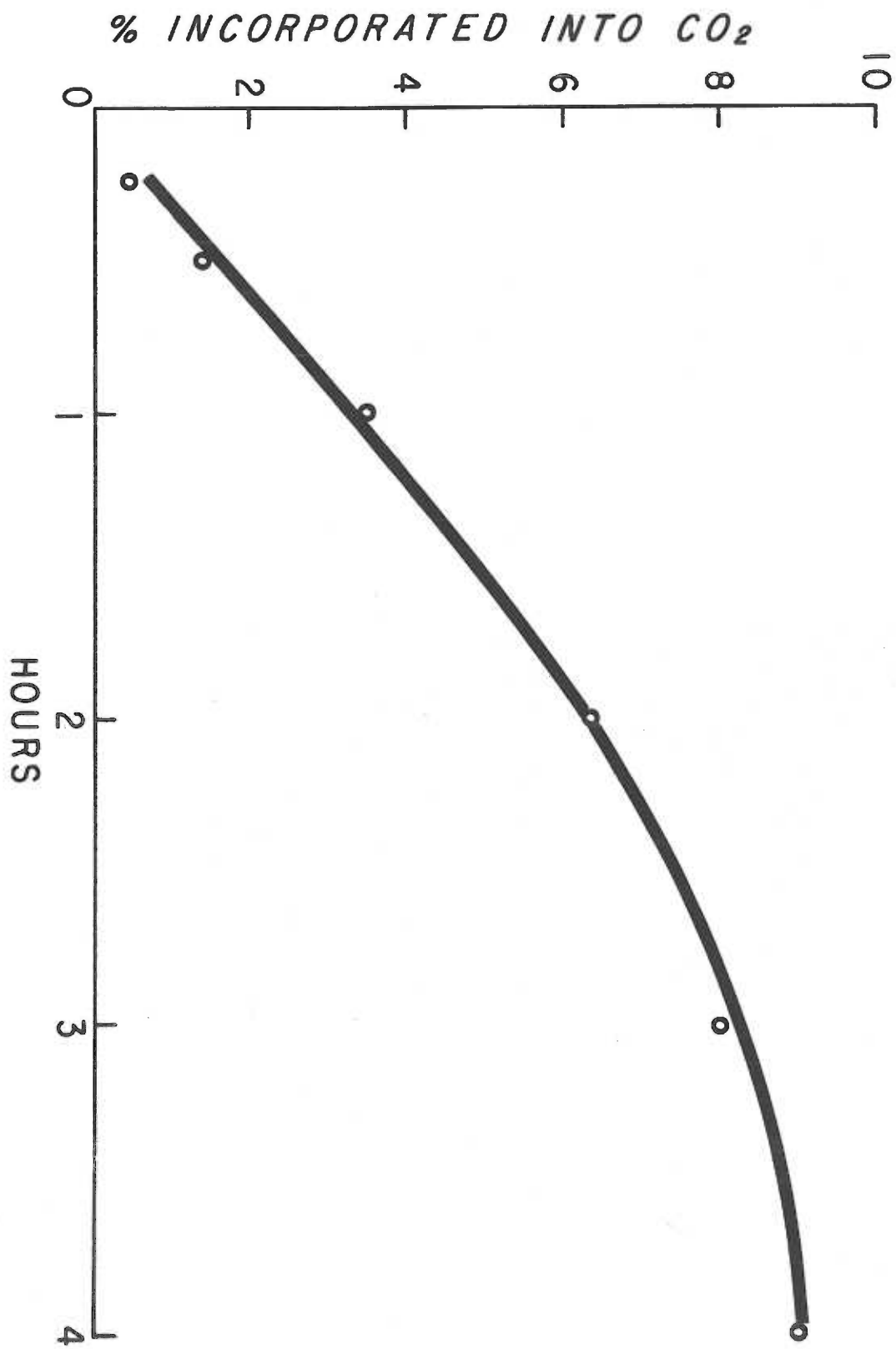


Table IX. Time course of cholesterol labeling from MVA-2-C^{14}

Post-Inject. Time	% of labeled body cholesterol in				
	Liver	Gut	Carcass	Skin	Brain
15 min.	25.0	6.8	63.2	2.2	0.3
30 min.	21.0	7.5	70.3	2.9	0.1
1 hour	17.3	8.3	72.0	2.5	--
2 hour	11.2	13.2	77.0	2.7	--
3 hour	10.1	14.4	73.0	2.3	0.2
4 hour	8.4	17.3	71.2	2.9	0.3
24 hour	4.4	11.7	81.0	2.9	--

Because of the heterogeneity of the carcass fraction regarding types of tissue, it was felt desirable to better define the participation of the several tissues. Table X records the values obtained on the total body distribution of labeled cholesterol 2 hours post injection. The results for the liver, gut, and skin fractions agree well with the previous data (Table IX) for 2 hour animals. A most interesting finding is the relative amount of radioactive cholesterol found in kidney tissue. The other fractions of the carcass contribute relatively little of the radioactivity of the newly formed cholesterol when compared to the kidney. The adrenal gland has an appreciable concentration of radioactive sterol, but it represents a small part of the total carcass sterol.

With the finding that at 2 hours the kidney contained the greater part of the radioactivity of the carcass fraction, it appeared

Fig. 5: Turnover of liver cholesterol.

The per cent of labeled body cholesterol found in the liver has been plotted with respect to time after injection of mevalonic acid-2-C^{14}. The straight lined curves were obtained by the method of least squares after the transformation of numbers to their logarithmic function. The difference in slopes of the two lines were compared and found to differ statistically. The half-live of the fast component ($t_{\frac{1}{2}}$) = 1.5 hours and the slower component ($t_{\frac{1}{2}}$) = 7.5 hours.

Fig. 6: Turnover of gut cholesterol.

The per cent of labeled body cholesterol found in the gut has been plotted with respect to time after injection of mevalonic acid-2-C^{14}. The evaluation of the lines of the slopes were as listed in Fig. 5 above. The time necessary for the gut to double its radioactivity is also seen to be biphasic. The more rapid component has a half-life ($t_{\frac{1}{2}}$) = 1.3 hours and the slower component ($t_{\frac{1}{2}}$) = 7.0 hours.

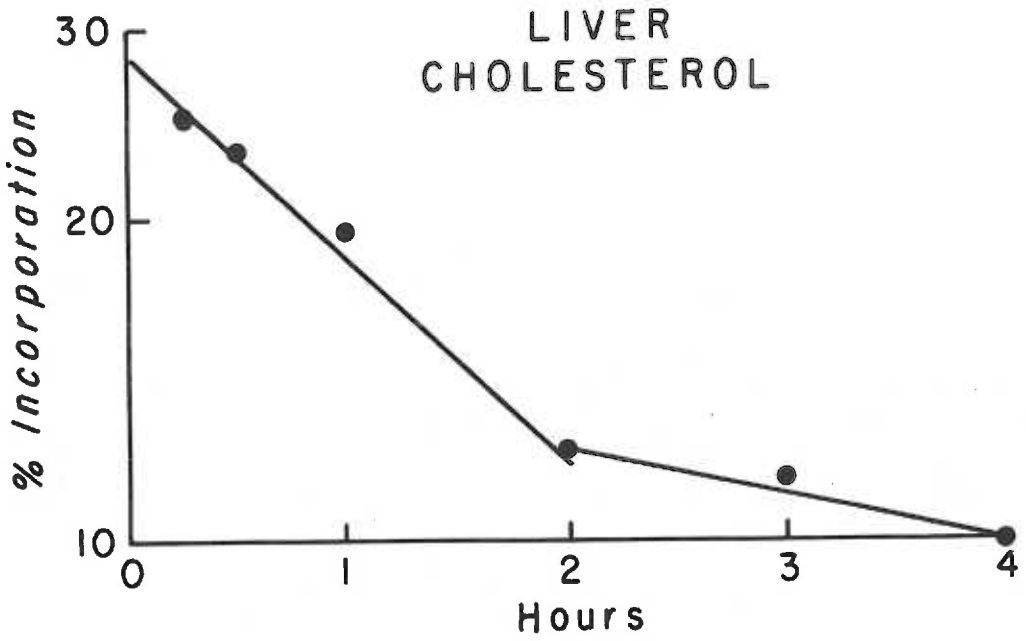


Figure 5

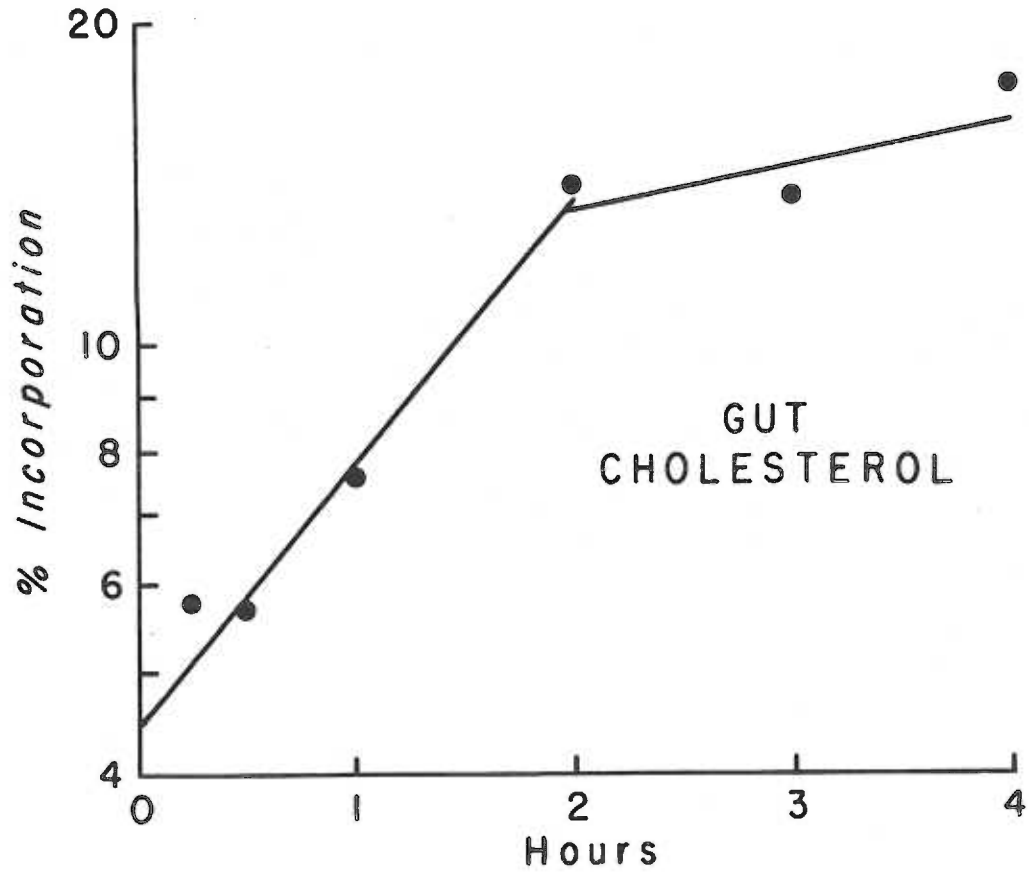


Figure 6

desirable to better define the role of the kidney in cholesterogenesis. To this end, a special time course study was made to follow the metabolism of cholesterol in kidney tissue. From the data of Table XI, it is clear that at the 15 minute time the kidney contains some 79 per cent of the radioactivity generally attributed to the carcass cholesterol fraction. The activity of the kidney cholesterol fraction rises during the first hour, but it is not known if this increase is significant. In any event, kidney sterol radioactivity remained high during the 4 hour period of study. Some 40 per cent of the total carcass acidic fraction was also found in kidney tissue.

Table X. Total body distribution of labeled cholesterol 2 hours post-injection.

Fraction	No. of Animals	% of labeled cholesterol	Fraction	No. of Animals	% of labeled cholesterol
Kidney	4	57.5**	Lung	3	0.7
Carcass*	4	11.5	Spleen	3	0.6
Gut	4	11.9	Muscle	4	0.3
Liver	4	14.1	Diaphragm	3	0.2
Skin	4	2.5	Heart	2	0.3
Adrenal	2	.2	Testes	3	0.2
Epididymal fat pads	3	.2			

* Muscle mass and body structure.

** As percentage of the total activity found in the entire animal.

Table XI. Kidney time course data

Time	No. of animals	% inc. of MVA-2-C^{14} into cholesterol fraction	% inc. of MVA-2-C^{14} into acidic fraction	% of total animal cholesterol-C^{14} present in kidney	% of total animal acidic-C^{14} present in kidney
15 min	2	12.5	0.9	79.1	36.0
1 hour	2	17.9	1.5	75.2	39.5
2 hour	4	15.8	0.6	75.2	25.0
3 hour	1	15.6	0.9	71.0	27.3
4 hour	2	13.0	0.6	72.2	16.7

Series IV: In vitro metabolism of kidney tissue

The following group of experiments was an in vitro approach to the problem of cholesterol and fatty acid synthesizing pathways in the normal and diabetic kidney slice. The animals used were trained to feeding, weighed 200 - 225 grams at sacrifice, and were fasted for 3 hours prior to use. The three C^{14} compounds, acetate-1-C^{14}, aceto-acetate-3-C^{14}, and mevalonic acid-2-C^{14}, were utilized in tracing various metabolic sequences.

The kidney slice studies were carried out to compare kidney and liver tissues in their utilization of the above mentioned tracer compounds. The study was also designed to compare the normal kidney slice and the alloxan diabetic kidney slice in the areas of CO_2 production, oxygen utilization, lipid content of tissue, and the incorporation of label from tracer compounds into CO_2, fatty acids, and cholesterol.

The amounts of oxygen utilized per hour per gram of tissue of the normal and alloxan diabetic kidney sliceare listed in Table XII,

as well as the amounts of CO_2 produced. The p values associated with the comparison of these preparations are also listed.

Table XII. Respiratory gas measurements for kidney tissue

Condition	No. Animals	No. Samples	umoles per hour per gram of tissue	
			oxygen utilized	CO_2 produced
Normal	10	10	88.6 ± 5.4*	114.8 ± 6.5
Alloxan Diabetic	12	17	88.4 ± 5.4	117.5 ± 7.1
"p" values			>0.10	>0.10

* Mean ± standard error

It is seen from Table XII that the amounts of oxygen utilized by the normal and diabetic animals were the same. A similar finding is observed for the CO_2 produced. There is also seen to be an increased amount of CO_2 produced per volume of O_2, yielding an R.Q greater than 1.

The amounts of lipid present in the kidney of normal and diabetic animals were determined and the results of these analysis are listed in Table XIII. The comparison is made between the fatty acid present per gram of kidney tissue in the two preparations, normal and diabetic, as well as the amount of cholesterol present. It is apparent from Table XIII that the lipid content of the kidney tissue is not altered by the condition of experimental alloxan diabetes, for as can be seen, the mg. of fatty acid and cholesterol per gram of kidney tissue remain

the same in the normal and diabetic. The findings of a lack of alteration in lipid composition of the kidney tissue is similar to that obtaining in the liver slice (Series I).

Table XIII. Lipid analysis of kidney tissue

Condition	No. Animals	mg of lipid per gram of tissue	
		Fatty Acids	Cholesterol
Normal	6	48.1 ± 7.8	4.3 ± 0.3
Alloxan Diabetic	6	47.8 ± 6.1	4.1 ± 0.3
"p" values		> 0.10	> 0.10

* Mean ± standard error

Table XIV lists the comparisons that were made between normal and diabetic kidney preparations as regards the activity of this tissue in incorporating acetate-1-C^{14}, the labeled compound, into respiratory CO_2, fatty acids, and cholesterol.

Table XIV. Acetate-1-C^{14} incorporation into kidney tissue

Condition	No. Animals	Per cent incorporation per hour per 100 mg		
		Fatty Acids	Cholesterol	CO_2
Normal	7	0.16 ± 0.04*	0.11 ± 0.05	53.4 ± 1.90
Alloxan Diabetic	6	0.15 ± 0.04	0.09 ± 0.02	45.0 ± 0.30
"p" values		> 0.10	> 0.10	< 0.01

* Mean ± standard error

The finding of a lack of alteration in the incorporation of acetate-1-C^{14} into the two fractions, fatty acid and cholesterol, differs from the findings presented in Series I for liver tissue. The diabetic liver slice was shown to have a decreased incorporation of acetate-1-C^{14} into fatty acids as well as cholesterol. The amount of acetate-1-C^{14} oxidized to CO_2 by the diabetic kidney slice as compared to the normal kidney is seen to be decreased.

When acetoacetate-3-C^{14} is added to the suspending medium of incubating kidney slices, there is seen to be a different response in handling of this tracer than is elicited by liver tissue. Table XV presents the data obtained from the incorporation of acetoacetate-3-C^{14} into CO_2, fatty acids and cholesterol of normal and alloxan diabetic kidney slices.

Table XV. Acetoacetate-3-C^{14} incorporation into kidney tissue

Condition	No. Animals	Per cent incorporated per hour per 100 mg		
		Fatty acids	Cholesterol	CO_2
Normal	9	0.21 ± 0.06*	0.09 ± 0.02	50.5 ± 2.0
Alloxan Diabetic	10	0.15 ± 0.03	0.09 ± 0.02	55.8 ± 2.6

* Mean ± standard error.
The "p" values for the above table are all >0.10.

The information for acetoacetate-3-C^{14} utilization resembles that for acetate-1-C^{14} for there is seen to be a similar magnitude of labeling of the 3 fractions by these two tracer compounds. There is

also seen to be little difference in the amount of acetoacetate-3-C^{14} oxidized to CO_2, incorporated into fatty acids and/or cholesterol when the normal and diabetic preparations are compared.

The final observations concerning kidney tissue were obtained utilizing mevalonic acid-2-C^{14} as the tracer molecule. The results of mevalonic acid-2-C^{14} incorporation into CO_2, fatty acids and cholesterol are listed in Table XVI.

Table XVI. Mevalonic acid-2-C^{14} incorporation into kidney tissue

Condition	No. Animals	Per cent incorporation per hour per 100 mg		
		Fatty Acids	Cholesterol	CO_2
Normal	7	0.45 ± 0.10*	5.2 ± 0.82**	3.7 ± 0.89
Alloxan Diabetic	7	0.67 ± 0.12	4.6 ± 0.23	2.3 ± 0.30
"p" values		> 0.10	> 0.10	> 0.10

* Mean ± standard error

** As per cent of the active isomer.

Mevalonic acid-2-C^{14} labeling of CO_2, fatty acids and cholesterol is similar in the normal and diabetic kidney slice preparations. The findings obtained from the liver slice with mevalonic acid as tracer is analagous to the findings obtained from the kidney, i.e. there is normal incorporation of the tracer in both preparations.

CHAPTER IV

DISCUSSIONS

Because the experimental results were presented in a sequence of four interrelated series, it seems appropriate to discuss the findings in a similar fashion. However, prior to such a discussion, there are some general remarks to be made which pertain to the overall problems of oxidation, lipogenesis, and cholesterogenesis of the experimental animal under consideration, i.e. the rat.

In the mammal, acetyl-S-CoA is the focal point for several metabolic sequences so that the size of, and the flux through, the acetyl-S-CoA pool are dependent on a variety of interrelated reactions.

The relationship of a number of the key metabolites are indicated in Fig. 7. The intermediates and end products with which this thesis are primarily concerned are shown without brackets.

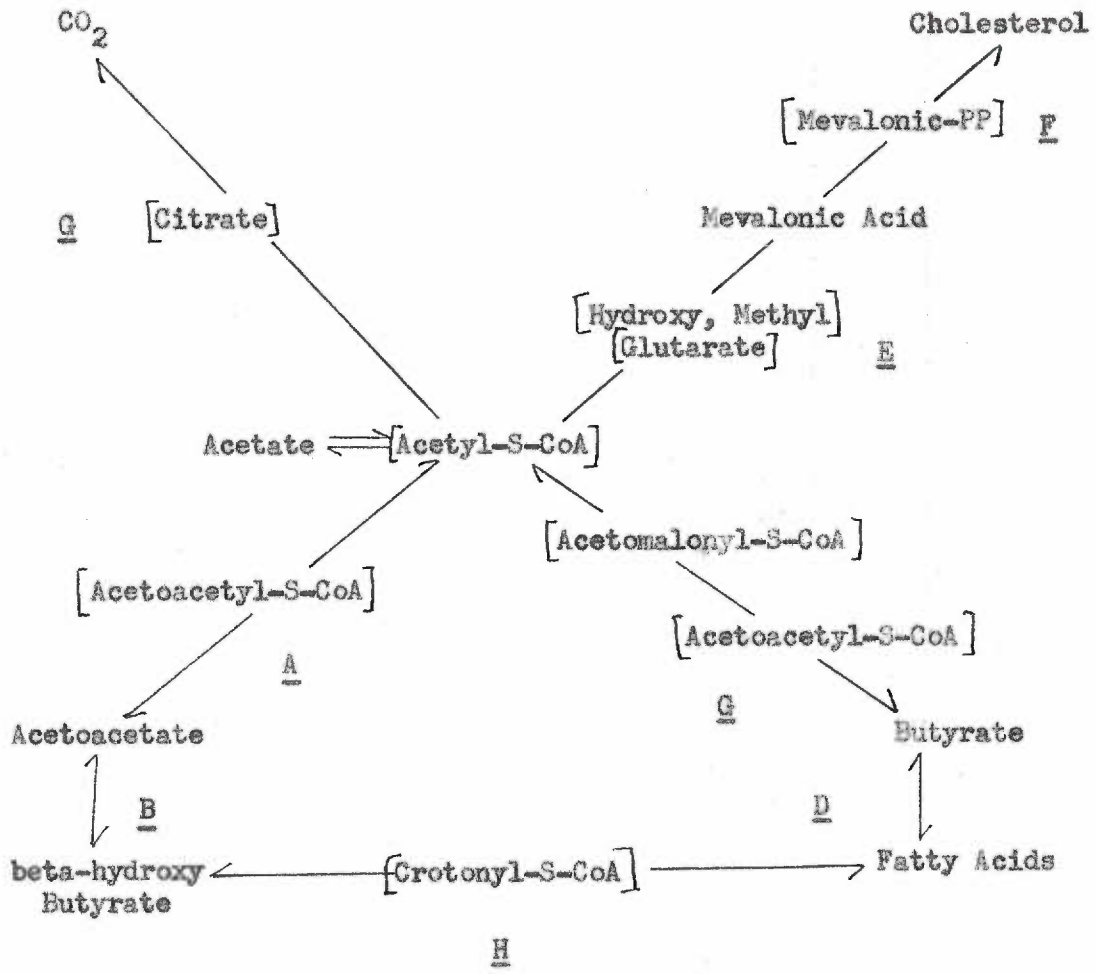
The decreased lipogenesis of the alloxan diabetic rat has been considered due to variations in a number of regulatory factors (24, 32,119,140). Some investigators have felt that the availability of oxalacetate was the limiting factor while others have proposed the need of glycolytic intermediates for the normal synthesis of fat. The decrease in fatty acid labeling in diabetic rats has been postulated also to be due to decreased availability of TPNH which is normally kept at optimum levels by carbohydrate metabolism via the hexose monophosphate shunt (99). Cofactor requirements for the DPN system have been shown at B,C and G and for the TPN system at C, D, E, F, and G, Fig. 7, (24,42,61,65,70).*

*Recent evidence by Chaikoff et al suggests that TPN/TPNH ratios are not altered in the diabetic and therefore TPNH supplies are not the controling factor in fatty acid synthesis(147).

Fig. 7: Relationship of some key metabolites in lipogenesis.

The schematic representation listed is an attempt to show the interrelationship of a number of intermediates in lipid metabolism and also to show the central position of acetate.

Fig. 7



Cholesterol labeling from acetate-1-C^{14} has also been shown to be diminished in the intact diabetic (119), as well as in diabetic liver slices as reported in this thesis. The observation of decreased cholesterol labeling in the diabetic is at variance with the findings of Hotta and Chaikoff (34) who observed increased cholesterol labeling from acetate. Gurin et al (50) showed no difference in cholesterol labeling between normal and diabetic liver tissue.

Some of these discrepancies have arisen as a consequence of comparing <u>in vitro</u> work from one laboratory with <u>in vivo</u> results from

from another. It was felt desirable, therefore, to conduct *in vitro* versus *in vivo* responses in the same laboratory using similar conditions of feeding, fasting, etc., and this has been attempted. The present results deal with the *in vitro* utilization of various tracer molecules as well as the comparison with *in vivo* information obtained in this laboratory.

Series I: *In vitro* metabolism of acetate in normal and diabetic rat liver.

As one would attempt to assay the utilization of acetate in mammalian systems, as a measure of lipogenesis, it is necessary to make available to the cell some labeled acetate. The 2 main routes of dosage administration for the intact animal are intravenous and intraperitoneal. For the slice technique, added tracer to the suspending medium is the usual practice for following the fate of the label. In all of these methods, it is a primary need to move the acetate from the extracellular environment into the cell. After entry into the cell, the first enzymatic step in acetate metabolism is undoubtably its "activation" by combination with coenzyme A to give a high energy thiol ester linkage which we call acetyl-S-CoA. After activation, this molecule has several routes available to it (see Fig. 7), and the pathways of choice will depend on the conditions prevailing in the cell at that time. Factors influencing the metabolic routes of acetate have been shown to be starvation and experimental alloxan diabetes. In Series I, the attempt was made to assay and evaluate the influence of the diabetic preparation on acetate utilization for fatty acid synthesis, cholesterol synthesis, and for oxidative metabolism.

The diabetic preparations utilized a decreased amount of O_2 and produced a decreased amount of CO_2 in the experimental period, but the CO_2 formed contained a significantly greater amount of the tracer carbon than did the control preparations (Table I). This observation, that the diabetic slice preparation produced a decreased amount of CO_2, is again not in accord with the conclusions of Hotta and Chaikoff (34). These workers, on the basis of an increased amount of $C^{14}O_2$, concluded that the diabetic slice oxidized acetate to a slightly greater degree than did their normals. Foster and Villee (127) reported that muscles of diabetic rats oxidized acetate at a reduced rate. The data on the actual amounts of CO_2 produced, together with the data on the radioactivity of the CO_2, indicates that the diabetic rat produces less CO_2, but converts more of the labeled acetate to $C^{14}O_2$. An explanation of the above findings may reside in the suggestion that as less CO_2 is produced in the face of more label becoming incorporated, it is reasonable to predict a decreased acetate pool in the diabetic liver to account for the increased specific activity. A dilution decrease, i.e. increase in acetyl-S-CoA specific actitivity, greater than the decreased flux would result in the production of an increased amount of $C^{14}O_2$ in the presence of a decreased CO_2 production. The present findings on the CO_2 fraction are in partial accord with _in vivo_ findings from this laboratory (117). Although the group of intact rats did not produce less CO_2, it did produce more $C^{14}O_2$ than did the normals. It would appear that the diabetic liver slice has adequate capacity to utilize exogenous tracer acetate. A ten-fold increase in acetate concentration made available

to the cell shows essentially the same responses, indicating that permeability of acetate across the cell membrane, or acetate activation, is not a limiting factor. It is not likely that the depressed labeling of fatty acid and cholesterol fractions is due to an increased dilution of the acetate-C^{14} by endogenous acetate, for as shown, the CO_2 information would suggest a decreased rather than an increased pool size in the in vitro diabetic liver slice preparation.

The large decrease seen in fatty acid labeling (Table II) is similar to that previously reported from this laboratory (119, 140) and also by others (32, 50), and may be due to several factors, such as pyridine nucleotide requirements, and acetate availability. It is likely that the increased mobilization of depot lipides that occurs in diabetes can account for the maintenance of lipid levels in spite of the decreased lipogenesis.

The data presented for the cholesterol fractions (Table II) reveals that the amounts of sterol were maintained, but that cholesterogenesis was depressed in the diabetic, the lipogenic and cholesterogenic reactions being depressed to about the same degree. This depression in hepatic cholesterogenesis is greater than originally reported by this laboratory for the intact rat (140), but is in agreement with later studies (119). These later studies were carried out on acute diabetic preparations and involved the use of a larger number of animals and the statistical treatment of data. It is not yet clear how the liver sterol levels have been maintained in the face of depressed cholesterogenesis. It is probable that the animals used in the present study had the same degree of hypercholesterolemia as the

animals used in the in vivo study (119), and that the levels of liver cholesterol may be related to the "turnover" of the sterol as well as sterol synthesis (126).

The decrease seen in total ketone bodies of the diabetic liver slice seems to be at variance with the general concept of the hyperproduction of ketones by the diabetic liver. It has been shown (1), however, that liver slices of the fasting diabetic rat did not cause an increased accumulation of ketones over that of normals, and it is well known that the addition of carbohydrate to the diet will surpress ketonuria and direct acetoacetate carbon to oxidative pathways (135). The rats of the present series were fed prior to use. The recent findings of Scow et al (97,98) with totally pancreatectomized rats on the close correlation between ketone body formation and the amount of liver lipid appear to be related to this problem. A number of workers have reported fatty livers of alloxan diabetic rats, and this has also been observed in this laboratory. However, this condition is most often found in rats diabetic over six months, rather than the relatively young diabetics used in these experiments. The requirement of severe insulin deficiency, elevated liver lipid, and cortical stimulation for maximum ketogenesis, appear not to be met by our preparations. The suggestion of Wertheimer (139) that non-acidotic animals are only partially diabetic may be pertinent. Although certain of these facts tend to make the alloxanized rat, that can be maintained without insulin, less desirable as an experimental animal, it is to be noted that we have shown alloxanized animals to be equivalent to conventionally pancreatectomized animal in

several parameters of comparison (119,126), and that both of these preparations show metabolic lesions not seen in control animals.

At this point we would recall that we have suggested a decreased acetate pool size in the diabetic liver. Since energy production is a prime function of an organism, one would expect oxidation to preferentially use available acetate. If the pool size is low, there would be less acetate available for synthesis, which might well be reflected in a decreased amount of label appearing in fatty acids and cholesterol. At the same time, there would be found more label in the expired CO_2. To test this hypothesis, it would only be necessary to look at the activity of some other metabolic pool which lies on the pathway of both fatty acid and cholesterol synthesis to see if label is arriving at this point from the acetate pool. Since others have felt the block in fatty acid and cholesterol synthesis to be due to a TPNH defeciency, one would need look at a metabolic step following acetate activation, but preceding any TPNH dependent reactions. These requirements are met by the acetoacetic-S-CoA molecule.

The liver has an effecient beta-keto thiolase enzyme which converts acetoacetyl-S-CoA to free acetoacetic acid. It is this reaction which is felt to give rise to the condition of ketosis when acetyl-S-CoA is being formed in the system in increased amounts, such as during fatty acid oxidation following starvation or during the condition of diabetes. The observation that the amount of label incorporated from acetate is reduced 38 per cent in the ketone bodies of the diabetic (Table III) would support the hypothesis that less label is available for synthetic purposes in the diabetic than in the normal.

The fact that there is not a marked ketosis in the diabetic liver would also lend support to this hypothesis.

The finding that the diabetic liver slice preparation produced as much acetoacetate as did the normals is of interest in the face of the postulated decrease in acetyl-S-CoA pool size. However, this seemingly increased ketogenesis is more than off-set by the decreased formation of beta-hydroxy butyric acid resulting in a decreased total ketone body production. The decrease in C^{14} label incorporation into the total ketone bodies is similar in magnitude to the decrease seen in beta-hydroxy butyrate production. Assuming that acetoacetate is the precursor of beta-hydroxy butyrate, it then becomes necessary to explain these findings on the basis of decreased synthesis of aceto-acetate because of decreased supplies of acetyl-S-CoA. However, an additional cause of depressed beta-hydroxy butyrate formation may be the presence of a break in the reductive conversion of acetoacetate to beta-hydroxy butyrate by the DPN linked dehydrogenase enzyme as:

Acetoacetate + DPNH ⟷ beta-hydroxy Butyrate + DPN

That such a break may be due to decreased supplies of DPNH seems possible for in addition to the depressed glycolysis of the dia-betic we have shown that when the C^{14} acetate dosage to the liver slice is increased there is an increase in acetoacetate production (both in mass and in radioactivity), but there is not a correspond-ing increase in beta-hydroxy butyrate production. Maintenance of a C=O/C-OH ratio would appear to be controlled by factors other than the acetoacetate concentration.

Fig. 8: Reactions associated with acetate metabolism.

A number of reactions associated with acetate metabolism are shown, and it is apparent that the source of, and the fate of, acetate in metabolic systems is a complex phenomenon.

Figure 8 outlines a number of the reactions associated with the metabolism of acetate. When the sequence and the interdependence of these reactions are considered, it appears unwise to attribute to a single step in the scheme the complete control of the system.

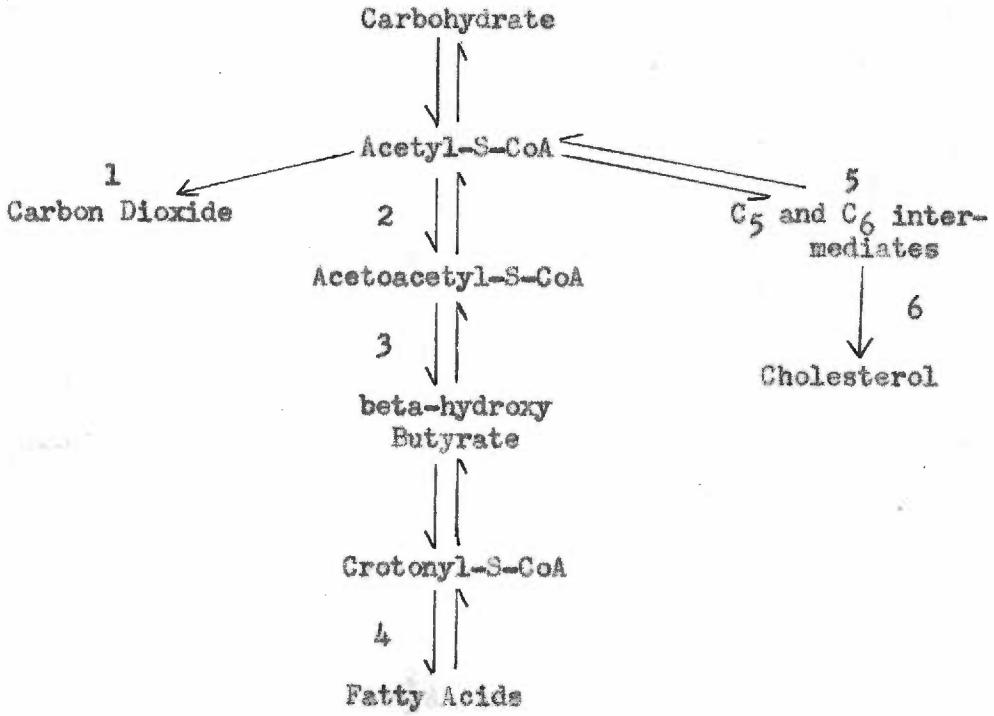


Fig. 8. General reaction sequences

Although the TPNH requirement of reaction 4 has been well documented (61,99), the presence of crotonyl-S-CoA is directly connected to the supply of beta-hydroxy butyryl-S-CoA which in turn is coupled through DPNH (reaction 3) and the dehydrogenase enzymes associated with the interconversion of the beta-keto/beta-hydroxy forms. The supplies of keto form are again dependent upon supplies of acetyl-S-CoA and to the diversion of this metabolite to

either oxidative or synthetic pathways. Although the phosphogluconate pathway does indeed produce TPNH (61,135,90) required at 4, 5, and 6, (Fig. 3), glycolysis and oxidative reactions of carbohydrate metabolism produce DPNH required at 3. In fact, stimulation of the citric acid cycle with the addition of isocitrate, as is customarily done, produces both DPNH and TPNH. These remarks are intended to direct attention to the need for consideration of the necessarily involved requirements of a complete physiologic system, requirements not always present in refined systems concerned chiefly with the enzymic characteristics of a particular reaction. In our diabetic slice preparations, the decreased beta-hydroxy butyrate formation may have been due to a decrease in DPNH availability with consequent slowing of reaction 3. In addition, the postulated decreased availability of acetyl-S-CoA may account for depression of reaction 2, which is of course, a key reaction prior to the need for the reductive intermediates DPNH and TPNH. The magnitude of depression of reactions 2 and 3 may be approximated as follows: there was found an 80 per cent to 90 per cent decrease in lipid labeling, but only some 40 per cent decrease in acetoacetate/beta-hydroxy butyrate labeling. It is possible then that depression in either reaction 2 and/or 3 may account for about half of the observed depression in fatty acid labeling. If acetyl-S-CoA concentration is limiting in reaction 2, then a similar decrease would be predicted for reaction sequences 5 and 6, a prediction verified by the experimental findings.

Series II: Metabolism of acetate, acetoacetate, butyrate and mevalonate.

As was shown in the previous section, the diabetic liver slice preparations from this laboratory show decreased fatty acid and cholesterol synthesis as measured by C^{14} incorporation from acetate-1-C^{14}. Also demonstrated was a reduction in the formation of ketone bodies. This decrease was shown to be due to a decrease in the beta-hydroxy butyrate fraction, for normal amounts of acetoacetate were found. Decreased conversion of the keto acid to the hydroxy acid may be due to DPNH deficit as detailed previously. However, our findings of an increase in $C^{14}O_2$ specific activity and a decrease in CO_2 production led us also to suggest a decreased acetyl pool size and decreased availability of acetyl-S-CoA for synthetic purposes. It is recognized that the interconversions of a number of the C_4 acids are most complex (49,65), and as yet not clearly understood. Studies of the present series may add additional information as to the reactions proceeding under conditions of the liver slice technique.

The fact that acetate, after conversion to acetyl-S-CoA, is an intermediate in multiple pathways, Fig. 7, somewhat limits its usefullness as a tracer of specific pathways. For this reason, in the present series, butyrate-1-C^{14} was chosen as a tracer to better define the fatty acid synthesizing pathway. As butyryl-S-CoA, this molecule is seemingly peculiar to fatty acid synthesis. It was felt that by elimination of the reactions preceding its formation, information would be obtained regarding its role as a precursor of fatty acids in both normal and diabetic preparations. Acetate-1-C^{14} and acetoacetate-3-C^{14} were also chosen because of their apparent common

roles in both fatty acid and cholesterol synthesis. Mevalonic acid-2-C^{14}, because of its unique property as a precursor of cholesterol, was utilized to better define the cholesterol synthesizing pathways. All four of these tracers were chosen in an attempt to better elucidate the sites of "metabolic lesions" in the diabetic.

Table V presents evidence that acetate, acetoacetate and butyrate are used to about the same extent for lipid synthesis in the liver slice system. The similarity between acetate and butyrate utilization is even closer, for in both cases the tracer C^{14} was found in all three metabolic fractions to the same extent. If the added butyrate, either free or as its CoASH derivative, could equilibrate directly with the cellular intermediates of fatty acid synthesis, then the fatty acid fraction should contain a disproportionate amount of the label, e.g. the ratio of incorporation into CO_2, fatty acids, and cholesterol would be altered for butyrate as compared to acetate. The essentially identical labeling from acetate and butyrate strongly suggests that the major part of butyrate carbon converted to CO_2, fatty acids, and cholesterol, enters these fractions via acetyl-S-CoA as an intermediate. Whether butyrate per se cannot enter the synthetic systems readily, due to a low dissociation of some form of this C_4 acid from the enzyme surface, or whether oxidation is the preferential pathway, is not demonstrable from the present data. That cell permeability is not limiting is suggested by the finding of the same amounts of acetate and butyrate being utilized. In the absence of selective labeling of the fatty acids, it is concluded that the fate of exogenous butyrate is catabolism to acetyl-S-CoA.

The observation that butyrate oxidation by the diabetic produced a CO_2 of higher specific acitivity than the normals indicates that the catabolic reactions from butyrate to acetate are not diminished in the diabetic liver slice as are the anabolic reactions. With the recent developments that have taken place since the inception of this work, showing that fatty acid synthesis and fatty acid breakdown are mediated by enzymes and enzyme systems qualitatively dissimilar and moreover geographically separated at the cellular level, it is not surprising then that these findings on butyrate production and use should appear to differ.

Table V also presents the finding that only one-sixth as much of the radioactivity of the acetoacetate-3-C^{14} as the acetate-1-C^{14} appeared in CO_2, while about as much of the acetoacetate label appeared in the lipid fractions as did the acetate label, strongly suggests that acetoacetate equilibrates with acetoacetyl intermediates of lipid synthesis either before or after activation.

Mevalonate-2-C^{14} was converted to CO_2, fatty acids, and cholesterol in a pattern quite different from the other intermediates. The major part of the mevalonate used appeared in the cholesterol fraction. The 5.8 per cent incorporation into the sterol fraction appears to be considerably lower than the figures usually given for mevalonate conversion to cholesterol by homogenates, but Gould and Popják (85) also found slices to be less active than homogenates in cholesterol synthesis.

The amounts of mevalonate-2-C^{14} appearing in the CO_2 and fatty acid fractions deserve further comment. If the C^{14} activity found

in the CO_2 and fatty acid fractions had been derived from a breakdown product of mevalonate metabolism such as acetyl-S-CoA, it would be expected that the CO_2 fraction would have a considerably greater amount of C^{14} than the fatty acid fraction. Since this was not the case, and since Popják (88) was unable to identify any labeled acetohydroxamate from his experiments on mevalonate metabolism, it is concluded that mevalonate is not converted to acetate or acetoacetate.

It is likely that the C^{14} activity found in the fatty acid fraction may be due to various higher acids (54,63,81,90), for as shown earlier, mevalonic acid per se does not appear in the fatty acid fraction to a significant degree.

The C^{14} activity found in the CO_2 fraction is not too different from that expected. In the conversion of mevalonate to sterol as outlined below, some 8.3 per cent of the starting C^{14} of the DL or 16.6 per cent (1/6) of the active form can be expected to appear as $C^{14}O_2$ if there is complete utilization of the isotope.

1. 6 DL mevalonate-2-C^{14} $\longrightarrow$ lanosterol-C^{14} + 6 CO_2
2. lanosterol-C^{14} $\longrightarrow$ cholesterol-C^{14} + 2 CO_2 + 1 $C^{14}O_2$

The 5.8 per cent incorporation of C^{14} into cholesterol represents the use of 0.0133 umoles of the active form (reaction 1 and 2). This is, however, only 5/6 of the true amount of mevalonate used because of the loss of $C^{14}O_2$ (reaction 2). Correction for this loss yields a figure of 0.0155 as the umoles of mevalonate utilized. It is expected that 1/6 of the C^{14} of the 0.0155 umoles of mevalonate i.e. 0.0026 umoles of $C^{14}O_2$, would be produced. Our finding that 0.0031

umoles (1.34 per cent of the starting 0.23 umoles) of $C^{14}O_2$ were produced indicates the presence of some 20 per cent more $C^{14}O_2$ than was expected. These results tend to agree with those of Popják who found "only a little more than may be expected" of mevalonate-C^{14} in CO_2 (88). It is not yet known, however, if this 20 per cent higher recovery of $C^{14}O_2$ is a significant deviation from the amount predicted by current concepts of mevalonate metabolism.

A comparison of the data of the control and the diabetic animals reveals that the previously reported defect in hepatic lipid synthesis in the diabetic (Series I), using acetate-1-C^{14} as tracer, is also demonstrable with both acetoacetate-3-C^{14} and butyrate-1-C^{14}. The metabolic defect associated with cholesterol synthesis would appear to be localized at reaction sequences E, Fig. 7, for mevalonate alone of the four substances was not hindered in conversion to sterol. The absence of a block in mevalonate conversion to cholesterol in the diabetic may be closely related to a similar finding as shown by Bucher (29) for fasted preparations. The block of acetate conversion to sterol may reside in the postulated decrease in acetate availability of the diabetic rat liver, and the decreased conversion of acetoacetate to cholesterol may reside in this same defect as well as in the suggested defect in availability of the reduced forms of coenzymes I and II (70,99). TPNH has been shown to enhance cholesterol synthesis in fractionated liver systems (17). If this requirement is present in the liver slice system, it would appear that both normal and diabetic preparations contained adequate amounts of TPNH, at least for tracer mevalonate incorporation into cholesterol.

Defective fatty acid synthesis, C and D in Fig. 7, may at least in part be due to a decrease in supplies of reducing coenzyme, for the diabetic slices showed decreased activity in the incorporation of acetoacetate into fatty acids. As suggested above, acetoacetate may equilibrate with fatty acid precursors and not require conversion to acetyl-S-CoA. If malonyl-S-CoA is the only required precursor of fatty acids, and if the prime condensation product is acetomalonyl-S-CoA which is then converted to butyryl-S-CoA, it is difficult to understand the selective participation of acetoacetate in fatty acid synthesis. That this participation is probably not due to complete conversion of the keto acid to acetyl-S-CoA has been suggested above. It may be possible that acetoacetate either free or activated can condense with malonyl-S-CoA. Whatever the mechanism of the keto acid utilization, it in turn is susceptible to a block in the diabetic condition.

Series III: In vivo metabolism of mevalonate.

The intraperitoneal injection of mevalonic acid-2-C^{14} into rats, with subsequent time course evaluations of cholesterol, fatty acid, blood, urine, and CO_2 radioactivity of various tissues has led to some very interesting findings. It was, of course, impossible to follow the turnover of biosynthesized cholesterol in all extrahepatic tissues, but preliminary studies gave a clue that the kidney might have an important role in the handling of body cholesterol.

This laboratory has previously separated the intact rat into four tissue fractions. The distribution of cholesterol in these four main fractions of rat tissue, namely, liver, gut, carcass, and skin has been followed and reported from this laboratory in previous

publications (119,140). The present investigation has shown the total carcass to be a metabolically heterogeneous system. The adrenal gland is shown to contain the highest concentration of color producing sterol. The brain contains by far the highest total amount of sterol. Liver, gut, skin, kidney, testes, lung, spleen, and heart contain lesser amounts of cholesterol per gram of tissue, but of this group, kidney contains the highest amount (Table VII). In contrast to the organ structures, muscle contains only small amounts of cholesterol. However, since the muscle mass is large, it does contribute a considerable amount to the total body cholesterol.

The information presented in Table VIII shows that 30 minutes after the intraperitoneal injection of mevalonic acid-2-C^{14}, 22.7 per cent of the label is found in cholesterol, 3 per cent is found in the saponifiable fraction, 23.3 per cent is in the urine, 5 per cent in the blood and 1.44 per cent in the CO_2 giving a total recovery of 55.4 per cent of the injected dose. In the 30-minute to 1 hour period, an additional 30 per cent of the dose is recovered in the urine, giving a total recovery of 86 per cent. It is apparent that within 30 minutes, the animal utilized the injected mevalonic acid. In other words, cholesterol synthesis from this precursor would appear to be complete after 30 minutes so that any major change in the activity of the tissue cholesterol radioactivity during the next few days would constitute transport and/or metabolism of sterol.

The radioactivity in the saponifiable fraction (acidic) was originally thought to consist of intermediates of cholesterol synthesis, perhaps farnesoic acid, but the maintenance of this activity in the total animal over the four hour period would tend to rule

this out. If the activity was present in obligate precursors of cholesterol, these should have diminished in amount of label remaining in the acidic fraction after the 30 minute time period due to conversion of these compounds into cholesterol. It can be concluded that the activity found constitutes some product(s) not on the direct cholesterol metabolic pathway and perhaps similar to the fatty acid described by Langdon and Olgilvie (63,81) or to the acidic fraction of Bucher (29). It is possible that the acidic fraction activity could arise from the breakdown of labeled cholesterol to bile salts which would be recovered in the saponifiable fraction. However, were this to happen, the gut should be increasing in its acidic component as the labeled compounds were excreted from the liver, but this was not the case. Also, one would not expect to find bile salts in the kidney.

It was found during the course of these studies that the urinary excretion of radioactivity is a good indication of a successful intraperitoneal injection of the mevalonate tracer. If the injection is made incorrectly, i.e. into the gut, there is a delayed urinary excretion of activity. The occasional animal which excreted less than 40 per cent of the labeled dose in the first hour, was not used for the present series because cholesterol labeling in these animals directly paralleled the faulty excretion of the dose up to a maximum of 23 per cent incorporation.

The radioactivity of the blood diminishes rapidly during the first 30 minutes, likely reflecting the combination of rapid tissue uptake and urinary clearance during the same period. The blood

activity falls to a value of 2.5 per cent during the subsequent 3-hour period. The activity in the blood during the first half hour probably is due chiefly to both D and L-MVA. A detailed study of the blood radioactivity was beyond the scope of the present investigation. The information reported in Table IX on the amount of label in the various fractions, represents the results of both synthesis and mobilization of sterol. The activity present in the liver declines at a rapid rate during the first 2-hours, the liver apparently losing approximately 1/2 of its newly formed cholesterol in this period. This loss is seen to be made up of at least two processes, the more rapid having a half life of about 1.5 hours (Fig. 5). It is not possible to determine if all of the cholesterol of the liver is this labile. It may well be that only a small pool of sterol is in this most rapid state of turnover. It is also possible that cholesterol synthesized from MVA may then be excreted without preliminary storage. The slower component of the liver sterol turnover curve has a half life of 7 hours (Fig. 5). This latter value is still less than the previously published half times determined in this laboratory. Using longer term time course studies (126), there was shown to be two components of decay, one with a half life of 0.63 days, and a slower component of 8.9 days. The two rapid components described in the present study were, of course, not detectable in the previous long term experiment. When the two studies are considered together, it is clear that metabolism of cholesterol in the liver must be described as the resultant of a minimum of at least four processes, having half times of 1.5, 7, 15 and 210 hours.

The gut fraction is seen to increase in labeled sterol during the 4-hour interval (Table IX). The plot of Fig. 6 shows a biphasic

type curve, the half life of the fast component being 1.3 hours and that of the slow component being 7.0 hours. It can be seen from the data of Table IX that in the 1- to 4- hour time interval, the liver fraction lost one half of its sterol radioactivity and that the gut fraction radioactivity doubled in amount. Considering the sizes and the sterol concentrations of these two tissues, it is not likely that the gut was the recipient of one half of the total liver sterol. It would seem more likely that at least a portion of the newly synthesized sterol was not in equilibrium with total liver sterol, and was in fact, selectively secreted from the liver. The 6.8 per cent of the tracer present in labeled sterol in the gut at the 15 minute time interval would seem to indicate appreciable synthesis from the labeled precursor. It has been shown in this laboratory that the use of the intraperitoneal route of tracer acetate administration resulted in gut tissue containing more of the label than from the intravenous route of dosage. As yet it is not clear if this is due to a local utilization of tracer before systemic distribution is effected or whether it is due to a selective distribution of the label to certain tissues.

The skin appears to synthesize a small amount of cholesterol during the initial time when mevalonic acid is available to it. However, no further increase or decrease is seen in the activity during the times studied. In previous studies (119), skin sterol activity increased in the first 4 days after injection. This slower response may not be detectable in the short time of the present experiments. The brain was quite inert as regards synthesis from mevalonic acid or in storage of newly formed cholesterol. Actually,

the activity given here for brain may represent residual blood activity not removed at section.

An interesting finding of the present study was that 60 per cent (Table X) of the label of the "carcass fraction" was contained in the kidneys. The other organs of the carcass fraction contributed little to the total labeling. The muscle mass was low in unit activity, but because of its total weight, it accounted for around 12 per cent of the total sterol radioactivity of the animal.

It is clear that the kidney is the carcass tissue which has the apparent maximum cholesterol synthesis from mevalonic acid in these rats; about 75 per cent of the labeled carcass cholesterol being found in the kidney at the end of 15 minutes. The activity of the sterol fraction remained essentially constant during the 4 hour period under investigation.

These findings on the kidney tissue should not be directly interpreted as representing an unduly high rate of synthesis of cholesterol. The intact kidney may collect and concentrate the labeled mevalonic acid dose and in this fashion increase the amount of label directed to kidney biosynthesis reactions. Approximately 35 per cent of the activity in the total acidic fraction is found in the kidney at the 1- and 2- hour time intervals. This also would indicate the increased availability of label to kidney tissue. Unfortunately, without knowledge of the specific activity of the precursor pools of biosynthesis, it is not possible to directly compare the rates of synthesis of various tissues. However, <u>in vitro</u> studies (next section) confirm the fact of active cholesterol and acid component labeling in kidney tissue from mevalonic acid-2-C^{14}.

From previous work in this laboratory (119), it can be calculated that 15.8, 35.2, 34.9, and 14.0 per cent of the labeled cholesterol was in liver, gut, carcass, and skin respectively 2 hours following injection of acetate-1-C^{14}. The present work lists values of 11.2, 13.2, 77.0, and 2.7 per cent for similar fractions labeled with mevalonic acid-2-C^{14}. It becomes evident that the labeling pattern of body cholesterol is grossly different when mevalonic acid-2-C^{14} is the precursor as compared to acetate-1-C^{14}.

Series IV: In vitro metabolism of kidney tissue.

Alloxan is known to be a general tissue poison as well as causing the destruction of the islet cells in the pancreas. Tassoni et al (107) have shown histologically in the hamster that 95 per cent of the beta cells of the pancreas have degenerated and been replaced by mononuclear cells 5 - 24 hours following injection of alloxan. These workers also found early kidney changes which were characteristic of hyperactivity and high pressure in the tubules, but were not degenerative, and in the liver, glycogen was severely diminished and nucleic acids were greatly reduced.

The experiments considered in the final series were designed to elucidate the response of kidney slices to various metabolic tracers. The purpose of these investigations were three-fold; first it was desired to follow the utilization of acetate-1-C^{14}, acetoacetate-3-C^{14}, and mevalonic acid-2-C^{14} by rat kidney slices; and secondly to compare these responses in normal and alloxan diabetic kidney tissue. The third area of interest was to compare the variations in response, if any, between liver and kidney alices, in normal and in diabetic tissue.

The kidney slice is seen to have a grossly different pattern of respiratory activity than that previously shown for the liver slice (Table I and XII). The amount of oxygen consumed by the kidney tissue and the amount of CO_2 produced is considerably higher than that observed for the liver per unit weight of tissue. The normal and diabetic kidney slice preparations are seen not to differ, a finding dissimilar to the liver, where a decreased oxygen consumption or CO_2 production were observed for the diabetic.

The lipids of the kidney were determined and the mg of fatty acid and cholesterol found per gram of tissue are listed in Table XIII. The finding of similar amounts of lipid in the normal and diabetic kidney compare well with the findings for liver tissue (Table II), for here, alloxan diabetics showed no change in the amount of lipid contained in the liver tissue. It is interesting, however, that the amounts of fatty acid and cholesterol present in the kidney of the rat is higher than that in the liver tissue. It is also seen that the 200 gram animals used in Series IV contained the same amount of cholesterol per gram of tissue as did the 80 - 100 gram animals used in Series III (Table VII). The fatty acid levels were less in the smaller animals, possibly indicating a more rapid growth rate with less storage of fat.

The incorporation of acetate-1-C^{14} into CO_2, fatty acids and cholesterol of the kidney slice showed marked differences when compared to liver tissue. Approximately half the dose of acetate-1-C^{14} presented to kidney tissue was oxidized to $C^{14}O_2$ in a one hour period (Table XIV). This is in contrast to the 8 per cent figure found for liver tissue. Masoro et al (77) have indicated that the kidney was the most active in oxidizing ethanol to CO_2 when compared to liver,

lung, diaphragm, and brain. It is difficult and hazardous to compare rates of reactions in different tissues when the precursor pool sizes are unknown. However, the comparison of alterations in normal versus diabetic of the liver and kidney tissue may be valid. Whereas the liver tissue of the diabetic oxidized more of the acetate-1-C^{14} to $C^{14}O_2$ than did the normal, the kidney tissue shows a slightly decreased conversion of acetate-1-C^{14} to $C^{14}O_2$. This finding of a decreased $C^{14}O_2$ may be related to an altered activation or permeability reaction in the diabetic kidney slice.

The utilization of acetate-1-C^{14} by kidney tissue for fatty acid and cholesterol synthesis is listed in Table XIV. An interesting finding is that the kidney and liver tissues incorporate the same amount of the acetate dose into fatty acids per unit weight of tissue. The liver would appear to incorporate more of the labeled acetate to cholesterol than does the kidney, however, as mentioned previously, comparing two different tissues as to rates of synthesis from isotope data should be done with caution when precursor pools and related reaction rates cannot be directly measured. The kidney has considerably more cholesterol per gram of tissue than does the liver, which might influence the rate of synthesis. Westerfeld and Shulman (142) compared the utilization of acetate-1-C^{14} by liver and kidney slices with the conclusion that the liver and kidney both carry out fatty acid and cholesterol synthesis from the C_2 unit, but that the liver converts more of the label into these products than does the kidney.

The kidney tissue shows no decrease in lipogenesis in the diabetic as was observed for liver tissue. The alloxan diabetes does not appear to alter the cellular components necessary for fat and cholesterol synthesis in the kidney. It would also appear that the

citric acid cycle functioning is adequate. The maintainance of liver lipid levels in the diabetic where fatty acid and cholesterol synthesis were reduced was felt to be due to the increased mobilization of depot lipids which occurs in diabetes. Since the levels of lipid in the diabetic kidney are not altered, nor is there a decreased synthesis, it would seem logical to infer that the mobilization of depot lipid does not influence the levels in the kidney as it does in the liver.

Acetoacetate-3-C^{14} was used in an attempt to ascertain the role of this molecule in the kidney in relation to oxidation and lipid synthesis. Table XVI lists the per cent incorporation of acetoacetate-3-C^{14} into CO_2, fatty acids and cholesterol with a comparison between normal and diabetic preparations. The incorporation of this C^{14} acid into CO_2 is seen to bear a striking similarity to that observed for acetate-1-C^{14}; approximately one-half the dose appearing in the respired CO_2 in 1 hour. This observation of 50 per cent conversion of acetoacetate to CO_2 by kidney tissue is considerably different that the response of the liver to this molecule. One reason for this difference resides in the apparent ease of activation of acetoacetate by the kidney compared with the small activity of the activating enzyme present in liver. The kidney tissue of the diabetic shows no altered ability to convert acetoacetate to CO_2. Weinhouse (137) has shown that acetoacetate formation is lower in the kidney slice than in the liver, but that its rate of oxidation is far higher. In this same work he concluded that extra hepatic tissues do indeed produce acetoacetic acid, but that its rate of utilization is great enough to eliminate any accumulation of ketone bodies. This is

contrary to the situation prevailing in the liver, for here formation exceeds utilization.

The utilization of acetoacetate-3-C^{14} for synthetic pathways by the kidney tissue (Table XV) is again similar to that found for acetate-1-C^{14}. In contrast to the large difference in $C^{14}O_2$ production by the liver and kidney from acetoacetate, both tissues incorporate the same amount of label into the fatty acid fraction. The incorporation of label into cholesterol is shown to be higher for liver than for kidney tissue as was also true when acetate was the tracer. In all of the parameters of measurement, there is seen to be little difference in utilization of acetoacetate-3-C^{14} and acetate-1-C^{14} by kidney slices. The diabetic kidney slice is again shown to have an unaltered metabolic pattern when compared to normal tissue. It appears likely that the initial reactions of acetoacetate in the kidney slice are activation and cleavage to form acetate or acetyl-S-CoA, which then enters the machinery of the cell. Since acetoacetate conversion to acetate and hence to CO_2 in the diabetic is not altered it is difficult to explain the decrease observed in $C^{14}O_2$ production from acetate-1-C^{14}. However, the fact that acetate-1-C^{14} is added exogenously and the acetate arising from acetoacetate is endogenous may be a partial explanation of these findings and related to intra-cellular location and/or orientation.

The preferential pathway of mevalonic acid conversion to cholesterol in liver tissue and yeast is now certain. It was felt of interest to determine if kidney tissue would utilize mevalonic acid in a manner similar to the liver, and if so, whether or not the condition of diabetes would alter these reaction sequences.

The three areas investigated were as before, $C^{14}O_2$ production, fatty acid and cholesterol synthesis. The per cent of mevalonic acid-2-C^{14} converted to kidney cholesterol is not significantly altered in the normal as compared to the diabetic kidney slice (Table XVI). Also of interest is the fact that the kidney tissue and liver tissue show the same per cent incorporation of this precursor. The large labelling of cholesterol noted in the in vivo studies (Series III) would appear to be due to the ability of the kidney to concentrate the dose of mevalonic acid and so increase cholesterol labeling. It may also be due to its ability to maintain its labeled cholesterol content during a four hour period.

The only known origin of $C^{14}O_2$ from metabolic reactions involving mevalonic acid-2-C^{14}, at the writing of this thesis, is in the demethylation of lanosterol formed from this tracer. Therefore, if cholesterol labeling is the same in the normal and diabetic kidney tissue, and is also the same in kidney and liver tissue, one would anticipate that the per cent conversion to $C^{14}O_2$ would also be the same. This hypothesis is verified by the experimental findings.

Fatty acid labeling is also observed to be the same in the normal and diabetic kidney slice from mevalonic acid-2-C^{14}, and when compared to liver tissue also appears to be similar. It is concluded, therefore, that the conversion of mevalonic acid-2-C^{14} to $C^{14}O_2$, fatty acids, and cholesterol is not altered by experimental alloxan diabetes in either the rat kidney or liver slice.

The finding of a normal lipogenic response of the kidney tissue was unexpected. That experimental diabetes would allow conditions

suitable for decreased synthesis of fatty acids and cholesterol in the liver and not in the kidney seems to be somewhat at variance with our present concept of the controlling factors associated with this pathological condition. However, similar results have been obtained for heart tissue utilizing acetate-1-C^{14} and acetoacetate-3-C^{14} as the tracer compounds (76). Teleologically, one might infer from this information that the kidney and heart tissue have preferential access to the nutrients being made available from the blood supply. It is also of interest that these two organs both utilize acetoacetic acid very readily. The liver, on the other hand, would appear to be a tissue which is supplying the blood stream with products such as acetoacetic acid and during the condition of experimental diabetes is in a more catabolic state than an anabolic one with the result that decreased lipogenesis and cholesterogenesis are manifested.

A concluding statement.

The relation of the present studies to human welfare may tend to be obscured by the many details presented above. That these investigations may in fact have a bearing upon human physiology seems likely in view of changing concepts of disease. Atherosclerosis is no longer considered a product of aging, per se. The overall atherogenic response is felt to be conditioned by metabolic factors, both local and systemic. Some investigators have gone so far as to state that atherosclerosis is a disease of lipid metabolism and transport and cholesterol metabolism has certainly been associated with the condition of atherosclerosis. It has been reported that arteriosclerotic disease has become the chief cause of death in diabetes,

accounting for 75 per cent of all the deaths. Cardiovascular disease appears to be one of the clinical areas where the application of biochemical and physiological techniques may prove fruitfull in the early diagnosis and treatment of a disease process. It is hoped that the investigations reported in the preceding thesis may add a small portion to the existing knowledge of the dynamics of lipid metabolism in the normal and experimentally prepared condition of alloxan diabetes.

CHAPTER V

CONCLUSIONS

Series I.

1. The amount of CO_2 produced and of O_2 utilized per hour per gram of liver slice was found to be decreased in the diabetic. The incorporation of acetate-1-C^{14} into CO_2 was increased by some 70 per cent in the diabetic.

2. The amount of fatty acid per gram of liver slice was the same for normal and diabetic, but the incorporation of acetate-1-C^{14} into fatty acids was reduced 83 per cent in the diabetic.

3. The amount of non-saponifiable matter (cholesterol) per gram of liver was the same for normal and diabetic, but the amount of beta-hydroxy butyric acid formed by the diabetic was decreased. The incorporation of acetate-1-C^{14} into the C_4 fraction was decreased 38 per cent in the diabetic.

4. A decreased availability of acetate for CO_2 and lipid formation and a decreased "acetate pool" size is indicated for these diabetic preparations.

5. The decreased flux of acetate to lipids in the diabetic slice is shown to be able to account for about half of the decrease observed in lipid synthesis.

Series II.

1. The four tracer molecules, acetate-1-C^{14}, acetoacetate-3-C^{14}, butyrate-1-C^{14}, and mevalonate-2-C^{14} have been used in liver slice

studies of CO_2, fatty acid, and cholesterol formation.

2. The data suggest that butyrate is first converted to acetyl-S-CoA before labeling of the three fractions occurs. Acetoacetate is seen to equilibrate with acetoacetate precursors of fatty acid and cholesterol synthesis, seemingly without complete conversion to acetyl-S-CoA.

3. Mevalonate is shown to label cholesterol selectively, the amount of C^{14} of mevalonate-2-C^{14} found in CO_2 being about 20 per cent higher than expected.

4. The diabetic rat liver slice preparation is shown to have metabolic lesions that influence acetate, acetoacetate and butyrate metabolism. Mevalonate is handled by diabetic tissue in the same quantitative way as it is by normal tissue.

Series III.

1. Mevalonate-2-C^{14} was given intraperitoneally to young white rats for the purpose of following cholesterol synthesis and turnover. Within 30 minutes of injection, the total body cholesterol fraction contained 23.8 per cent of the injected DL tracer or 47.6 per cent of the active isomer. Time course data suggest that at the 30 minute time after injection, utilization of the tracer mevalonic acid was essentially complete.

2. Some 3 per cent of the dose was recovered in the acidic fraction. Since this amount remained quite constant throughout four hours, it is concluded that acidic fraction activity represents metabolites not on the direct route to cholesterol.

3. Urinary radioactivity accounted for slightly more than one half of the dose and presumably represents excretion of the biologically inactive isomer.

4. The total recovery of label was almost 90 per cent within the first hour post injection.

5. Liver and gut cholesterol turnover are suggested to be reciprocally related, the processes having half times of about 1.5 and 7 hours.

6. Kidney tissue accounted for the greater part of the label that was utilized by "carcass tissue". The high degree of kidney sterol labeling may be related to active cholesterogenesis and/or to a magnified labeling of a less significant rate of synthesis due to the concentration of tracer mevalonic acid-2-C^{14} by kidney tissue.

Series IV.

1. The kidney slice was found to have a different pattern of respiratory activity than that shown previously for liver tissue. The O_2 consumption and the CO_2 production wereshown to be considerably higher in the kidney tissue than in the liver.

2. The normal and diabetic kidney slice do not differ in the quantities of respiratory gases either utilized or produced.

3. Acetate-1-C^{14} is oxidized to $C^{14}O_2$ very rapidly in kidney tissue, 50 per cent of the dose being converted per hour. There was a slight decrease in the diabetics ability to oxidize the labeled precursor.

4. The utilization of acetate-1-C^{14} for fatty acid synthesis and cholesterol synthesis is seen not to be impaired by the condition of experimental diabetes. This observation is dissimilar to that observed for liver tissue.

5. Acetoacetate-3-C^{14} is also readily oxidized to $C^{14}O_2$ by normal and diabetic kidney slices; there being no difference between the two preparations. Acetoacetate oxidation is shown to be similar to that obtained with acetate. The conversion of acetoacetate to acetate prior to oxidation and/or synthesis is suggested.

6. The incorporation of acetoacetate-3-C^{14} into fatty acids and cholesterol is the same for both fractions as from acetate, and the alloxan diabetic kidney slice showed the same metabolic responses as did the normal.

7. Mevalonic acid-2-C^{14} was converted to $C^{14}O_2$, fatty acids, and cholesterol in a similar manner for both normal and diabetic kidney tissue.

8. When compared to liver tissue, the kidney is seen to convert less label to the cholesterol fraction when the precursors are acetate-1-C^{14} or acetoacetate-3-C^{14}. However, mevalonic acid-2-C^{14} is shown to be incorporated in similar amounts by liver and kidney tissue.

9. The condition of experimental alloxan diabetes does not appear to alter the lipogenic or cholesterogenic responses in the kidney tissue as is observed for liver.

BIBLIOGRAPHY

1. Beatty, C. H., West, E. S., & Bocek, R. M. Effect of succinate, fumarate, and oxalacetate on ketone body production by liver slices from non-diabetic and diabetic rats. J. Biol. Chem., 1958, 230, 725.

2. Berg, P. Acyl adenylates: An enzymatic mechanism of acetate activation. J. Biol. Chem., 1956, 222, 991.

3. Berg, P. Role of magnesium in acetyl coenzyme A formation by acetothiokinase. Science, 1959, 129, 895.

4. Berg, P. Acyl adenylates: The synthesis and properties of adenyl acetate. J. Biol. Chem., 1956, 222, 1015.

5. Bessman, S., & Anderson, M. Estimation of citric acid and ketone bodies by the salicylaldehyde-acetone reaction. Federation Proc., 1957, 16, 154.

6. Block, K., & Rittenberg, D. The biological formation of cholesterol from acetic acid. J. Biol. Chem., 1942, 143, 297.

7. Block, K., & Rittenberg, D. On the utilization of acetic acid for cholesterol formation. J. Biol. Chem., 1959, 145, 625.

8. Block, K., Borek, E., & Rittenberg, D. Synthesis of cholesterol in surviving liver. J. Biol. Chem., 1946, 162, 441.

9. Block, K. Recent progress in hormone research, 6, 111. New York: Academic Press , 1951.

10. Block, K., & Langdon, R. G. The biosynthesis of squalene. J. Biol. Chem., 1953, 200, 129.

11. Block, K., & Clayton, R. B. Biological synthesis of lanosterol and agnosterol. J. Biol. Chem., 1956, 218, 305.

12. Block, K., Schneider, P. B., & Clayton, R. B. Synthesis of lanosterol in vivo. J. Biol. Chem., 1957, 224, 175.

13. Block, K., & Tchen, T. T. On the conversion of squalene to lanosterol in vitro. J. Biol. Chem., 1957, 226, 921.

14. Block, K., & Tchen, T. T. On the mechanism of enzymatic cyclization of squalene. J. Biol. Chem., 1957, 226, 931.

15. Block, K., Olson, J. A., Jr., & Lindberg, M. On the demethylation of lanosterol to cholesterol. J. Biol. Chem., 1957, 226, 941.

16. Block, K., & Gautschi, F. On the structure of an intermediate in the biological demethylation of lanosterol. J. Am. Chem. Soc., 1957, 79, 684.

17. Block, K., & Tchen, T. T. On the conversion of squalene to lanosterol in vitro. J. Biol. Chem., 1957, 226, 921.

18. Block, K., & Johnston, J. P. In vitro conversion of zymosterol and dehydrozymosterol to cholesterol. J. Am. Chem. Soc., 1957, 79, 1145.

19. Block, K., & Gautschi, F. Synthesis of isomeric 4, 4-dimethyl-cholestenols and identification of a lanosterol metabolite. J. Biol. Chem., 1958, 233, 1343.

20. Block, K., Chaykin, S., Low, J., Phillips, A. N., & Tchen, T. T. Phosphorylated intermediates in the synthesis of squalene. Proc. Natl' Acad. Sci. U. S., 1958, 44, 998.

21. Block, K., Chaykin, S., & Phillips, A. N. Phosphorylated intermediates in squalene synthesis from mevalonic acid. Federation Proc., 1959, 18, 193.

22. Bonner, J., & Arreguin, B. Radioactive beta-hydroxy isovaleric acid in rat liver. J. Am. Chem. Soc. 1955, 77, 1295.

23. Bonner, J., & Millard, A. Z. Acetate activation and acetoacetate formation in plant systems. Arch. Biochem. Biophys., 1954, 49, 343.

24. Brady, R. O., Mamoon, A., & Stadtman, E. R. The effects of citrate and coenzyme A on fatty acid metabolism. J. Biol. Chem., 1956, 222, 795.

25. Brady, R. O. The enzymatic synthesis of sphingosine. J. Biol. Chem., 1958, 233, 1072.

26. Brady, R. O. The enzymatic synthesis of fatty acids by aldol condensation. Proc. Natl. Acad. Sci. U.S., 1958, 44, 993.

27. Brady, R. O. The enzymatic carboxylation of acetyl-CoA. J. Am. Chem. Soc., 1959, 81, 752.

28. Bruesch, F. L. The biochemistry of fatty acid catabolism. Adv. in Enzym., 1948, 8, 404.

29. Bucher, N. L., McGarrahan, I., Gould, E., & Loud, A. V. Cholesterol biosynthesis in preparations of liver from normal, fasting, X-irradiated, cholesterol-fed, triton, or 4'-cholesten-3-one treated rats. J. Biol. Chem., 1959, 234, 262.

30. Burton, K. The free energy change associated with the hydrolysis of the thiol ester bond of acetyl coenzyme A. Biochem. J., 1955, 59, 44.

31. Chaikoff, I. L., Masoro, E. J., Chernick, S. S., & Felts, J. M. Previous nutritional state and glucose conversion to fatty acids in liver slices. J. Biol. Chem., 1950, 185, 845.

32. Chaikoff, I. L., Baker, N., & Shusdeck, A. Effect of fructose on lipogenesis from lactate and acetate in diabetic liver. J. Biol. Chem., 1952, 194, 435.

33. Chaikoff, I. L., Lyon, I., & Masri, M. S. Fasting and hepatic lipogenesis from C^{14}-acetate. J. Biol. Chem., 1952, 196, 25.

34. Chaikoff, I. L., & Hotta, S. Cholesterol synthesis from acetate in the diabetic liver. J. Biol. Chem., 1952, 198, 895.

35. Chevallier, F. Thèse présentées a la faculté des sciences de l' universite de paris pour obtenir le grade de Docteur es Sciences Naturelles., 1 re Thèse - Concertion nouvelle concernant l'état dynamique du cholestérol chez le rat. 2e Thèse - Propositions données par la Faculté., 1955.

36. Cornforth, J. W. Biosynthesis of fatty acids and cholesterol considered as chemical processes. J. Lipid Research, 1959, 1, 3.

37. Creamer, R. M. Modified spectronic 20 having high stability. Anal. Chem., 1957, 29, 1722.

38. Eisenberg, M. A. The tricarboxylic acid cycle in Rhodospirillum rubrum. J. Biol. Chem., 1953, 203, 815.

39. Elwood, J. C. Thiol acyls and acetate metabolism. Unpublished Master's dissertation, Univ. of Oregon Medical School, 1958.

40. Hellman, L., Rosenfeld, R. S., & Gallagher, T. F. Cholesterol synthesis from C^{14}-acetate in man. J. Clin. Invest., 1954, 33, 142.

41. Gould, R. G. Lipid metabolism in atherosclerosis. Am. J. Med., 1951, 11, 209.

42. Green, D. E., Dewan, J. G., & Leloir, L. F. The beta-hydroxy butyric dehydrogenase of animal tissues. Biochem. J., 1937, 31, 934.

43. Green, D. E., Crane, F. L., Mii, S., Hauge, J. E., & Beinert, H. On the mechanism of dehydrogenation of fatty acyl derivatives of CoA. 1. The general fatty acyl coenzyme A dehydrogenase. J. Biol. Chem., 1951, 218, 701.

44. Green, D. E., Beinert, H., Hale, P., Hift, H., Koff, R. W., & Ramakrishman, J. The acetate activating enzyme system of heart muscle. J. Biol. Chem., 1953, 203, 35.

45. Green, D. E., Goldman, D. S., & Beinert, H. The acetoacetic acid activation and cleavage enzyme system. J. Biol. Chem., 1953, 202, 137.

46. Green, D. E. Fatty acid oxidation in soluble systems of animal tissues. Biol. Reviews, 1954, 29, 330.

47. Green, D. E. Oxidation and synthesis of fatty acid in soluble enzymes system of animal tissues. Clin. Chem., 1955, 1, 53.

48. Green, D. E. The synthesis of fat. Scientific American, 1960, 202, 46.

49. Greenberg, D. M., & Adamson, L. F. The significance of certain carboxylic acids as intermediates in the biosynthesis of cholesterol. Biochim. Biophys. Acta, 1957, 23, 472.

50. Gurin, S., & Brady, R. O. Biosynthesis of labeled fatty acids and cholesterol in experimental diabetes. J. Biol. Chem., 1950, 187, 589.

51. Gurin, S., & Rabinowitz, J. L. Biosynthesis of cholesterol from beta-hydroxy beta methyl glutaric acid by extracts of liver. J. Biol. Chem., 1954, 208, 307.

52. Gurin, S., & Rabinowtiz, J. The biosynthesis of radioactive cholesterol, beta-methyl glutaconic acid and beta-methyl crotonic acid by aqueous extracts of liver. J. Am. Chem. Soc., 1954, 76, 5168.

53. Gurin, S., Shaw, W. N., & Dituri, F. Lipogenesis in particle-free extracts of rat liver. II. Experimental diabetes. J. Biol. Chem., 1957, 226, 417.

54. Gurin, S., Dituri, F., Rabinowitz, J. L., & Hullin, R. P. Precursors of squalene and cholesterol. J. Biol. Chem., 1957, 229, 825.

55. Hile, P. The acetate activity enzyme of beef heart. J. Biol. Chem., 1954, 206, 671.

56. Isler, O., Ruegg, J., Wursch, J., Gey, K. F., & Pletscher, A. Zur biosynthese des cholesterins aus beta,gamma-dihydroxy-beta-methyl-valeriansoure. Helv., Chemica Acta, 1957, 40, 2369.

57. Johnson, L. N. Lipid techniques and acetate dosage studies. Unpublished master's dissertation, Univ. of Oregon Medical School, 1958.

58. Kornberg, H., & Pricer, W. E., Jr. Enzymatic synthesis of the coenzyme A. Derivatives of long chain fatty acids. J. Biol. Chem., 1953, 204, 329.

59. Krebs, H. A., & Henseleit, K. Untersuchungen uber die harnstoff-bildung in tierkorper. Zeit. fur Physiol. Chemie, 1932, 210, 33.

60. Krebs, H. A., & Eggleston, J. Metabolism of acetoacetate in animal tissues. Biochem. J., 1945, 39, 408.

61. Langdon, R. G. The biosynthesis of fatty acids in cell-free preparations of mammalian liver. In I. H. Page (Ed.) Chemistry of lipides as related to atherosclerosis. Springfield, Ill.: Charles C. Thomas, 1958, pp. 291-309.

62. Langdon, R. G. The biosynthesis of fatty acid in rat liver. J. Biol. Chem., 1957, 226, 615.

63. Langdon, R. G., & Ogilvie, J. W. The enzymatic conversion of mevalonic acid-2-C^{14} to an olefinic acid. J. Am. Chem. Soc., 1959, 81, 754.

64. Lehninger, A. L., & Greville, G. D. The enzymic oxidation of D- and L-beta-hydroxy butyrate. Biochem. Biophys. Acta, 1953, 12, 188.

65. Lehninger, A. L. Oxidation of fatty acids. In I. H. Page (Ed) Chemistry of lipides as related to atherosclerosis. Springfield, Ill.: Charles C. Thomas, 1958, pp. 265-290.

66. Lipmann, F. Enzymatic synthesis of acetyl phosphate. J. Biol. Chem., 1944, 155, 55.

67. Lipmann, F., Stadtman, E. R., & Novelli, G. D. Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem., 1951, 191, 365.

68. Lipmann, F., & Chow, T. C. Extract separation of acetyl transfer enzymes in pigeon liver. J. Biol. Chem., 1952, 196, 89.

69. Lynen, F., Reichert, E., & Rueff, L. Biological degradation of acetate acid, isolation and chemical nature of activated acetic acid. Ann. Chem., 1951, 374, 1.

70. Lynen, F., Wessely, L., Wieland, O., & Rueff, L. Beta oxidation of fatty acids. Angew. Chem., 1952, 64, 687.

71. Lynen, F., & Seubert, W. Enzymes of the fatty acid cycle. II. Ethylene reductase. J. Am. Chem. Soc., 1953, 75, 2787.

72. Lynen, F., Knappe, J., Eggerer, H., Henning, U., & Aranoff, B. W. Biosynthesis of terpenes. Federation Proc., 1959, 19, 1099.

73. Lynen, F. New aspects of acetate incorporation into isoprenoid precursors. In G. E. W. Wolstenholme and M. O'Connor (Eds) Biosynthesis of terpenes and sterols. Boston, Mass.: Little, Brown and Company, 1959, pp. 95-118.

74. Lynen, F., Agranoff, B. W., Eggerer, H., & Henning, A. Isopentenol pyrophosphate isomerase. J. Am. Chem. Soc., 1959, 81, 1254.

75. Lynen, F., Eggerer, H., Henning, U., & Kessel, J. Anger. Chem., 1959, 70, 738.

76. Marcó, A. E. Studies on the metabolism of acetate and acetoacetate. Unpublished master's dissertation, Univ. of Oregon Medical School, 1959.

77. Masaro, E. J., Abramonitch, H., & Birchard, J. R. Metabolism of C^{14} ethanol by surviving rat tissues. Am. J. Physiol., 1953, 173 37,

78. Ochoa, S., & Stern, J. R. Enzymatic synthesis of citric acid. 1. Synthesis with soluble enzymes. J. Biol. Chem., 1951, 191, 161.

79. Ochoa, S.,Stern, J. R., & Schneider, M. C. Enzymatic synthesis of citric acid. II. Crystalline condensing enzyme. J. Biol. Chem., 1951, 193, 691.

80. Ochoa, S., Stern, J. R., Shapiro, B., & Stadtman, E. R. Enzymatic synthesis of citric acid. III. Reversibility and mechanism. J. Biol. Chem., 1951, 193, 703.

81. Ogilvie, J. W. The degradation and structure of a terpenoid acid enzymatically synthesized from mevalonic acid. J. Am. Chem. Soc., 1959, 81, 756.

82. Popjak, G. Chemistry, biochemistry and isotopic tracer technique. Lectures, monographes, and reports, #2. The Royal Inst. of Chem. Cambridge, England: W. Heffer and Sons, Ltd., 1955, p. 59.

83. Popjak, G., & Tietz, A. Biosynthesis of fatty acids in cell-free preparations. 3. Coenzyme A dependent reactions in a soluble system of mammory gland. Biochem. J., 1955, 60, 155.

84. Popjak, G., Beinert, H., Green, D. E., Hele, P., Hoffmann-Ostenhof, O., Lynen, F., Ochoa, S. & Ruyssen, R. Nomenclature of the enzymes involved in fatty acid metabolism. Science, 1956, 124, 614.

85. Popjak, G., & Gould, R. G. Biosynthesis of cholesterol in vivo and in vitro from DL-beta-hydroxy-beta-methyl-gamma-(2-^{14}C)-valero lactone. Biochem. J., 1957, 66, 51P.

86. Popjak, G., Cornforth, J. W., Cornforth, R. H., & Gore, I. Y. Studies on the biosynthesis of cholesterol. 5. Biosynthesis of squalene from DL-3-hydroxy-3-methyl-(2-C^{14}) pentano-5-lactone. Biochem. J., 1958, 69, 146.

87. Popjak, G., Gosselin, L., Gore, I., & Gould, Y. Coenzyme requirements of liver enzymes for synthesis of squalene and of sterol from DL-3-hydroxy-3-methyl-(2-^{14}C)-pentano-5-lactone. Biochem. J., 1958, 69, 238.

88. Popjak, G. Biosynthesis of cholesterol and related substances. In Annual Review of Biochemistry, 1958, 27, 533.

89. Popjak, G., & Levy, H. R. Liver Mevalonic Kinase. Biochem. J. 1959, 72, 35P.

90. Popjak, G. Biosynthesis of squalene and of cholesterol from mevalonic acid. In G. E. W. Wolstenholme and M. O'Connor (Eds) Biosynthesis of terpenes and sterols. Boston, Mass.: Little, Brown and Company, 1959, 148-172.

91. Rabinowitz, J. The biosynthesis of radioactive senecioic acid (beta-methyl crotonic acid) in particle free extracts of rat liver. J. Am. Chem. Soc., 1954, 3037, 76.

92. Rabinowitz, J. The biosynthesis of radioactive beta-hydroxy isovaleric acid in rat liver. J. Am. Chem. Soc., 1955, 77, 1295.

93. Rudney, H. The synthesis of beta-hydroxy-beta-methyl glutaric acid in rat liver homogenates. J. Am. Chem. Soc., 1954, 76, 2595.

94. Rudney, H. The synthesis of beta beta dimethyl acrylic acid in rat liver homogenate. J. Am. Chem. Soc., 1955, 77, 1698.

95. Rudney, H. The biosynthesis of beta-hydroxy-beta-methyl-glutaryl coenzyme A and its conversion to mevalonic acid. In G. E. W. Wolstenholme and M. O'Connor (Eds) Biosynthesis of terpenes and sterols. Boston, Mass.: Little, Brown and Company, 1959, 75-94.

96. Schwenk, E., Alexander, G. J., Fish, C. A., & Stoudt, T. H. Federation Proc., 1955, 14, 752. Biosynthesis of substances which accompany cholesterol.

97. Scow, R. O., Chernick, S. S. Early Effects of "Total" pancreatectomy on fat metabolism in the rat. Am. J. Physiol., 1959, 196, 125.

98. Scow, R. O., Chernick, S. S., Guarco, B. A. Ketogenic action of pituitary and adrenal hormones in pancreatectomized rats. Diabetes, 1959, 8, 132.

99. Siperstein, M., Role of glycolysis in fatty acid and cholesterol synthesis in normal and diabetic rats. Science, 1957, 126, 1012.

100. Siperstein, M. Glycolytic pathways their relation to the synthesis of cholesterol and fatty acids. Diabetes, 1958, 7, 181.

101. Skeggs, H. R., Wright, L. D., Folkers, K., & Aldrich, P. E. Discovery and elucidation of mevalonic acid. In G. E. W. Wolstenholme and M. O'Connor (Eds) Biosynthesis of terpenes and sterols. Boston, Mass.: Little, Brown and Company, 1959, 20-45.

102. Somogyi, M. Determination of blood sugar. J. Biol. Chem., 1945, 160, 69.

103. Stern, J. R., & Del Campillo, A. Enzymatic reaction of crotonyl coenzyme A. J. Am. Chem. Soc., 1953, 75, 2277.

104. Stern, J. R., & Del Campillo, A., Enzymes of Fatty acid metabolism II. Properties of crystalline crotonase. J. Biol. Chem., 1956, 12, 188.

105. Stern, J. R., Coon, M., & Del Campillo, A. Enzymes of fatty acid metabolism III. Breakdown and synthesis of beta-keto fatty acids. J. Biol. Chem., 1956, 221, 1.

106. Stockes, W. M., Hickey, F. C., & Fish, W. A. Sterol metabolism I. The occurrence of desmosterol (24-dehydrocholesterol) in rat skin and its conversion in vivo to cholesterol. J. Biol. Chem., 1958, 232, 347.

107. Tassoni, J. P., House, E. L., & Nace, P. F. Alloxan Diabetes in the hamster; organ changes during the first day. Endo., 1956, 59, 433.

108. Tavormina, P. A., Gibbs, M. H., Huff, J. W. The utilization of beta-hydroxy beta-methyl gamma-valerolactone in cholesterol biosynthesis. J. Am. Chem. Soc., 1956, 78, 4498.

109. Tavormina, P. A., Gibbs, M. H. The metabolism of beta,gamma dihydroxy-beta-methyl valeric acid by liver homogenates. J. Am. Chem. Soc., 1956, 78, 6210.

110. Tchen, T. T. Mevalonic kinase; purification and properties. J. Biol. Chem., 1958, 233, 1100.

111. Teng, Ching Tseng. Studies on carbohydrate metabolism in rat kidney slices II. Effect of alloxan diabetes and insulin administration on glucose uptake and glucose formation. Arch. BioC. BioPhys., 1954, 48, 415.

112. Tepperman, J., Tepperman, H. M. Effects of antecedent food intake pattern on hepatic lipogenesis. Am. J. Physiol., 1958, 193, 55.

113. Van Bruggen, J. T., Claycomb, C. K., Hutchens, T. T. Semi-micro synthesis of C^{14}-labeled acetic acid. Nucleonics, 1950, 3, 38.

114. Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., Cathey, W. J. Preparation of $BaCO_3$ plates by centrifugation. Nucleonics, 1950, 3, 41.

115. Van Bruggen, J. T., Claycomb, C. K., Hutchens, T. T. Techniques in the use of C^{14} as a tracer. Nucleonics, 1950, 3, 38.

116. Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., Cathey, W. J., West, E. S. The tissue effect of fasting upon lipogenesis in the intact rat. J. Biol. Chem., 1952, 196, 389.

117. Van Bruggen, J. T. Cage Hood assembly for small animals. Nucleonics, 1952, 10, 64.

118. Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K. Cathey, W. J., West, E. S. The effect of fasting upon lipogenesis in the intact rat. J. Biol. Chem., 1954, 208, 115.

119. Van Bruggen, J. T., Wong, R. K. Lipid Metabolism in the diabetic rat I. Acetate metabolism and lipid synthesis in vivo. J. Biol. Chem., 1960, 235, 26.

120. Van Bruggen, J. T., Hutchens, T. T., Atomic Energy Commission Release AECU 3128.

121. Van Bruggen, J. T. Metabolism of $NaHC^{14}O_2$ Effects of anesthesia and route of dosage. Proc. Soc. Exptl. Biol. Med., 1956, 91, 140.

122. Van Bruggen, J. T., & Emerson, R. J., Acetate metabolism; effects of tracer concentration. Arch. Biochem. Biophys., 1958, 77, 467.

123. Van Bruggen, J. T., & Cockburn, R. M. Acetate metabolism in vivo; effect of refeeding. J. Biol. Chem., 1959, 234, 431.

124. Van Bruggen, J. T., Emerson, R. J., Bernards, W. C. Acetate metabolism in vitro; effect of refeeding. J. Biol. Chem. 1959, 234, 435.

125. Van Bruggen, J. T., Marcd, A., Scott, J., & Elwood, J. C. Radioassay of aqueous samples. Anal. Chem., 1959, 31, 1746.

126. Van Bruggen, J. T., & Wong, R. K. Lipid Metabolism in the diabetic rat II. cholesterol turnover studies. J. Biol. Chem., 1960, 235, 30.

127. Villee, C. A., & Foster, J. M. Pyruvate and acetate metabolism in the isolated rat diaphragm. J. Biol. Chem., 1959, 211, 797.

128. Wakil, S., Gibson, D. M. Jacob, M. F., & Porter, J. W. Biosynthesis of fatty acids by soluble enzyme fractions. Biochem. biophys. Acta, 1952, 23, 219.

129. Wakil, S., Mahler, R. H., & Bock, R. M. Studies on fatty acid oxidation i. Enzymatic activation of fatty acids. J. Biol. Chem., 1953, 204, 453.

130. Wakil, S., Gibson, D. M., Titchsner, E. B. Requirement for bicarbonate in fatty acid synthesis. J. Am. Chem. Soc., 1958, 80, 2908.

131. Wakil, S., A malonic acid derivative as an intermediate in fatty acid synthesis. J. Am. Chem. Soc., 1958, 80, 6465.

132. Wakil, S., Gibson, D. M., Titchsner, E. B. Studies on the mechanism of fatty acid synthesis. V. Bicarbonate requirement for the synthesis of long chain fatty acids. Biochem. et Biophys. Acta, 1958, 30, 376,

133. Wakil, S. On the mechanism of fatty acid synthesis. Federation Proc., 1959, 18, 1371.

134. Wakil, S., Gaugly, J., On the mechanism of fatty acid synthesis. J. Am. Chem. Soc., 1959, 81, 2597.

135. Weinhouse, S., Millington, R. H., & Friedman, B. The effect of carbohydrate on the oxidation of fatty acids by liver slices. J. Biol. Chem., 1948, 181, 489.

136. Weinhouse, S., Medes, S., Thomas, A. Nutritional factors in fatty acid synthesis by slices of tissue in vitro. J. Biol. Chem., 1952, 197, 181.

137. Weinhouse, S. Factors involved in the formation and utilization of ketone bodies. In Major Metabolic Fuels, Brookhaven Symposia in Biology No. 5. Brookhaven National Laboratories. 1952, 201-221.

138. Weinhouse, S., & Wenner, C. E. An isotope tracer study of glucose catabolism pathways in liver. J. Biol. Chem., 1955, 219, 691.

139. Wertheimer, E., Tuerkischer, E., Alloxan Diabetes and liver glycogen. Endo., 1948, 5, 229.

140. West, E. S., Van Bruggen, J. T., Yamada, P., & Hutchens, T. T. Lipogenesis of the intact alloxan diabetic rat. J. Biol. Chem., 1954, 209, 635.

141. West, E. S., Beatty, C. H., & Bocek, R. M. Effect of succinate and oxaloacetate on ketone body production by liver slices from non-diabetic and diabetic rats. J. Biol. Chem., 1957, 230, 725.

142. Westerfeld, W. W., Shulman, M. P., & Zurek, M. The pathway of alcohol metabolism. In H. E. Himwich (Ed) Alcoholism, Basic aspects and treatment. Publication No. 47 of the American Association for the advancement of science. 1957, 19-37.

143. Westerfeld, W. W., & Shulman, M. P. An unidentified product of ethanol metabolism. Federation Proc., 1959, 18, 1265.

144. Wolf, D. E., & Hoffman, C. H. Beta-hydroxy-beta-methyl-gamma-valero lactone (divalonic acid) a new biological factor. J. Am. Chem. Soc., 1956, 78, 4499.

145. Wright, L. D., & Cleland, M. Factoss influencing incorporation of mevalonic acid into cholesterol by rat liver homogenates. Proc. Soc. Exp. Biol. Med., 1957, 96, 219.

146. Zlatkis, A., & Zak, B., & Boyle, A. J. A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med., 1953, 41 486.

147. Chaikoff, I. L., Matthes, K. J., & Abraham, S. Role of TPNH in fatty acid synthesis from acetate by normal and diabetic rat-liver homogenate fractions. Biochim. Biophys. Acta, 1959, 36, 556.